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THESE

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Biologie de la reproduction, phylogéographie et diversité de l'arbre à beurre *Pentadesma butyracea* Sabine (Clusiaceae) - implications pour sa conservation au Bénin

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A
Viviane, Joas et Abdias

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RÉSUMÉ GÉNÉRAL

Pentadesma butyracea Sabine est l'une des quatre espèces du genre *Pentadesma* endémique de l'Afrique. Elle est distribuée de la Sierra Léone au Gabon dans deux grands types d'habitats : les forêts denses humides discontinues du domaine guinéo-congolais (Haute- et Basse-Guinée) et le domaine soudanien du couloir sec du Dahomey (assimilé à une barrière à l'échange de gènes et d'espèces entre les deux blocs guinéo-congolais). Dans ce dernier, l'espèce se retrouve dans des galeries forestières et occupe une place capitale dans le développement socio-économique des communautés locales en raison des multiples biens et services que procurent ses produits (alimentation, médecine et pharmacopée traditionnelle, etc.). Cependant, des pressions d'origines multiples, telles que le ramassage des graines pour fabriquer du beurre, la fragmentation de l'habitat et sa destruction en faveur du maraîchage, les pratiques culturales inadaptées, les incendies, font peser de lourdes menaces sur l'espèce.

Le but de ce travail est d'acquérir les connaissances requises pour la conservation et la gestion durable des ressources génétiques de l'espèce. Trois objectifs ont été définis : (i) étudier la phylogéographie de l'espèce, (ii) étudier sa variabilité morphologique et génétique au Bénin et (iii) caractériser sa biologie de reproduction. En amont de ces travaux, nous avons développé onze marqueurs microsatellites nucléaires chez *P. butyracea* (chapitre 2). Ils ont été utilisés pour l'étude de la phylogéographie et la diversité génétique de *P. butyracea* (chapitres 3 et 5), ainsi que pour étudier la dépression de consanguinité et les paramètres de son système de reproduction (chapitre 7).

La caractérisation de la répartition spatiale des lignées génétiques de régions intergéniques de l'ADN chloroplastique et de l'ADN ribosomal (ITS) a détecté deux lignées génétiques allopatriques entre le Haut et le Bas-Guinéen, indiquant une forte différenciation génétique et un signal phylogéographique. L'analyse des microsatellites détecte trois pools géniques correspondant aux trois régions étudiées (Haute Guinée, Dahomey Gap et Basse Guinée). La diversité génétique est faible dans le Dahomey Gap, modérée dans le Haut-Guinéen et élevée dans le Bas-Guinéen. Ces résultats indiquent une séparation très ancienne des populations d'Afrique centrale et d'Afrique de l'ouest, alors que celles du Dahomey Gap pourraient résulter des forêts denses humides de l'Afrique de l'ouest lors de la période Holocène humide africaine. Dans ce couloir sec, les populations ont subi une forte dérive génétique, potentiellement due à des évènements de fondation. Au Bénin, deux groupes éco-morphologiques ont été détectés suivant un gradient nord-sud, contrastant avec deux pools géniques présentant une distribution est-ouest.

P. butyracea est une espèce auto-compatible majoritairement allogame. La corrélation de paternité est plus élevée aux niveaux intra-fruit vs. inter-fruits, et au sein d'une population de petite taille vs. de grande taille. Les principaux pollinisateurs au Bénin sont deux oiseaux (*Cyanomitra verticalis, Cinnyris coccinigastrus*) et trois abeilles (*Apis mellifera, Meliponula togoensis, Hypotrigona* sp.). La productivité totale en fruits augmente en fonction de l'âge de l'arbre et varie en fonction de l'année, atteignant un pic pour les arbres ayant un diamètre de 60-80 cm. Les graines sont récalcitrantes et ont une teneur en eau de 42.5 ± 2.9 %.

L'analyse des paramètres de reproduction et de diversité génétique, associés aux facteurs écogéographiques, nous a permis de proposer un échantillon de neuf populations représentatives de la diversité à l'échelle du Bénin, dans la perspective d'une conservation *in situ*. Le succès de celle-ci dépendra des efforts conjugués des communautés locales, de la recherche forestière et de la définition d'un cadre législatif par le politique pour la protection des habitats. La conservation *ex situ* est envisagée sous forme d'un verger rassemblant diverses origines, présentant l'intérêt supplémentaire de permettre d'étudier les contributions de la diversité génétique et de la plasticité phénotypique à la variation phénotypique.

GENERAL SUMMARY

Reproductive biology, phylogeography and diversity of the butter tree *Pentadesma butyracea* Sabine (Clusiaceae): implications for its conservation in Benin

Pentadesma butyracea Sabine is one of the four species of the endemic genus *Pentadesma* in Africa. The species is distributed from Sierra Leone to Gabon in two major types of habitats: the discontinuous and dense Guineo-Congolian rainforests (Upper and Lower Guinea) and the Sudanian domain of the dry corridor of Dahomey (considered as a barrier to the exchange of genes and species between Upper and Lower Guinea). In the latter, the species is found in gallery forests and plays a vital role in the socio-economic livelihood of local communities due to the various resources and services that provide its products (food, medicine and traditional, etc.). However, pressure from many sources including the collection of seeds to make butter, habitat fragmentation and its destruction for market gardening, inadequate agricultural practices, fires, are serious threats to the species.

The aim of this work was to acquire appropriate knowledge for the conservation and sustainable management of genetic resources of the species. Three objectives were defined (i) study the phylogeography of the species; (ii) evaluate its morphological and genetic variability in Benin; and (iii) characterize its reproductive biology. In a preliminary work, eleven nuclear microsatellite markers of *P. butyracea* were developed (Chapter 2). They were used for the study of phylogeography and genetic diversity of *P. butyracea* (chapters 3 and 5), and to study the inbreeding depression and parameters of its breeding system (Chapter 7).
The characterization of the genetic lineages and their spatial distribution using intergenic regions from chloroplast DNA and ribosomal DNA (ITS) region detected two allopatric genetic lineages between Upper and Lower Guinea, indicating a high genetic differentiation and a phylogeographic signal. Microsatellite markers allowed us to detect three genepools matching with the three studied regions (Upper Guinea, Dahomey-Gap and Lower Guinea). Genetic diversity was low in the Dahomey Gap, moderate in Upper Guinea and high in Lower Guinea. These results indicate an ancient separation of populations from Central and West Africa, while those from Dahomey Gap could originate West African rainforests (Upper Guinea) during the African humid Holocene period. In this dry corridor, populations experienced high genetic drift, possibly due to founding events. In Benin, two eco-morphological groups were detected following a north-south gradient, contrasting with two gene pools presenting an east-west distribution.

Pentadesma butyracea is a self-compatible, mainly allogamous species. The correlation of paternity was higher within-fruit vs. among-fruits, and in population of small size vs. large size. The main pollinators in Benin are two birds (*Cyanomitra verticalis, Cinnyris coccinigastrus*) and three bees (*Apis mellifera, Meliponula togoensis*, *Hypotrigona* sp.). Total productivity in fruit increases with tree age and varies yearly, reaching a peak for trees of 60-80 cm of diameter class. Seeds are recalcitrant (i.e. they cannot be conserved at low temperature), having a water content of 42.5 ± 2.9% at maturity.

The analysis of reproduction and genetics parameters, associated with eco-geographic factors, enabled us to select nine populations representative of the diversity in Benin, from the perspective of *in situ* conservation. The success of the latter will depend on combined efforts of local communities, forest research and an adequate legislative framework for the protection of habitats. *Ex situ* conservation is envisaged as an orchard assembling various origins, and would have the additional advantage of allowing to study the contribution of genetic diversity and phenotypic plasticity to phenotypic variation.

ACRONYMES ET DÉFINITION DE QUELQUES EXPRESSIONS UTILISÉES (*traduction en anglais*)

ADN (*DNA*)**:** acide désoxyribonucléique ; double chaîne de nucléotides liés entre eux (ayant comme sucre constituant un désoxyribose). Molécule fondamentale dont sont constitués les gènes.

AFLP (*Amplified Fragment Length Polymorphism*)**:** polymorphisme de longueur de fragments amplifiés. Méthode très sensible pour détecter des polymorphismes dans l'ADN. L'ADN est d'abord digéré par deux enzymes de restriction peu spécifiques, puis un sous-ensemble de fragments d'ADN obtenus est sélectionné pour amplification par PCR ; les fragments de différentes tailles sont séparés selon leur poids moléculaire et visualisés. La variation entre individus est détectée par la présence ou absence de fragments de différentes tailles. L´avantage des AFLP est de ne pas avoir besoin d´information préalable sur le génome du taxon en question.

Allèle (*allele*)**:** une des formes alternatives d'un gène qui peut exister à un locus unique.

Allozyme (*allozyme*)**:** isozyme dont la synthèse est contrôlée par des allèles codominants d'un gène.

Amorce (*primer*)**:** petit fragment d'ADN ou d'ARN qui se lie sur un ADN simple-brin et auquel d'autres nucléotides peuvent être ajoutés par l'ADN polymérase. Une paire d'amorces permet d'amplifier (démultiplier) toute la région ADN située entre elles grâce à une PCR.

ARN (*RNA*)**:** acide ribonucléique, acide organique qui contient des unités répétées de nucléotides: adénine (A), guanine (G), cytosine (C) et uracile (U), et dont les composés ribose sont liés par des liaisons phosphodiester.

Autogamie ou **autopollinisation** (*selfing*)**:** transfert de pollen de l'anthère d'une fleur vers le stigmate de la même fleur ou d'une fleur de la même plante.

Banque d'ADN (*DNA library*): collection de clones d'ADN obtenus d'un ADN donneur.

Banque d'ADN (*Gene bank*): installation établie pour la conservation *ex situ* d'individus (semences), de tissus ou de cellules reproductrices de plantes ou d'animaux.

Nucléotide (*nucleotide*)**:** unité chimique ou constituant élémentaire de l'ADN et de l'ARN, composé d'un sucre (désoxyribose ou ribose), de phosphates et d'une base azotée. Dans l'ADN les bases présentes sont l'adénine (A), la guanine (G), la thymine (T) et la cytosine (C). Dans l'ARN, les bases sont l'adénine, la guanine, l'uracile (U) and la cytosine.

Caractère (*trait*)**:** trait, attribut (génétique, morphologique) des individus dans une espèce pour lequel peuvent être définies des différences héritables.

Chromosome (*chromosome*)**:** molécule d'ADN linéaire, avec des protéines et de l'ARN associés, constituant une unité élémentaire dédoublée et transmise entre cellules-filles lors de divisions cellulaires.

Codominance (*codominance*)**:** situation dans laquelle un individu hétérozygote présente un phénotype où les effets des deux allèles d'un gène particulier sont exprimés.

Conservation *ex situ* (*ex situ conservation*)**:** méthode de conservation des ressources génétiques (semences, pollen, sperme, organisme individuel, …) en dehors de l'habitat original ou environnement naturel de l'espèce.

Conservation *in situ* (*in situ conservation*)**:** méthode de conservation qui vise à conserver l'intégrité des ressources génétiques d'une espèce au sein même de son habitat. Cela permet en particulier une évolution dynamique de l'organisme dans son habitat d'origine.

Délétion (*deletion*)**:** type particulier de mutation de l'ADN impliquant la perte d'un ou plusieurs nucléotides.

Dépression de consanguinité (*inbreeding depression*)**:** baisse de la survie et/ou de la fertilité des descendants d'individus apparentés, liée à l'accumulation d'allèles délétères récessifs s'exprimant au sein de ces individus consanguins.

Dérive génétique (*genetic drift*)**:** évolution des fréquences alléliques d'une population d'une génération à l'autre à l'intérieur par le simple fait du hasard. Cela est dû à l'échantillonnage aléatoire des gènes d'une génération à l'autre, ce qui est inévitable dans des populations de taille finie. Plus petite est la population, plus grande est la dérive génétique, avec comme conséquence la perte de certains allèles, et la réduction de la diversité génétique.

Dichogamie (*dichogamy*): séparation temporelle de la maturation des organes de reproduction mâles (anthères) et femelles (stigmate).

Ecotype (*ecotype*)**:** population ou lignée d'un organisme adaptée à un habitat particulier.

Flux de gènes (*gene flow*)**:** échange de gènes au sein ou entre populations d'une même espèce. Chez les plantes, il s'effectue par l'intermédiaire du pollen et des graines.

Fragment de restriction (*restriction fragment*)**:** fragment d'ADN qui a été coupé par une enzyme de restriction reconnaissant une courte séquence d'ADN spécifique.

Fréquence allèlique (*allelic frequency*)**:** mesure de la fréquence à laquelle un allèle est trouvé dans une population.

Gène (*gene*)**:** unité physique et fonctionnelle de l'hérédité, transmise d'une génération à l'autre. En général, c'est un segment d'ADN incluant une section transcrite et des éléments régulateurs qui permettent sa transcription.

Génome (*genome*)**:** effectif complet du matériel génétique d'un organisme.

Génotype (*genotype*)**:** composition allélique spécifique soit de la cellule entière ou, plus souvent, d'un certain gène ou ensemble de gènes.

Germoplasme (*germplasm*)**:** variabilité génétique disponible pour une population d'organismes, représentée par des cellules sexuelles, des semences, etc.

Goulot d'étranglement (*bottleneck*): réduction brutale de la taille d´une population qui conduit généralement à une dérive génétique forte, entraînant une diminution de sa diversité génétique d´origine.

Haplotype (*haplotype*)**:** constitution allélique spécifique à un certain nombre de locus, dans un bloc de liaison défini.

Equilibre de Hardy-Weinberg (*Hardy-Weinberg equilibrium*)**:** théorie de génétique des populations, qui postule l'existence d'un équilibre des fréquences allélique et génotypique d'une génération à l'autre au sein d'une population idéalisée (taille infinie, espèce diploïde et reproduction sexuée, panmixie, absence de migration, de mutation, de sélection et générations non chevauchantes).

Herkogamie (*herkogamy*): séparation spatiale entre anthères (organe reproducteur mâle) et stigmate (organe reproducteur femelle) permettant de réduire l'autopollinisation au sein d´une même fleur.

Hérédité (*heredity*)**:** processus par lequel les caractères génétiques sont transmis des parents à leur descendance.

Hétérozygotie (*heterozygosity*)**:** gène représenté par deux allèles différents sur les deux copies chromosomiques d'un individu diploïde.

Homoplasie (*homoplasy*) : similitude d'un état de caractère entre deux individus d'une même espèce ne provenant pas d'un ancêtre commun.

Homozygotie (*homozygosity*)**:** gène représenté par deux allèles identiques sur les deux copies chromosomiques d'un individu diploïde.

Hybride (*hybrid*)**:** individu issu de la descendance d'un croisement entre parents de génotypes très différents, généralement considérés comme appartenant à des (sous-)espèces différentes.

Insertion (*insertion*)**:** type particulier de mutation de l'ADN impliquant le gain d'un ou plusieurs nucléotides

Isolement par la distance (*isolation by distance*)**:** tendance des individus spatialement plus proches à être plus apparentés que ne le sont les individus éloignés et/ou augmentation de la différentiation génétique entre populations avec la distance spatiale, découlant d'un flux de gènes spatialement restreint.

Isozyme (*isozyme*)**:** formes multiples d'une enzyme dont la synthèse est contrôlée par plus d'un gène.

Liaison génétique (*genetic linkage*)**:** proximité physique entre gènes sur un chromosome avec la tendance d´être hérité ensemble.

Ligase (*ligase*)**:** type d'enzyme capable de reconstituer une liaison phosphodiester rompue dans un acide nucléique.

Ligation (*ligation*)**:** réaction enzymatique qui conduit à joindre deux fragments d'ADN ou plus ensemble.

Locus (*locus, loci*)**:** emplacement physique spécifique sur un chromosome où est localisé un gène ou un fragment d'ADN donné.

Marqueur génétique (*genetic marker*)**:** caractère ou produit d'une méthode de biologie moléculaire héritable et utilisé pour caractériser expérimentalement le génotype d'un individu.

Microsatellite (*microsatellite*) ou **SSR:** type d'ADN répété dont les unités de base sont de très courtes séquences comme des dinucléotides, des trinucléotides ou des tétranucléotides. Leur haut taux de mutation génère un polymorphisme élevé, ce qui en fait des marqueurs génétiques intéressants pour de nombreuses applications.

Multiplex PCR (*multiplex PCR*)**:** amplification PCR simultanée de plusieurs locus d'ADN différents permettant de réduire le temps de manipulation et les frais de produits.

Mutation (*mutation*)**:** modification de l'information génétique dans le génome d'une cellule ou d'un virus. C'est donc une modification de la séquence de l'ADN, ou bien dans l'ARN pour un virus à ARN. On peut distinguer plusieurs types de mutations. On parle de mutation de transition lorsqu'il y a substitution d'une base purique à une autre base purique (ou d'une base pyrimidique à une autre base pyrimidique). Au contraire, une mutation de transversion est une mutation causée par le remplacement d'une base purique par une base pyrimidique (ou d'une base pyrimidique par une base purique). Les mutations silencieuses sont des mutations qui ne modifient pas la séquence d'une protéine, à cause de la redondance du code génétique (le nouveau triplet code le même acide aminé que le triplet original), ou parce qu'elle touche une région non codante de l'ADN, ou un intron.

nSSR (*nuclear Simple Sequence Repeats*)**:** marqueurs microsatellites du génome nucléaire.

Nucléotide (*nucleotide*)**:** molécule composée d'une base azotée (Adénine, Thymine, Guanine, Cytosine dans le cas de l'ADN ou Uracile dans le cas de l'ARN), d'un sucre et d'un groupement phosphate. Les nucléotides sont les unités de construction des acides nucléiques formant l'ADN.

Oligonucléotide (*oligonucleotide*)**:** petit segment d'ADN synthétisé artificiellement.

Paire de bases (*base pair*)**:** deux bases nucléotidiques sur les différents brins d'une molécule d'acide nucléique maintenues ensemble par des liaisons hydrogène: l'adénine avec la thymine (ADN) ou l'uracile (ARN), et la guanine avec la cytosine (ADN et ARN).

Panmixie (*panmixy*)**:** mode de croisement aléatoire entre individus (suppose équiprobabilité des gamètes des adultes, rencontre des gamètes au hasard ou formation aléatoire des couples, ségrégation aléatoire des gamètes lors de la méiose).

Patrimoine génétique (*gene pool*): somme totale des gènes, avec toutes leurs variations, possédée par une espèce particulière ou un sous-ensemble particulier à un moment donné.

PCR (*Polymerase Chain Reaction*)**:** amplification en chaîne par la polymérase. Une méthode pour produire un nombre élevé de copies d'une région de l'ADN, en utilisant une enzyme polymérase thermostable et des amorces spécifiques permettant de focaliser l'amplification sur la région désirée de l'ADN.

Phénotype (*phenotype*)**:** soit (1) la forme prise par un caractère (ou ensemble de caractères) chez un individu particulier; ou (2) l'apparence externe détectable d'un génotype spécifique.

Plasmide (*plasmid*)**:** molécule d'ADN extrachromosomique qui est capable de se répliquer de façon autonome.

Polymorphisme (*polymorphism*)**:** apparition de différentes formes associées avec des allèles variés d'un gène ou homologues d'un chromosome.

Population (*population*)**:** groupe d'individus inter-fertiles de la même espèce vivant dans une aire géographique délimitée.

RAPD (*Random Amplified Polymorphic DNA*)**:** ADN polymorphe amplifié au hasard. Méthode très sensible pour détecter des polymorphismes dans l'ADN. Des fractions d'ADN anonymes sont amplifiées au hasard en utilisant la PCR avec des amorces arbitraires, puis les fragments d'ADN amplifiés sont séparés selon leurs poids moléculaires et visualisés. . Cette technique a été presque complètement remplacée par les AFLP à cause des problèmes de reproductibilité.

Recombinaison (*recombination*)**:** réorganisation des combinaisons alléliques au sein des génomes (source de variation génotypique) au cours d'une meiose. Au sein d'un chromosome, elle est due à des *crossing over*. Chez les eucaryotes, elle se produit par l'échange réciproque d'ADN entre chromatides non-sœurs à l'intérieur d'une paire de chromosomes homologues, pendant la prophase de la première division méiotique. La recombinaison permet le réarrangement du matériel génétique des chromosomes, augmentant ainsi le potentiel de diversité génétique.

Ressources génétiques (*genetic resources*)**:** composante de la biodiversité (gènes de plantes ou d'animaux) utilisée par l'homme à diverses fins (agriculture, médecine, industrie, environnement, spiritualité, culture, écologie, etc.).

RFLP (*Restriction Fragment Length Polymorphism*)**:** Polymorphisme de longueur des fragments de restriction. Méthode pour détecter des polymorphismes dans l'ADN. Une région spécifique de l'ADN est amplifiée par PCR en utilisant des amorces spécifiques, puis le produit de PCR est digéré avec une enzyme ; les fragments résultants sont séparés selon leurs poids moléculaires et visualisés. Cette méthode est presque abandonnée à cause des techniques avancées de séquençage.

Sélection (*selection*)**:** changement de la fréquence allélique d'un gène lorsque celui-ci agit sur le succès reproducteur des individus qui le porte.

Séquençage (*sequencing*)**:** détermination de l'ordre des nucléotides dans une molécule d'ADN ou ARN, ou l'ordre des acides aminés dans une protéine, à l'aide de techniques de biologie moléculaire.

Séquence d'ADN (*DNA sequence*)**:** ordre des bases nucléotidiques dans la molécule d'ADN qui, dans le cas des gènes, contient l'information utilisée par la cellule pour produire des protéines.

CHAPITRE I.-

INTRODUCTION GÉNÉRALE

I 1.- La perte des ressources génétiques : une problématique internationale

Les forêts tropicales humides se situent de part et d'autre de l'équateur, et sont les biomes continentaux les plus riches et diversifiés de notre planète. Au cours des derniers siècles, plus du tiers de ces écosystèmes ont été convertis par l'Homme en d'autres formes d'utilisations des terres, comme l'agriculture, les plantations, l'exploitation minière, l'urbanisation etc. (FAO 2010, Corlett et Primack 2011). En raison de l'ampleur et de l'irréversibilité des dégâts subis par les forêts tropicales, la perte effective et potentielle des espèces et de leurs ressources génétiques constitue l'une des plus grandes perturbations environnementales de notre temps. Le plan d'action mondial pour une utilisation durable des ressources phytogénétiques pour l'alimentation et l'agriculture (FAO, 1996) met en exergue l'importance des ressources phytogénétiques pour la sécurité alimentaire mondiale. Ce sont des composantes de la biodiversité utilisées par l'homme à diverses fins (agriculture, médecine, industrie, environnement, spiritualité, culture, écologie, etc.). Elles possèdent de ce fait une valeur économique importante au niveau local et national et sont capitales pour la survie de l'humanité.

En Afrique, les forêts tropicales hébergent d'innombrables produits forestiers sauvages riches en éléments nutritifs recherchés et consommés par toutes les classes d'âge. Cependant, ces ressources et leurs habitats naturels font l'objet d'une exploitation incontrôlée et subissent les conséquences du changement global, aggravé par la crise économique mondiale et la croissance démographique galopante. Selon la FAO (2002), les forêts africaines connaissent une réduction annuelle de leur surface de 5,3 millions d'hectares soit l'équivalent de 0,78% de leur superficie totale actuelle. Dans les forêts humides d'Afrique Centrale par exemple, on enregistre une augmentation constante de la dégradation des forêts due au développement des cultures de rente, à l'urbanisation, à l'exploitation commerciale du bois d'œuvre et à l'exploitation minière. Cette destruction est considérée comme la principale cause de réduction de la diversité biologique. Celle-ci a des conséquences économiques et biologiques graves, telles que la réduction du nombre d'espèces et de leur diversité génétique, les perturbations des interactions biotiques et des flux de nutriments et des processus dynamiques des écosystèmes (climat, rétention de l'eau, conservation des sols, séquestration du carbone, pollinisation…) (Bawa & Dayanandan 1998, Ouédraogo 1999).

I 1.1.- Situation en Afrique de l'Ouest

En Afrique de l'Ouest, l'exploitation forestière est forte également et la déforestation est encore aggravée à cause des incendies de forêts. Dans les habitats secs où les espèces forestières doivent s'adapter à des conditions écologiques de faible disponibilité en eau, ces incendies font des ravages. C'est le cas dans le Dahomey Gap (entre 0 et 3° de longitude Est incluant les pays Bénin, Togo et l'Est du Ghana), où les conditions climatiques sèches de l'Holocène récent ont provoqué une rupture de la forêt dense humide remplacée par la savane qui atteint la côte (Salzmann & Hoelzmann 2005). L'intensité de la dégradation forestière y est si forte que certaines espèces sont déclarées localement menacées pendant que d'autres sont déjà inscrites sur la liste rouge de l'IUCN (Neuenschwander *et al.* 2011). Ce couloir sec est considéré comme une barrière écogéographique à l'échange d'espèces entre les deux blocs forestiers guinéo-congolais (Adomou 2005) et joue un rôle important pour la conservation de la biodiversité et pour la compréhension des processus de l'évolution des espèces forestières d'Afrique tropicale.

La Convention des Nations Unies sur la Diversité Biologique, à laquelle plus de 180 pays ont adhéré, représente un cadre juridique international unique pour la protection et l'utilisation rationnelle de la diversité biologique. Elle reconnaît que la conservation de la diversité biologique est une préoccupation commune à l'humanité et fait partie intégrante du développement socio-économique durable. Dans ce contexte, depuis les années 2000, le programme des ressources phytogénétiques forestières en Afrique sub-saharienne (SAFORGEN) encourage et soutient les activités de recherches axées sur les espèces ligneuses utilisées pour l'alimentation, les produits médicinaux et aromatiques, le bois et les fibres ainsi que le fourrage en Afrique. Il a pour objectif principal l'étude du statut des ressources génétiques des espèces ligneuses sauvages ou semi-domestiquées ainsi que des menaces pesant sur elles, et de fournir des informations pratiques sur l'utilisation durable et la conservation des espèces ligneuses alimentaires sauvages. Ces axes de recherche portent sur la taille efficace des populations de ces espèces ainsi que leur taille minimale viable en vue de leur conservation et leur gestion à long terme. Ils visent à déterminer la variation génétique intra-spécifique et à localiser les sources importantes de cette variabilité, à élaborer des protocoles pour le stockage *ex situ* des espèces à graines récalcitrantes (qui ne supportent pas la conservation *ex situ* à long terme), à identifier les espèces pollinisatrices, à analyser les flux polliniques efficaces, l'efficacité de la dispersion des graines et le degré de leur dépendance vis-à-vis d'animaux rares ou menacés.

I 1.2.- Ressources phytogénétiques au Bénin

Au Bénin, les petites surperficies de forêts denses humides semi-décidues disséminées au sud du pays bénéficient pour la plupart d'actions de conservation. Par contre, de nombreux parcs agroforestiers, forêts denses sèches et galeries forestières au centre et au nord du pays hébergent aussi d'importantes espèces ligneuses sauvages alimentaires (de forêt et de savane) très utiles aux populations, sans bénéficier de cette protection. Certaines de ces espèces font l'objet d'un commerce national et international, comme le karité (*Vitellaria paradoxa)*, le baobab (*Adansonia digitata)*, le tamarinier (*Tamarindus indica)*, l'arbre à beurre *(Pentadesma butyracea)* ou le néré (*Parkia biglobosa)*. Parmi ces espèces, le cas de *Pentadesma butyracea* (considéré comme un fruitier alimentaire négligé, http://www.prota4u.org/protav8.asp?fr=1&p=Pentadesma+butyracea) est alarmant et nous intéresse particulièrement dans ce travail. C'est un arbre sauvage à usages multiples. Les populations extraient de ses graines du beurre pouvant être conservé jusqu'à trois ans, et qui se substitue au beurre de karité dans l'alimentation humaine, en pharmacopée et en cosmétique. Ce beurre jaune a les mêmes propriétés que celui du karité. Il est utilisé pour la fabrication de cosmétiques par la firme L'OREAL. Actuellement, la demande en noix de karité est croissante à cause de l'autorisation par l'Union européenne d'utiliser le beurre de karité dans l'industrie du chocolat, comme alternative au beurre de cacao, jusqu'à la hauteur de 5% (CIRAD/GRET/MFAE 2002). Cette augmentation de la demande pour l'exportation devrait être une opportunité pour le Bénin pour compenser la baisse de productivité croissante du karité à cause des hémi-parasites et s'inscrit dans le cadre de la politique de diversification et de relance des produits agricoles de l'Etat béninois. La domestication de *Pentadesma butyracea* comme espèce de reboisement ou d'agroforesterie peut être envisagée dans cette optique.

Pentadesma butyracea figure sur la liste des dix espèces ligneuses alimentaires prioritaires pour la conservation au Bénin et au Togo à cause de leur importance alimentaire et des menaces avérées sur leurs ressources (Eyog-Matig *et al.* 2002). Il bénéficie aussi d'un statut particulier du programme DIRECTS (Darwin Initiative Research Exercice on Community Tree Seeds) qui vise la compréhension des facteurs impliqués dans la détérioration des semences lors du stockage, la tolérance à la dessiccation, les dommages d'imbibition et la durée du stockage à des températures basses en dessous de zéro degré, le rôle des antioxydants etc. (Sacandé *et al.* 2006-2007, Sama et Sacandé 2007). Il figure sur la liste des espèces vulnérables de l'IUCN (Neuenschwander *et al.* 2011).

Dans un tel contexte, la prise de mesures visant la protection de cette espèce est urgente.

I 2.- Apports de la génétique des populations et des marqueurs génétiques à la problématique de conservation des ressources phytogénétiques

En général, les populations naturelles de plantes présentent une variabilité génétique intraspécifique. Celle-ci contribue à une meilleure survie de l'espèce face aux fluctuations des conditions du milieu, lorsqu'elle implique des caractères adaptatifs. Elle a également l'avantage de fournir une gamme diversifiée de potentialités aux niveaux de caractères morphologiques ou d'autres caractéristiques sous contrôle génétique, au sein desquelles l'Homme peut opérer des choix pour satisfaire ses besoins.

La variabilité phénotypique entre individus peut-être la conséquence de la plasticité phénotypique, c'est-à-dire la capacité d'un génotype à donner divers phénotypes en fonction des conditions du milieu, et/ou d'un polymorphisme sous-jacent au niveau des gènes, plus précisement, de la coexistence de plusieurs allèles en un locus. La mutation est la seule source d'allèles nouveaux : elle transforme un allèle en un autre, nouveau ou déjà présent dans la population. En général, le taux de mutation est faible et les mutations ont un rôle négligeable sur l'évolution de la fréquence des allèles. Une fois qu'un nouvel allèle est apparu, ce n'est plus la mutation qui détermine son devenir. L'évolution ultérieure et les fréquences alléliques au sein des populations sont modelées par d'autres forces évolutives.

La génétique des populations est la science qui mesure la variation génétique, décrit le modèle d'organisation de la diversité, tente d'expliquer le maintien de cette variation génétique et son évolution sous l'effet de la mutation, la sélection, la dérive génétique, la recombinaison et les flux de gènes. Pour parvenir à ses objectifs, la génétique des populations s'appuie sur des marqueurs moléculaires utilisés comme indicateurs de la variabilité génétique entre individus, populations et espèces. Ils permettent, entre autres, d'estimer la variabilité génétique d'une population, d'observer la répartition géographique de la diversité génétique et d'inférer des informations sur la capacité de dispersion des individus. Lorsque les populations sont démographiquement stables et échangent des gènes de façon constante, il est possible de prédire leur structure génétique spatiale à partir de modèles théoriques. En effet, à des échelles spatiales fines, les structures génétiques spatiales se traduisent par une agrégation d'individus apparentés et le taux d'apparentement entre individus décroit avec la distance géographique séparant les individus, un patron dénomé isolement par la distance (Vekemans and Hardy 2004).

I 2.1.- Marqueurs moléculaires et diversité génétique

Au cours du développement de la génétique des populations ces trois dernières décenies, les isoenzymes ont été les premiers marqueurs largement exploités pour estimer la diversité génétique et les paramètres des systèmes de reproduction des plantes (Diallo 2001, Sina 2006). Ces marqueurs ont un pouvoir de discrimination plus élevé que les traits morphologiques et constituent toujours une méthode relativement simple et peu coûteuse pour obtenir des informations génétiques. Mais leur application est limitée par le faible nombre moyen d'allèles et de locus d'isoenzymes, par l'occurrence de mutations silencieuses (c'est-à-dire non détectables), par l'expression de certains enzymes en fonction du stade de développement, par leur faible niveau de variabilité chez certaines espèces et par le fait qu'ils ne révèlent la variation que dans des gènes codant des protéines et donc potentiellement influencés par la sélection.

La mise au point de méthodes basées sur les séquences nucléotidiques a permis de surmonter les contraintes liées au nombre de locus en fournissant des outils pour étudier les variations dans les régions codantes et non codantes, tant au niveau nucléaire que cytoplasmique. Ces marqueurs comprennent les RFLP (polymorphisme de longueur des fragments de restriction), AFLP (polymorphisme de longueur des fragments amplifiés), les microsatellites, et le séquençage. En fonction de l'objet d'étude, le matériel génétique utilisé peut être d'origine nucléaire ou cytoplasmique (ADN chloroplastique et mitochondrial).

I 2.2.- Intérêt des marqueurs microsatellites

I 2.2.1.- Estimation de la diversité génétique

Les marqueurs microsatellites sont des outils adéquats pour étudier la diversité génétique, la structure génétique à fine et large échelle des populations et les flux de gènes. Ce sont des segments d'ADN contenant de nombreux motifs courts et répétés en tandem. Les différences alléliques se traduisent par un nombre de répétitions variables d'un individu à un autre liées à un taux de mutation très élevé des microsatellites (10^{-3} à 10^{-4} par locus, par gamète et par génération). Il existe deux hypothèses sur le mécanisme générant les variations de taille des allèles des locus microsatellites. Soit elles proviennent d'erreurs de réplication de type "glissement" qui augmentent ou diminuent la longueur des allèles, soit elles sont dues à des recombinaisons entre chromosomes homologues, ces deux types d'erreurs ayant lieu lors de la méiose. Les locus microsatellites sont très utilisés à cause de leur forte variabilité et du fait qu'ils sont codominants, ce qui facilite la distinction entre hétérozygotes et homozygotes.

Cependant l'investissement pour obtenir un nombre suffisant de locus microsatellites opérationnels est lourd par rapport aux locus RFLP, RAPD et AFLP.

Deux types de marqueurs microsatellites existent et sont utilisés en fonction des buts poursuivis, les uns développés à partir de l'ADN nucléaire, les autres au niveau chloroplastique. Les microsatellites nucléaires sont transmis par la voie maternelle et paternelle tandis que les microsatellites chloroplastiques sont généralement transmis par voie maternelle chez les angiospermes.

Les divers paramètres révélés par les marqueurs moléculaires sont le pourcentage de locus polymorphes, le nombre moyen d'allèles par locus et l'hétérozygotie. Ils fournissent des valeurs plus précises sur le déficit en hétérozygotes et permettent d'inférer les coefficients de parenté et de consanguinité.

Les microsatellites nucléaires ont été utilisés avec succès pour l'étude de la diversité génétique sur des espèces de la même famille que *Pentadesma butyracea* comme *Symphonia globulifera* (Aldrich et al. 1998, Vinson *et al.* 2005), mais aussi sur divers autres arbres forestiers des zones tropicales humides (ex : White & Powell 1997, Hughes *et al.* 2002, Born *et al.* 2006). Ces diverses études montrent qu'un locus polymorphe peut présenter 2 à 15 allèles différents. Les locus microsatellites étudiés par ces auteurs révèlent une hétérozygotie très élevée pouvant atteindre 87% chez de nombreux arbres. En général, ces marqueurs révèlent que la diversité génétique intrapopulation est plus élevée que la diversité génétique entre les populations à cause des flux de gènes importants entre populations, en particulier via les mouvements du pollen. Ces flux sont généralement suffisants pour aboutir à une faible différenciation génétique.

I 2.2.2.- Estimation directe et indirecte de la dispersion du pollen et des graines à partir des microsatellites nucléaires

La dynamique d'évolution et de la structure spatiale de la diversité génétique dépend de l'efficacité de la dispersion des graines et du pollen. La connaissance de l'étendue de dispersion peut permettre de prédire l'évolution des populations.

Approche directe

Les microsatellites permettent d'analyser la paternité et d'évaluer les distances de dispersion du pollen. Sur la base du degré de ressemblance des génotypes, on peut évaluer l'apparentement et les relations parents-enfants. Les microsatellites permettent de retrouver le père de graines d'un arbre-mère connu, ou de retrouver simultanément les deux géniteurs.

Dans ce dernier cas, on fait souvent l'hypothèse que le parent le plus proche est l'arbre-mère, car les graines dispersent généralement moins loin que le pollen. Cette approche permet de déterminer directement l'étendue de dispersion des propagules en associant les relations de parenté avec les coordonnées spatiales des parents et des descendants étudiés. La distance moyenne entre plante-mère et descendants est évaluée à 12,4 m, 144 m et 194 m respectivement chez les chênes, l'Alisier et le Cèdre dont les pollens dispersent respectivement 25, 5 et 1 fois plus que les graines (Gerber *et al.* 2003).

Estimation indirecte

Elle se base sur la structure génétique par exemple à partir de l'indice de différenciation F_{ST} pour déterminer indirectement le nombre de migrants entre populations, ou à partir de l'évolution du coefficient de parenté en fonction de la distance. Les analyses à fine échelle conduites par Hardy *et al.* (2006) ont montré que le coefficient de parenté est inversement proportionnel au logarithme de la distance spatiale et que la dispersion des gènes de dix arbres tropicaux peut atteindre 150 à 1200 m en fonction du type de diaspores. D'après Born *et al.* (2008), les semences d'okoumé (*Aucoumea klaineana*), un grand arbre des forêts du Gabon, sont disséminées par le vent de 210 à 570 m.

I 2.2.3.- Estimation des paramètres du système de reproduction

Les microsatellites nucléaires intègrent deux jeux de chromosomes : l'un issu du père et l'autre maternel. Ainsi à l'instar des autres marqueurs moléculaires, ils sont utilisés pour déterminer la proportion de graines issues d'autofécondation ou d'allofécondation parmi les descendants d'un arbre-mère. Ces estimations sont souvent réalisées à l'aide de l'approche multilocus de Ritland (2002) basée sur la méthode d'estimation du maximum de vraissemblance des paramètres de reproduction (Ritland & Jain, 1981) et décrite pour deux régimes de reproduction des plantes (autofécondation et reproduction mixte associant autofécondation et allofécondation). Les hypothèses d'application sont :

- ségrégation des allèles aux différents locus ;
- homogénéité du pool pollinique entre arbres maternels ;
- les marqueurs génétiques utilisés ne sont affectés ni par la sélection, ni par la mutation entre la pollinisation et le temps d'échantillonnage des descendants.

Cette approche estime le taux d'allofécondation monolocus et multilocus calculé respectivement pour chaque locus et l'ensemble des locus.

I 2.3.- Intérêt de l'ADN chloroplastique

Pour reconstituer l'histoire démographique des populations, on utilise le plus souvent l'ADN cytoplasmique, et principalement l'ADN chloroplastique chez la plupart des angiospermes. Il s'agit d'un ADN circulaire qui comprend plusieurs gènes codants et des régions non codantes, tous liés. La structure de l'ADN chloroplastique du tabac (*Nicotiana tabacum*) est illustrée ci-dessous (Figure 1). C'est un ADN transmis généralement par la voie maternelle et donc dispersé par la graine chez les angiospermes. Il permet généralement de différencier des espèces et des populations végétales et il convient bien à l'étude de la phylogéographie de plantes.

En complément aux marqueurs microsatellites nucléaires, la variabilité du génome chloroplastique permet d'apprécier les processus évolutifs et variations environnementales historiques qui ont affecté la structure génétique des populations. C'est le domaine de la phylogéographie qui s'investit à connaitre les lignées génétiques et leur distribution spatiale pour inférer des informations sur l'histoire démographique des populations (Avise 2009). Elle suppose que les populations spatialement structurées sont génétiquement liées sous la dépendance d'obstacles comportementaux et/ou physiques aux échanges de gènes entre populations actuellement ou dans le passé. En effet, les variants génétiques ou haplotypes sont souvent spatialement localisés et lorsque deux haplotypes étroitement liés sont géographiquement plus proches que ne sont deux haplotypes très distants génétiquement on parle de « structure phylogéographique ». Au cours de l'évolution et du temps, il se produit des échanges occasionnels ou non de gènes et de lignées génétiques. Le but de la phylogéographie est d'utiliser les arbres généalogiques pour déduire l'histoire démographique et les facteurs qui ont généré l'architecture généalogique actuelle des populations. Les branches de l'arbre sont en rapport avec les évènements historiques survenus dans un contexte géographique donné.

Au cours de leur histoire, les forêts tropicales africaines ont connu plusieurs cycles climatiques caractérisés par des phases de glaciation et des phases de réchauffement au cours du Pléistocène, avec des conséquences majeures sur la végétation et des traces génétiques au sein des espèces. Les grandes conséquences ont été la fragmentation du couvert forestier (Vincens *et al.* 1999, Lezine 2007) et la fluctuation de la taille des forêts. Au niveau intra-spécifique, les conséquences sont la suppression de flux de gènes, l'augmentation de la différenciation génétique et l'isolement génétique des populations favorable à des lignées génétiques allopatriques. Ceci est la conséquence de l'accumulation de mutations de lignées

incompatibles adaptées aux conditions écologiques de différents sites, et de la dérive génétique. Ainsi les changements démographiques peuvent laisser des signatures dans la structure génétique des populations.

Chez les plantes, les marqueurs génétiques cytoplasmiques présentent une structuration génétique généralement plus forte que les marqueurs nucléaires, du fait de leur mode de transmission maternel impliquant généralement une plus faible dispersion par voie de graines comparée au pollen, ainsi qu'une plus forte dérive génétique (génome haploïde) (Dumolin-Lapegue *et al.* 1997, Cottrell *et al.* 2005, Petit *et al.* 2005). Ces marqueurs représentent un outil qui s'est révélé précieux pour étudier la distribution spatiale de la diversité génétique intra-spécifique dans les massifs forestiers d'Afrique tropicale (Catalano *et al.* 2008, Koffi 2010, Born 2007, Petit *et al.* 2003).

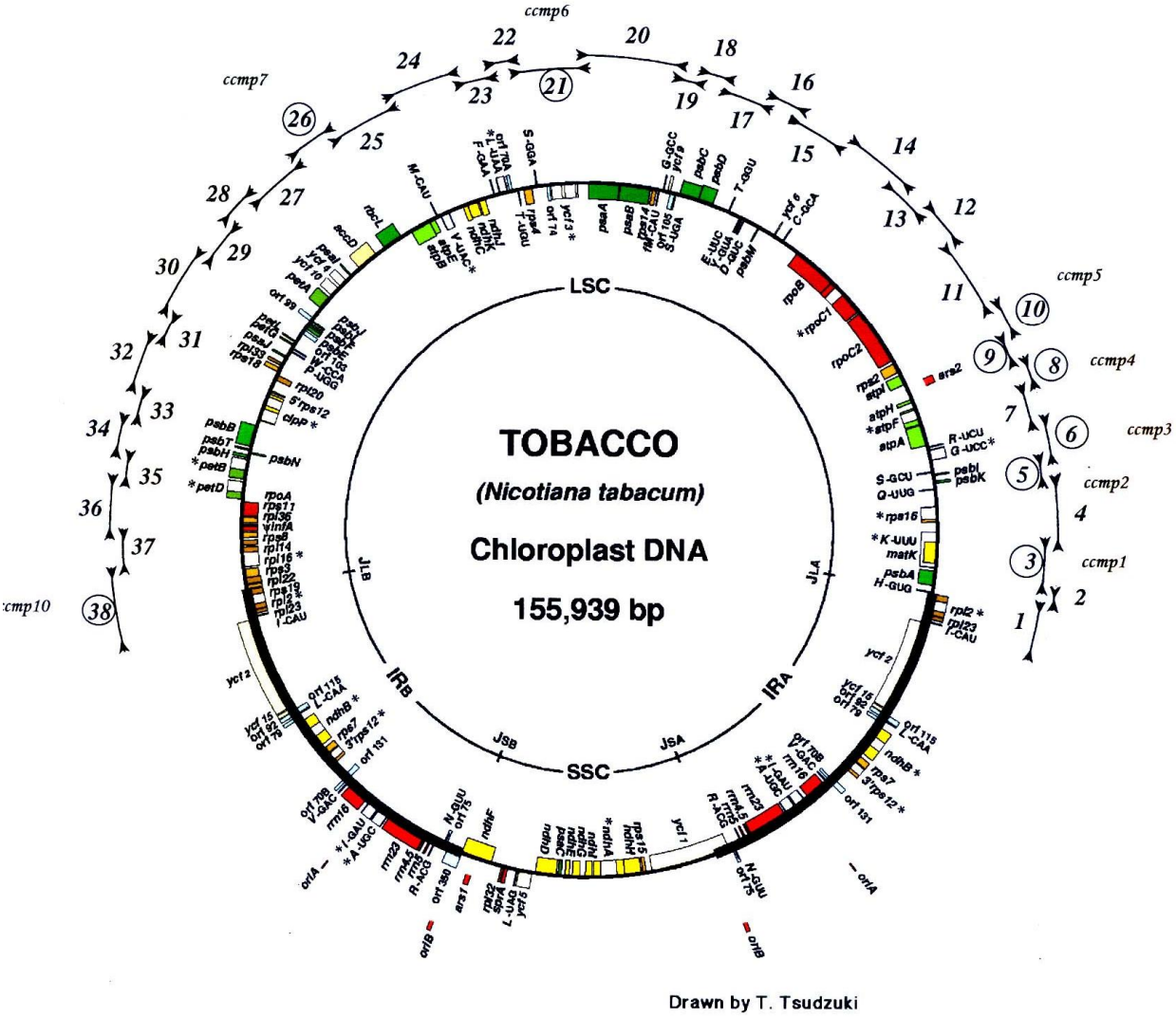

Figure 1 : Structure de l'ADN chloroplastique du tabac

I 3.- Justification et définition des objectifs

Malgré l'importance socio-économique et le statut de *P. butyracea* en Afrique de l'Ouest, les impacts de l'exploitation et de la fragmentation de l'habitat demeurent inconnus pour définir des stratégies de conservation dans le cadre d'une utilisation durable de l'espèce. Les paramètres de la biologie de la reproduction, de la diversité génétique et de l'histoire des populations d'une espèce sont des données biologiques préalables à la mise en place des stratégies efficientes de conservation des ressources génétiques. Les patrons de distribution spatiale de la diversité intra-spécifique sont en effet modelés par des paramètres intrinsèques à la biologie de l'espèce (mécanismes de reproduction, mode de dispersion du pollen et de la graine etc.) et par des facteurs stochastiques liés au hasard et anthropiques à l'origine du morcellement de l'habitat. Les études théoriques sur l'effet de cette fragmentation suggèrent que la dispersion restreinte de gènes découlant de l'autofécondation ou du croisement entre individus apparentés, réduit la taille effective de la population et induit la dépression de consanguinité au niveau des espèces allogames (Slatkin 1985, Alvarez-Buylla *et al.* 1996, Wang *et al.* 1999). En conséquence, la restriction de la dispersion de gènes peut devenir une menace à la viabilité des populations de ces plantes allogames. La variation du moment de floraison entre différentes saisons ou années, le synchronisme floral (ou chevauchement de stades de floraison entre arbres) et la durée de la floraison ainsi que les fluctuations d'effectif des pollinisateurs peuvent conduire à une variation de la distance de dispersion du pollen (Braga and Collevatti 2011).

Pour parvenir à la connaissance de ces paramètres et comprendre les processus dynamiques modelant la diversité intraspécifique de *P. butyracea*, trois objectifs principaux ont été définis.

(i) Etudier la phylogéographie de l'espèce.

(ii) Etudier la variabilité morphologique et génétique de l'espèce à l'échelle du Bénin.

(iii) Caractériser la biologie de reproduction de l'espèce.

Comme outil permettant d'atteindre chacun de ces objectifs, nous avons développé des marqueurs microsatellites nucléaires chez *P. butyracea* qui sont présentés au chapitre 2.

L'étude de la phylogéographie de *Pentadesma butyracea* à l'échelle de toute l'Afrique tropicale peut permettre de détecter l'origine des populations du Bénin, tenant compte de sa position azonale par rapport aux deux régions biogéograpgiques du domaine guinéo-congolais. Elle permet d'identifier les signes d'évènements passés de fragmentation et de recolonisation en rapport avec les crises climatiques de l'Holocène. Cette étude a été abordée

en utilisant des marqueurs moléculaires universels chloroplastiques et nucléaires, puis les marqueurs microsatellites nucléaires développés sur l'espèce. Les résultats obtenus ont fait l'objet du chapitre 3 de cette thèse.

L'étude de la variabilité morphologique et génétique vise à déterminer les traits morphologiques les plus discriminants et les indices de diversité génétique (richesse allélique, taux d'hétérozygotie) et de différentiation des populations susceptibles d'être sélectionnées dans le cadre de la conservation des ressources génétiques. La variabilité morphologique a été étudiée dans des populations naturelles, à travers la mesure de traits morphologiques quantitatifs et qualitatifs. Les résultats obtenus sont présentés dans le chapitre 4. Les résultats sur la variabilité génétique sont présentés au chapitre 5.

La biologie florale présentée au chapitre 6 a été caractérisée en milieu naturel au travers d'observations, de mesures des dimensions d'organes et produits floraux et d'essais en laboratoire (phénologie florale, organes floraux associés au régime de reproduction, pollinisateurs, teneur en eau des semences, effets de la conservation *ex situ*). Les paramètres de reproduction (notamment taux d'allogamie, corrélation de paternité) sont présentés dans le chapitre 7 et ont été estimés à partir des marqueurs microsatellites.

Enfin, l'ensemble des résultats est discuté dans le chapitre 8 pour en tirer une conclusion générale au chapitre 9.

I 4.- Matériel et méthode

I 4.1.- Modèle d'étude : *Pentadesma butyracea* Sabine

I 4.1.1- Taxonomie et distribution

Le genre *Pentadesma* appartient à l'embranchement des angiospermes, la classe des Dicotylédones, la sous-classe des Rosideae, l'ordre des Malpighiales, la famille des Clusiaceae, la sous-famille des Clusioideae, et la tribu des Symphonieae. Les séquences du gène chloroplastique codant *rbc*L suggèrent que la tribu des Symphonieae est paraphylétique (Gustafsson *et al.* 2002). Le nom de ce genre découle de la combinaison des mots grecs « penta » = cinq et « desma » = phalange ou faisceau (bundle en Anglais) en faisant allusion aux étamines groupées en cinq phalanges staminales. Il comprend quatre espèces, toutes d'origine africaine : (1) *P. butyracea* Sabine, très largement répandu dans le bloc guinéo-congolais ; (2) *P. grandifolia* E.G.Baker, spécifique du Cameroun et du Gabon ; (3) *P. lebrunii* Staner, connu en RDC et au Burundi ; et (4) *P. reyndersii* Spirlet, endémique du Rwanda. Cependant, selon van Meer (1965), *P. reyndersii* est synonyme de *P. grandifolia*. La figure 2 présente la carte de distribution de ces quatre espèces, réalisée à l'aide des coordonnées géographiques des échantillons extraits de la base de données *Brahms* à l'Herbier de Wageningen, aux notes de Bamps (1971) sur les Guttiferae d'Afrique tropicale, et de nos propres récoltes (674 individus provenant de 51 populations des sept pays Guinée, Côte d'Ivoire, Ghana, Bénin, Nigeria, Cameroun et Gabon).

Pentadesma butyracea est un fruitier appelé « Arbre à beurre » ou « Arbre à suif » en Français, « Butter tree » ou « Tallow tree » en Anglais. Ses noms locaux sont "Lami", "Krinda" ou "Tama", "Lorokiéré", "Ouotéra" et "Djréhé" (en Côte d'Ivoire), "Abotoasebie" (au Ghana), "Kpangnan, "Akoto" et "Sesseido" (au Bénin) et "Agnuhé" au Gabon.

Synonyme de *Pentadesma nigritiana* Baker f., *P. leucantha* A.Chev, et *P. kerstingii* Engl., *Pentadesma butyracea* Sabine est un arbre africain des forêts humides sempervirentes et de montagne (jusqu'à 700 m d'altitude) et des galeries forestières en milieu plus sec, sur terrain humide ou marécageux. La multiplicité des synonymes de ce taxon découlerait de la description de cette plante sur des collections restreintes à l'époque coloniale par divers auteurs (Anglais, Français, Allemands etc.) qui ont très peu communiqué entre eux. On le trouve également à l'état subspontané aux Seychelles où il aurait été introduit (Sinsin & Avocèvou 2007). Il prospère dans les zones à pluviométrie moyenne annuelle variant entre 1300 et 5000 mm d'eau avec une température moyenne annuelle de 25°C.

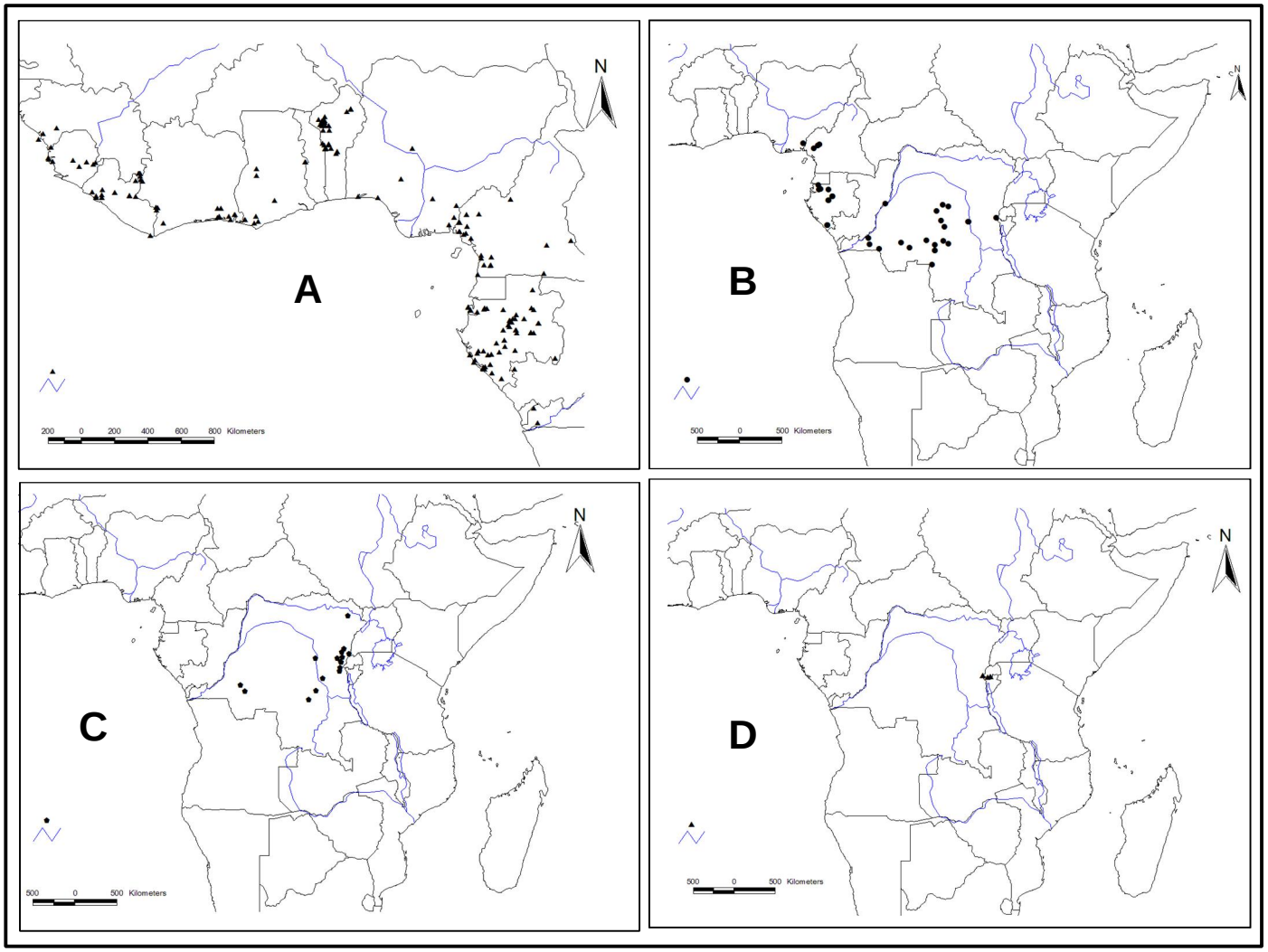

Figure 2 : Distribution des espèces du genre *Pentadesma* en Afrique tropicale (en bleu, les principaux cours d'eau)

A- *Pentadesma butyracea* ; B- *P. grandifolia* ; C- *P. lebrunii* ; D- *P. reyndersii* (source: Base de données *Brahms* à l'Herbier de Wageningen, Notes de Bamps (1971) sur les Guttiferae d'Afrique tropicale, et nos propres récoltes)

I 4.1.2- Biologie et écologie

L'arbre adulte mesure 10 à 35 m de hauteur et le tronc a un diamètre pouvant atteindre 130 cm à 1,30 m du sol. Son fût est cylindrique, droit, sans contrefort, avec ou sans racines échasses. Il présente un port caractéristique constitué de branches presque toujours horizontales, étendues perpendiculairement au tronc. L'écorce est de couleur rouille et fissurée longitudinalement et transversalement (Sinsin & Avocèvou 2007, Ouattara 1999, White et Abernethy 1996). Ses feuilles simples, entières, sans stipules et recouvertes de points translucides, oblongues, courtement et largement acuminées, cunéiformes à la base, coriaces et glabres sont opposées et mesurant 12 à 24 cm de long et de 4 à 7 cm de large, en touffes denses terminales. Une entaille dans le tronc laisse écouler un latex jaune (caractéristique des Clusiaceae) qui laisse une teinture indélébile sur les vêtements lorsqu'il sèche (White et Abernethy 1996, Aubreville 1959). La figure 3 et les photos 1 à 8 montrent différentes parties et produits de l'espèce.

L'inflorescence est terminale et comprend 1-7 grandes fleurs (6-7 cm de longueur), régulières, bisexuées, blanches et pentamères. Les fleurs fraîches tombées au sol libèrent aussi cette résine jaune. Les fleurs produisent une grande quantité de nectar. Les fruits sont des baies ellipsoïdes à ovoïdes, brun foncé, ressemblant à de grosses poires dont la base est entourée du calice, d'étamines et de glandes du disque persistants, l'apex est pointu. Ils peuvent atteindre 15 cm de longueur et 11 cm de largeur et contiennent 5 à 15 graines pyramidales, à côtés aplatis ou irrégulières, de 3-4 cm x 2,5-3 cm et noyées dans un mésocarpe épais et jaune imbibé de latex collant. Quand le fruit est encore vert, la pulpe est amère à cause de la grande quantité de tanins qu'elle contient, mais lorsqu'il est mûr, la pulpe est jaunâtre, sucrée et assez bonne à manger. Selon Ouedraogo (1999), un arbre adulte de 13 m de haut et 62 cm de diamètre produit en moyenne environ 500 fruits en un an avec un poids moyen du fruit de 0,59 kg contenant 0,12 kg de graines fraîches.

De nombreux aspects liés à la reproduction biologique de l'arbre à beurre tels que la floraison, la pollinisation, la compatibilité sexuelle ainsi que la fructification, sont très peu connus. Il existe un décalage spatio-temporel de floraison et fructification entre les populations de différentes régions biogéographiques, en relation avec les conditions climatiques. Les fleurs et les fruits précoces apparaissent respectivement en septembre et janvier au Bénin, mais elles se déroulent deux mois plus tôt dans les deux massifs de forêts denses humides de l'Afrique de l'Ouest (Côte d'Ivoire, Aubreville 1959) et de l'Afrique centrale (Gabon, White et Abernethy 1996). Les oiseaux souimangas et les petits primates visitent les fleurs pour se nourrir de nectar. A l'époque de la fructification, plusieurs espèces

de petits primates ainsi que les gorilles et les éléphants se nourrissent des fruits. Les fruits tombés à maturité se rompent facilement à la main en raison de leur tégument flexible et mou.

Pentadesma butyracea rejette bien de souches et a une régénération naturelle abondante dans les sites peu perturbés. Les jeunes plants semblent toutefois redouter le plein soleil et la régénération est plus abondante dans les secteurs ayant un léger ombrage (Houngbédji 1997). En dehors de l'Homme, seuls les éléphants consomment et dispersent les graines (White et Albernethy 1996). Les semences ont la particularité de porter plusieurs germes répartis sur l'ensemble de la surface de contact. Leur grosseur et le nombre élevé de germes pourraient être des facteurs favorables à une longue conservation en conditions naturelles. La germination est hypogée, l'épicotyle est rougeâtre avec des préfeuilles opposées.

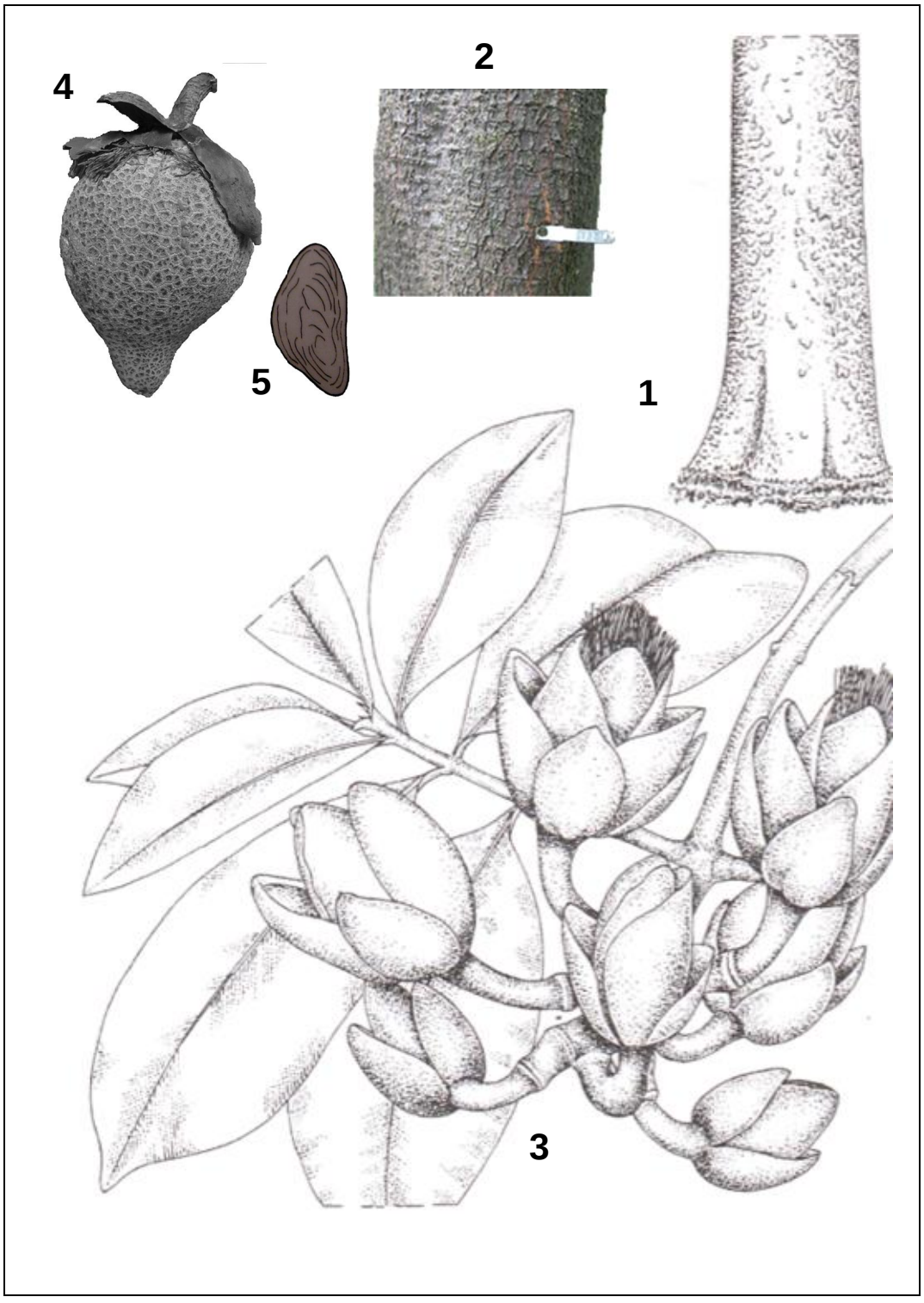

Figure 3 : Différentes parties de *Pentadesma butyracea*
(1, base du fût ; 2, écorce ; 3, rameau feuillé en fleurs ; 4, fruit ; 5, graine).

Photo 1 : Arbre adulte avec ses branches horizontales perpendiculaires à l'axe du tronc.

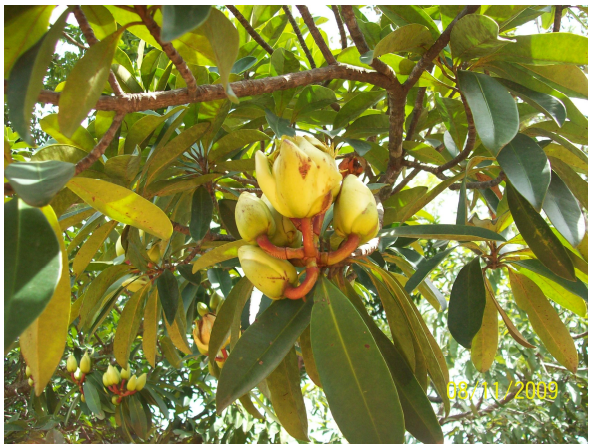

Photo 2 : Inflorescence montrant une fleur âgée et des boutons floraux

Photo 3 : Fruits non mûrs montrant un style et un calice persistants, leur surface est rugueuse et verruqueuse.

Photo 4 : Amandes fraîches.

Photo 5 : Plantules en pépinière.

Photo 6 : Amandes sèches débarassées de leur tégument pour être transformées en beurre.

Photo 7 : Beurre fabriqué de façon traditionnelle.

Photo 7 : Beurre fabriqué et commercialisé industriellement.

Photos montrant les différentes parties et produits de *Pentadesma butyracea* (Clusiaceae)

I 4.1.3- Usages

Pentadesma butyracea présente de multiples fonctions et constitue une source inestimable de biens et de services pour les communautés locales en Afrique de l'Ouest (Natta *et al.* 2010). Il est principalement reconnu pour la qualité de son bois et de ses graines (Avocèvou-Ayisso *et al.* 2011, Natta *et al.* 2010, Tchobo *et al.* 2007, Natta 2003, Sinsin & Sinadouwirou 2003). Diverses études scientifiques ont été consacrées à cette espèce concernant les aspects botaniques, socio-économiques, et les propriétés chimiques et biochimiques du beurre. Ces études sont discutées ci-dessous.

Les investigations menées sur le rabotage, la déformation, le sondage, la mortaison, le tournage et le ponçage ont montré que son bois possède de très bonnes propriétés mécaniques, semblables à ceux du caïlcédrat (*Khaya senegalensis*) et de l'iroko (*Milicia excelsa*) (Rachman *et al.* 1987).

Les graines servent à fabriquer un beurre alimentaire jaunâtre utilisé principalement comme matière grasse de préparations culinaires que les femmes substituent au beurre de karité car ce dernier connaît une baisse de productivité à cause d'une forte infection actuelle par des hémiparasites de la famille des Loranthacées (*Tapinanthus* spp) (Dah-Dovonon 2002). Contrairement au karité dont le beurre dégage une odeur désagréable, celui de *P. butyracea* présente un goût assimilé à celui de l'huile d'arachide. Les consommateurs de ce beurre sont surtout des personnes âgées. L'analyse socio-économique des activités de ramassage, de transformation et de commercialisation des produits de l'espèce a montré que les femmes sont les principales actrices du circuit de commercialisation et que le ramassage est plus profitable financièrement que la transformation en beurre. Les femmes qui ramassent les graines récupèrent 49 à 80% des prix payés par les consommateurs, en fonction de la longueur de chaîne de commercialisation et de la qualité du produit (Avocèvou-Ayisso *et al.* 2009). Selon ces auteurs, l'impact du ramassage est si important qu'il affecte significativement la régénération dans les habitats perturbés par le ramassage par rapport à ceux moins fréquentés (4200 ± 3810 vs. 13872 ± 7886 de semis et jeunes tiges à l'hectare, p-value < 0.001).

Les semences présentent une forte concentration en acides gras constitués de près de 96% d'acide stéarique et d'acide oléique, et une forte proportion de stigmastérol reconnu pour son faible taux de cholestérol (Tchobo *et al.* 2007). Selon ces auteurs, l'indice de saponification du beurre est NaOH : 191 ; KOH : 136 et sa température de fusion est 38-40°C. La composition en phytostérol varie très largement entre les échantillons de beurre du

Dahomey Gap et ceux des forêts denses de l'Afrique de l'Ouest (3% vs. 22% de Δ^7 stérol respectivement, Dencausse *et al.* 1995, Tchobo *et al.* 2007).

Ce beurre est aussi utilisé en médecine traditionnelle comme ingrédients dans le traitement d'affections comme la toux des enfants, les maux de côtes, etc. Il est également utilisé dans la fabrication du savon, le massage et la cosmétique (Sinsin & Sinadouwirou, 2003).

Le décocté des racines est utilisé dans la lutte contre les vers intestinaux et celui de l'écorce du tronc pour le traitement de la diarrhée et des hémorroïdes (Abbiw 1990 ; Akoegninou *et al.* 2006).

I 4.2- Cadre biogéographique

La biogéographie est l'étude de la distribution actuelle et passée des êtres vivants dans la biosphère, de leur adaptation aux influences locales dans le temps et dans l'espace, de leurs migrations et des associations qu'ils constituent (Dansereau 1987). Les forêts tropicales forment une large ceinture dense sempervirente autour de l'équateur entre 10°N et 10°S. Elles ont trois localisations disjointes (Amazonie, Bassin africain et Est de Madagascar, la région indo-malaise et l'Australie). Ces formations végétales incluent des forêts de montagnes et s'étendent du niveau de la mer jusqu'à une altitude de 4000 m (Corlett & Primack 2011). Mais de nos jours près du tiers de cette aire est détruite par les activités anthropiques.

Toutes les forêts tropicales humides ont une origine commune qui remonte à 150 millions d'années lorsque tous les continents étaient connectés dans le sud du Gondwana (Morley 2007). Cette interconnexion a favorisé la migration des espèces entre les aires continentales actuelles au Jurassique. Plus tard, au Crétacé, il y a 90 millions d'années, une séparation est intervenue entre l'Amérique du sud, l'Afrique, l'Inde et l'Australie. La séparation entre l'Afrique et Madagascar est intervenue au milieu de l'Eocène, il y a 50 millions d'années.

D'après des études palynologiques, *Pentadesma* aurait émergé à l'Eocène au Nigeria (Jan du Chêne *et al.* 1978) et à l'Oligocène-Miocène au Cameroun (Salard-Cheboldaerff 1975). Les forêts africaines ont connu des phases d'expansion et de contraction sous l'effet des changements climatiques à l'échelle géologique (Morley 2007). Au cours des deux derniers millions d'années, le climat était sec et le niveau de dioxyde de carbone atmosphérique était faible durant les périodes glaciaires. Ces changements ont considérablement réduit l'étendue des forêts tropicales (surtout en Afrique et en Australie) altérant aussi leur composition floristique. Beaucoup d'espèces (comme *Pentadesma butyracea*) ont trouvé

refuge dans les formations forestières restantes et dans les forêts galeries le long des rivières (Morley 2000, Corlett & Primack 2011). Les forêts denses humides africaines contemporaines doivent donc être perçues comme des reliquats de forêts plus humides et plus étendues sur le continent il y a 30 millions d'années (Plana 2004).

De nos jours, *P. butyracea* est largement réparti dans tout le domaine guinéo-congolais dans trois régions phytogéographiques discontinues (White 1979 ; cf. Fig. 4) : le Haut Guinéen incluant les forêts de l'Afrique de l'Ouest s'étendant du sud de la Guinée à l'Est du Ghana, le Bas Guinéen englobant l'Afrique centrale depuis le Nigéria jusqu'au Congo. L'espèce est cependant absente et remplacée par un taxon proche (*P. grandifolia*) dans le domaine congolais, principalement dans le bassin de la Rivière Congo. *P. butyracea* est également distribué dans le Dahomey Gap (Bénin, Togo, Est du Ghana), en zone plus sèche.

Selon Maley (1996), l'aire de distribution actuelle des espèces tropicales humides d'Afrique, parmi lesquelles figure *Pentadesma butyracea*, découle d'une expansion à partir de populations très réduites ayant survécu au dernier Maximum Glaciaire à la fin du Pléistocène. Dans ce contexte, *Pentadesma butyracea* aurait dû trouver des habitats avec une humidité favorable à son maintien lors des longues périodes sèches passées, ces habitats étant le plus souvent confinés aux zones montagnardes. La réduction drastique et la fragmentation des populations végétales à cette époque auraient été suivies d'une perte de diversité génétique. Ensuite, des différences génétiques se seraient accumulées entre les différentes populations. La fragmentation des populations se poursuit de nos jours et combinée aux dégâts causés aux arbres par les feux de végétation a pour effet que les effectifs des populations de *Pentadesma butyracea* se réduisent.

Contexte géographique du Bénin

Les précipitations annuelles varient dans tout le pays entre 900 et 1400 mm, avec près de sept mois de saison sèche incluant un à trois mois d'harmattan. Les populations de *Pentadesma butyracea* sont situées dans le domaine soudanien (White 1983) où la pluviométrie est inférieure à 1100 mm d'eau par an, sauf à l'extrême ouest autour de Djougou (09°45' N 01°38' E) où elle atteint 1380 mm (cf. Fig. 4). Du point de vue géomorphologique, deux formations géologiques bien distinctes auxquelles sont liées des formes particulières de reliefs se partagent le territoire béninois, à dominance cristalline au nord et sédimentaire au sud. Les formations cristallines représentent le bouclier africain sur lequel repose en discordance le bassin sédimentaire côtier dont la limite septentrionale se situe à environ 7° 30' de latitude

Nord. Les formations cristallines (au niveau desquelles se situe l'habitat de *P. butyracea*, cf. Fig. 4) sont constituées de roches éruptives et de roches métamorphiques plissées antécambriennes (Slansky 1962, Anonyme 1989). On y distingue le Dahoméyen occupant une bonne partie du domaine soudanien et formé de micaschistes, quartzites, gneiss variés et migmatites complexes ; l'Atacorien du côté Ouest et formé de schistes et de quartzites très durs qui ont donné naissance aux seuls reliefs élevés du pays, les massifs de l'Atacora, d'altitude néanmoins modeste (600 m). A l'extrême Est se trouvent les formations sédimentaires de Kandi, constituées d'un ensemble de grès argileux datant du Crétacé. La zone des formations cristallines est drainée par plusieurs cours d'eau, dont les plus importants sont la Sota, l'Alibori, la Mékrou qui sont des affluents du fleuve Niger et la Pendjari qui contourne les chaînons de l'Atacora pour se jeter dans la Volta.

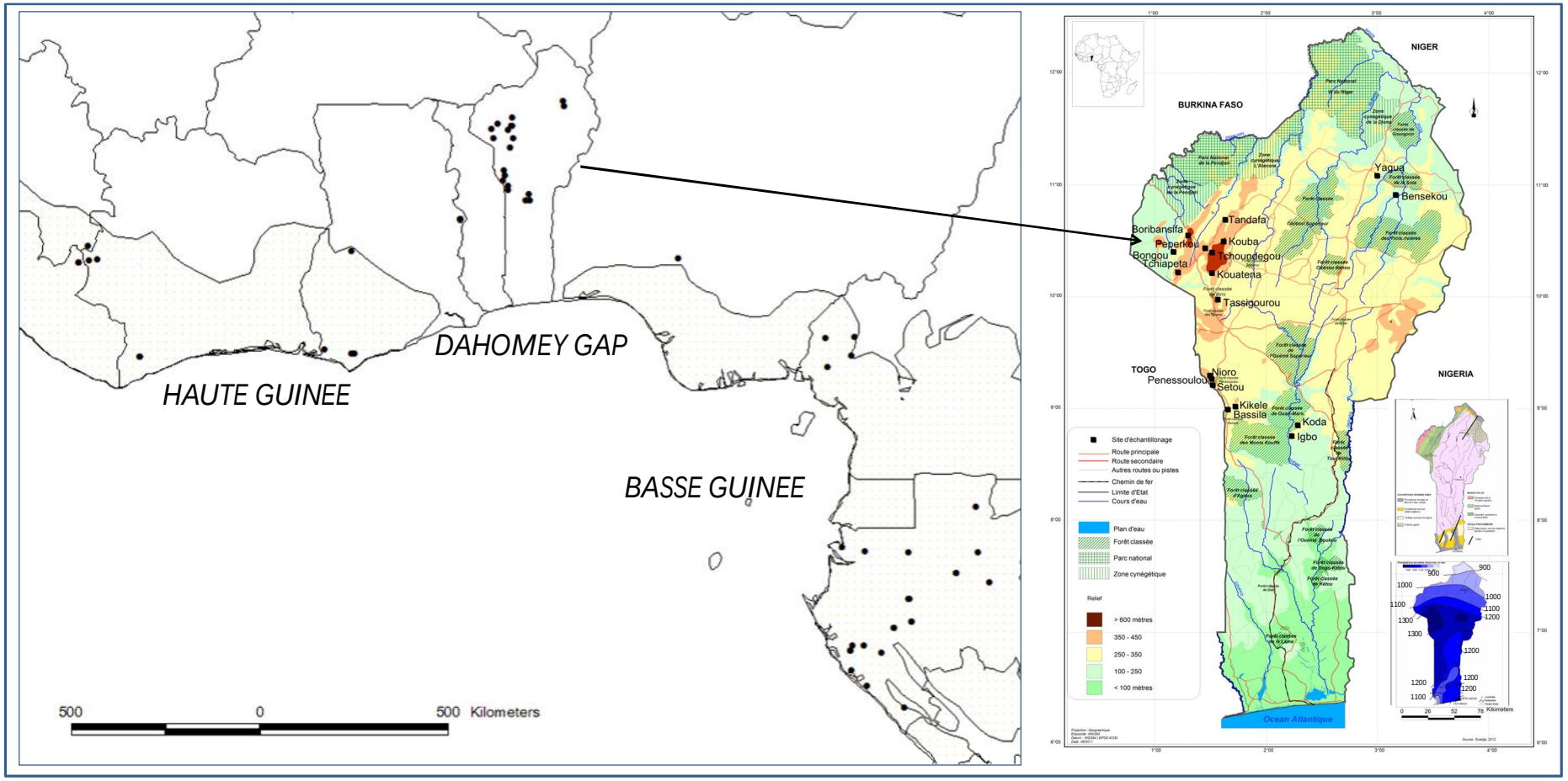

Figure 4 : Carte de répartition des populations étudiées dans le domaine guinéo-congolais (à gauche), avec en détail à droite les caractéristiques physico-climatiques et géomorphologiques du Bénin. (Source : données de terrain)

I 4.3- Organisation des travaux de thèse

Les travaux ont été conduits conformément à la formule d'un Doctorat mixte comprenant par an, un séjour local (pour la collecte des données de terrain, les expérimentations et certaines analyses de laboratoire) et un séjour belge (pour les analyses génétiques). Pour atteindre les trois objectifs de la thèse, diverses activités de terrain et de laboratoire puis de collecte d'échantillons de feuilles et/ou cambium ont été exécutées au Bénin de 2007 à 2011. Durant la même période, en Belgique, l'ADN a été extrait de tous les échantillons de feuilles et/ou cambium collectés dans toute l'aire de distribution de l'espèce en Afrique. Les analyses de génétique des populations ont été réalisées au Service Evolution Biologique et Ecologie (EBE) de l'Université Libre de Bruxelles.

Objectif 1 : Etude de la phylogéographie de l'espèce au service EBE. En complément aux marqueurs universels chloroplastiques et nucléaires utilisés, cette étude a nécessité l'isolement et la caractérisation des marqueurs microsatellites nucléaires de l'espèce depuis 2007 lors de notre formation doctorale. Ce n'est qu'en 2010 que nous avons réussi à isoler des microsatellites après deux essais infructueux avec des méthodes classiques (Hamilton *et al.* 1999). Le chapitre 2 présente les résultats de ce développement de marqueurs microsatellites.
La phylogéographie proprement dite a été étudiée à grande échelle au fur et à mesure que les échantillons de *P. butyracea* ont été collectés au Bénin ou dans le reste de son aire de distribution soit environ dans 73% des pays où l'espèce est présente (Fig. 3), entre les années 2007 et 2011. Notre échantillonnage à large échelle comprend des individus provenant de toute l'aire de distribution de *Pentadesma butyracea* dans les deux blocs forestiers du domaine guinéo-congolais. Pour ce faire, de nombreuses missions de terrain de l'équipe EBE ont été effectuées dans le cadre de plusieurs projets de recherche depuis 2006 au sein du bloc forestier du bassin du Congo et dans les forêts humides fragmentées de l'Afrique de l'Ouest étendues, depuis la Sierra Leone jusqu'au Ghana. Il s'agit de forêts denses humides sempervirentes physionomiquement similaires, mais de compositions spécifiques relativement différentes (White 1983). Les forêts les plus riches en espèces étant localisées dans les zones les plus arrosées et dans les forêts de montagnes (Ouest du Cameroun et du Gabon, Ouest de Côte d'Ivoire et Libéria, (Linder 2001)). Le total pluviométrique annuel varie entre 1600 et 3000 mm de pluie avec 3 mois de saisons sèches, le degré hygrométrique atmosphérique est élevé, dans ces deux blocs. Un total de 67 arbres (dont 56 génotypés) provenant de 33 populations a été échantillonné au sein des deux blocs forestiers, soit par le service EBE soit par des équipes qui collaborent avec ledit service dans le cadre de divers programmes de

recherche.. Au Bénin où nous avons effectué l'échantillonnage, l'effectif total d'arbres étudiés est 607 (dont 402 génotypés) provenant de 18 populations situées dans des galeries forestières dans la zone soudanienne où l'eau ne tarit pas en saison sèche. En général, les populations échantillonnées sont séparées par 15 à 200 km au sein de chaque pays. Chaque site de présence de *Pentadesma butyracea* est considéré comme une population. Toutefois au Bénin, deux populations (sites) représentées par 3 individus adultes chacune (Kouatena et Gbesse) ont été confondues, chacune, à une autre population plus proche et plus large. L'identification des sites a été faite sur la base d'un mémoire d'ingénieur (Sogbégnon 2008) mais aussi en présentant une photo de l'arbre, des fruits et amandes aux transhumants peuhls et populations locales (qui connaissent et utilisent les ressources de la plante) rencontrés progressivement lors de nos prospections. La figure 3 indique les populations étudiées ; leurs caractéristiques et l'effectif d'arbres utilisés pour chaque type d'analyse sont présentés dans le Tableau 1. Les résultats de la phylogéographie de *P. butyracea* sont indiqués au chapitre 3.

Objectif 2 : Etude de la variabilité morphologique et génétique à l'échelle du Bénin

La variabilité morphologique a été estimée en 2010 à partir de 14 descripteurs quantitatifs et 13 caractères qualitatifs mesurés sur des arbres, fruits et graines provenant de 12 populations. Les résultats sont présentés au chapitre 4.

Les marqueurs microsatellites ont été utilisés au cours des années 2010-2011 pour estimer la diversité et la différentiation génétique puis analyser la structure génétique de 16 populations échantillonnées dans toute l'aire de distribution de l'espèce au Bénin. Les résultats obtenus ont fait l'objet du chapitre 5.

Objectif 3 : Caractérisation de la biologie de reproduction de l'espèce

Dans un premier temps, la biologie de reproduction a été étudiée *in situ* au Bénin où nous avons étudié la biologie florale en recherchant les structures florales liées à la pollinisation, suivi le profil phénologique de la floraison et de la fructification au cours de deux années successives (2009 et 2010), recherché le ratio entre les nombres de grains de pollen et d'ovules produits par fleur, identifié les pollinisateurs, déterminé la teneur en eau des semences et réalisé des essais de germination et de stockage des graines. Le chapitre 6 présente les résultats obtenus. Dans un deuxième temps, nous avons estimé le taux d'allogamie et la corrélation de paternité à partir de familles de graines dans quatre populations dont la structure génétique à fine échelle a été également étudiée à partir des

marqueurs microsatellites. Les résultats obtenus sont indiqués au chapitre 7 du présent document.

Tous les résultats présentés dans les chapitres 2, 3, 4, 5, 6 et 7 de cette thèse sont rédigés sous forme d'articles en anglais dont deux publiés (Genetic Resources and Crop Evolution et Molecular Ecology Resources) mais chaque texte est précédé d'un résumé en français.

Tableau 1 : Caractéristiques des populations échantillonnées. N_T, effectif total d'arbres ; N_{PHY}, effectif d'arbres étudiés en phylogéographie ; N_{SSR}, effectif d'arbres et de plantules utilisés pour l'isolement et le développement des microsatellites nucléaires ; N_{MORP}, effectif d'arbres mesurés pour la variabilité morphologique ; $N_{DIV,}$ effectif d'arbres étudiés pour la diversité génétique au Bénin ; N_{REP}, effectif d'arbres étudiés pour la biologie de reproduction ; N_{ALL}, effectif d'arbres échantillonnés pour estimer la consanguinité, le taux d'allogamie et la corrélation de paternité ; ^{gr} nombre de familles de graines.

Population	N_T	N_{PHY}	N_{SSR}	N_{MORP}	N_{DIV}	N_{REP}	N_{ALL}^{gr}	Pays	Domaine	Longitude	Latitude
Diecke	5	5	-	-	-	-	-	Guinea	1	-8.28	7.54
Ziama	4	3	-	-	-	-	-	Guinea	1	-8.29	7.68
Dodo	4	4	-	-	-	-	-	Ivory Coast	1	-7.04	5.04
Mont Nimba	3	1	-	-	-	-	-	Ivory Coast	1	-8.26	7.34
Ankassa	1	1	-	-	-	-	-	Ghana	1	-2.65	5.21
Nueng South	1	1	-	-	-	-	-	Ghana	1	-1.93	5.11
Techiman	1	1	-	-	-	-	-	Ghana	2	-2.02	7.56
Sabu R valley	2	2	-	-	-	-	-	Ghana	2	0.57	8.29
Bassila	86	2	-	45	11	-	-	Benin	2	1.65	8.99
Bensekou	22	2	-	9	17	-	-	Benin	2	2.80	10.94
Bongou	19	2	-	12	9	-	-	Benin	2	1.62	10.40
Boribansifa	9	2	-	7	8	-	-	Benin	2	1.29	10.54
Igbo Aladja	90	4	6	29	85	26	85^{22}	Benin	2	2.22	8.74
Kikele	18	2	-	-	9	-	-	Benin	2	1.95	9.01
Koda	18	2	-	-	17	-	-	Benin	2	2.28	8.84
Kouatena	3	1	-	-	3	-	-	Benin	2	1.82	10.21
Kouba	91	2	-	20	64	-	64^{17}	Benin	2	1.61	10.49
Nioro	11	2	-	10	11	-	11^{8}	Benin	2	1.49	9.29
Penessoulou	27	3	-	-	10	20	-	Benin	2	1.82	9.26
Peperkou	37	4	-	27		31	-	Benin	2	1.45	10.43
Gbesse	3	-	-	-		-	-	Benin	2	3.15	10.94
Setou	144	2	-	115	103	-	103^{19}	Benin	2	1.81	9.20
Tandafa	43	3	-	34	15	-	-	Benin	2	1.63	10.68

Tassigourou	19	2	-	-	14	-	-	Benin	2	1.85	9.97
Tchiapeta	12	3	-	6	8	-	-	Benin	2	1.64	10.22
Tchoundegou	15	2	-	-	12	-	-	Benin	2	1.51	9.97
Yagua	41	2	-	34	11	-	-	Benin	2	3.00	11.08
Oka	1	1	-	-	-	-	-	Nigeria	3	5.72	7.37
Korup NP	2	2	-	-	-	-	-	Cameroun	3	8.86	5.07
Korup Plot	3	3	-	-	-	-	-	Cameroun	3	8.84	5.06
Park of Korup	1	1	-	-	-	-	-	Cameroun	3	8.87	5.05
Mamfe	1	1	-	-	-	-	-	Cameroun	3	9.24	5.48
Lokando	1	1	-	-	-	-	-	Cameroun	3	9.28	4.83
Fontem	2	2	-	-	-	-	-	Cameroun	3	9.93	5.50
Moukalaba Park	1	1	-	-	-	-	-	Gabon	3	10.57	-1.99
Minkebe Park	1	1	-	-	-	-	-	Gabon	3	12.80	1.49
Lopé	1	1	-	-	-	-	-	Gabon	3	11.57	-0.17
Milolé	2	2	-	-	-	-	-	Gabon	3	13.12	-0.31
Sogademin	1	1	-	-	-	-	-	Gabon	3	10.18	0.44
Waka Park	1	1	-	-	-	-	-	Gabon	3	11.29	-1.23
NKomi	1	1	-	-	-	-	-	Gabon	3	9.89	-1.80
Iguela	1	1	-	-	-	-	-	Gabon	3	9.83	-1.93
Waka	1	1	-	-	-	-	-	Gabon	3	10.86	-1.38
Haut-Abanga	1	1	-	-	-	-	-	Gabon	3	11.22	0.41
Mandji	1	1	-	-	-	-	-	Gabon	3	10.16	-1.80
CFAD of Rimbunan	2	2	-	-	-	-	-	Gabon	3	11.22	-0.69
Ivindo	1	1	-	-	-	-	-	Gabon	3	12.35	-0.07
Tchibanga	1	1	-	-	-	-	-	Gabon	3	11.12	-3.28
Cap Esterias	1	1	-	-	-	-	-	Gabon	3	9.33	0.54
NR848	1	1	-	-	-	-	-	Gabon	3	11.29	-1.23
Gamba	5	5	-	-	-	-	-	Gabon	3	10.22	-2.76
Luango	1	1	-	-	-	-	-	Gabon	3	9.64	-2.40

1, Haute Guinée ; 2, Dahomey-Gap ; 3, Basse Guinée

CHAPITRE II.-

Développement des loci microsatellites de *Pentadesma butyracea* (Clusiaceae) en utilisant la technologie reposant sur le pyroséquençage de fragments enrichis

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Développement des loci microsatellites de *Pentadesma butyracea* (Clusiaceae) en utilisant la technologie reposant sur le pyroséquençage de fragments enrichis

L'acquisition de marqueurs microsatellites s'est avérée nécessaire comme outil pour atteindre les trois objectifs de notre thèse (étude de la phylogéographie de l'espèce, étude de la variabilité morphologique et génétique de l'espèce à l'échelle du Bénin et caractérisation de la biologie de reproduction de l'espèce). Dans un premier temps, nous avons vérifié le statut de ploïdie de l'espèce en déterminant la taille du génome nucléaire que nous avons comparée avec celle du génome d'une espèce diploïde proche dans la tribu des Symphonieae (*Symphonia globulifera*) et en comptant le nombre de chromosomes. Le pommier nain (*Malus domestica* Mill. Rosaceae, 2n= 34, 770 Mpb) a été utilisé comme référence pour déterminer la taille du génome nucléaire. Dans un deuxième temps, nous avons isolé et caractérisé les marqueurs microsatellites de *Pentadesma butyracea* à partir de six extraits d'ADN provenant du site Igbo Aladja au Bénin.

L'isolement des locus a été effectué à partir d'un assemblage de fragments enrichis en microsatellites, les huit motifs utilisés étant $(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$. Ces fragments ont été séquencés sur une plateforme 454GS-FLX.

Pentadesma butyracea est vraissemblablement diploïde (2n =56); cependant la présence de plus de deux allèles à certains locus et la plus grande taille du génome (200 Mpb de plus) que celle de *Symphonia globulifera* suggère la présence de duplication de gènes à certains locus ou un ancien évènement de polyploidisation suivi d'une diploidisation de la majorité du génome. Onze microsatellites ont été isolés et neuf locus ont été amplifiés avec succès en deux multiplex de réactions PCR. Tous les locus sont polymorphes, montrant trois à neuf allèles par locus soit un nombre moyen de 4,7 allèles par locus. Huit locus de ces multiplex ont été aussi amplifiés avec succès et se sont avérés polymorphes chez deux espèces apparentées (*P. grandifolia* and *P. reyndersii*). Ces marqueurs seront utilisés pour l'étude de la diversité génétique de *P. butyracea* (chapitres 3 et 5), ainsi que pour étudier la dépression de consanguinité et les paramètres de son système de reproduction (chapitre 7).

Development of microsatellite loci in *Pentadesma butyracea* (Clusiaceae) using SSR-enriched pyrosequencing based-technology

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Abstract

Microsatellite markers were developed in *Pentadesma butyracea* (butter tree) using a combination of multiplex microsatellite enrichment and next-generation sequencing on a 454 GS-FLX Titanium platform. Eleven microsatellites were successfully isolated and nine could be amplified in two PCR multiplexed reactions. All loci were polymorphic, displaying three to eight alleles per locus and a mean value of 4.7 alleles per locus. In two congeneric species (*P. grandifolia* and *P. reyndersii*), PCR multiplexed reactions also successfully amplified eight loci that generally displayed polymorphism. These markers provide useful tools to conduct mating system analyses, and to assess the genetic structure of *P. butyracea* and congeneric species.

Keywords: Butter tree, Clusiaceae; microsatellites; next-generation sequencing; *Pentadesma butyracea*.

Introduction

Pentadesma butyracea Sabine (Clusiaceae) is a long-living African rainforest tree species occurring from Sierra Leone to Gabon (Bamps 1971). Three congeneric species also occur in Central Africa: *P. grandifolia* E.G. Baker, *P. lebrunii* Staner, and *P. reyndersii* Spirlet (Bamps 1971). In West Africa, the seeds of *P. butyracea* are exploited to make the "kanya" butter, which is widely used in cosmetics, food, and medicinal preparations, so that the species is considered as vulnerable, and included among the ten most important food tree species for conservation in Benin and Togo (Eyog-Matig *et al.* 2002).

Mangenot and Mangenot (1958) describe *P. butyracea* as a polyploid species. Then in a first time, we checked the ploidy status of the species by assessing its nuclear genome size using flow cytometry and by counting its chromosomes (karyotype). This allowed us to better understand the nuclear microsatellite patterns observed.

Concerning microsatellite isolation, ancient classical methods (Hamilton *et al.* 1999) were inefficient for the isolation of microsatellite primers in *P. butyracea*. In this study, we describe how the use of next generation sequencing technologies allowed us to develop 11 polymorphic microsatellite loci. These markers were used to assess the level of genetic variation in a population of *P. butyracea* in Benin and a group of samples collected from West African rainforests. Their transferability to the related species *P. grandifolia* and *P. reyndersii* was also tested.

Methods

Nuclear genome size and karyotype of *Pentadesma butyracea*

Flow cytometry

Flow cytometry allows a fast determination of nuclear DNA content. This analysis is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence intensity emitted by stained cell nuclei. To release cell nuclei, approximately 50 mg of *P. butyracea* young leaf tissue was chopped with a scalpel in a Petri dish containing a buffer from PARTEC CyStain UV Ploidy – 05-5001. The nuclei suspension, stained with the fluorochrome DAPI, was then analysed in a PARTEC PA equipment, quantifying the relative amount of DNA per nuclei. To determine nuclear DNA content in absolute units, fluorescence intensity of nuclei is compared with the fluorescence intensity of nuclei isolated from the common apple species *Malus domestica* (pommier nain) considered here as a standard (its nuclear genome size is ~770 Mbp on average, Arumuganathan and Earle 1991) and from

Symphonia globulifera, a diploid species phylogenetically close to *P. butyracea*. To this end, approximately 50 mg of young leaf tissue of *Malus domestica* and *Symphonia globulifera* were simultaneously chopped. The genome size of *S. globulifera* or *P. butyracea* was obtained by multiplying the genome size of *M. domestica* by the ratio of their fluorescence intensities.

Pre-treatment, fixation and chromosome preparation

Excised root tips were washed with tap water a few times, treated in 8-hydroxyquinolene (0.002M OQ aqueous solution) at 21°C for 3 hours to accumulate metaphases, fixed in absolute ethanol:glacial acetic acid (3:1) at 21°C for 3 hours and stored in absolute ethanol at -20°C until preparation. Enzyme-treated root tips described by Schwarzacher *et al.* (1980) were squashed on slides in a drop of 45% propionic acid with 2% carmine and covered with a coverslip. Photographs of metaphase chromosomes were taken on a Zeiss Axiophot microscope using a computer-assisted cooled CCD camera (Zeiss Axiocam HRC) employing Zeiss Axiovision software.

DNA extraction and microsatellite enriched library by the way of pyrosequencing technology

Total DNA was extracted from silica gel dried or fresh leaf using NucleoSpin Plant kits (Macherey-Nagel, Düren, Germany). A mixture of ca. 5 µg of DNA including six individuals sampled from the population Igbo Aladja in Benin (8.75 N, 2.23 E) was sent to Genoscreen (Lille, France) to isolate microsatellite loci following the protocol of Malausa *et al.* (2011) that associates multiplex microsatellite enrichment and pyrosequencing. Briefly, enriched libraries were constructed using the following eight motifs: $(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$. They were then sequenced on a 454 GS-FLX Titanium platform (see Malausa et al. 2011 for additional details). The sequencing run generated 22,338 reads which were analyzed using the bioinformatic program QDD (Meglécz et al. 2010): sequences <80bp or containing microsatellites with <5 repeats were discarded, as well as pairs of sequences with significant reciprocal BLAST hits but with flanking region identity levels below 90%. Sequences displaying reciprocal BLAST hits for which pairwise similarity between the complete overlapping part of the flanking regions was greater than 90% were grouped into contigs. Primer pairs were designed automatically by Primer3 (Rozen and Skaletsky 2000) within QDD with the criteria described in Malausa *et al.* (2011). Overall, 405 loci were identified by QDD as potentially appropriate to design primers for microsatellite markers (sequences available as DRYAD provisional repository DOI:10.5061/dryad.q0520).

From a total of 3,388 primer pairs generated for the 405 loci, we selected 24 primer pairs among loci presenting the longest di-, tri, and tetranucleotide repeats, and having more than 10 flanking nucleotides between microsatellite motifs and designed primers. To reduce the cost of dye-labeled primers, we opted for a M13-like labeling protocol, using four universal sequences as described in Micheneau *et al.* (2011) (named Q1-Q4 in Table 1). Briefly, the forward sequence of each selected locus was redesigned by incorporating to the 5′ end one of the four universal sequences (Table 1), after having checked that these newly designed sequences were suitable for primer mix and did not present any hairpin structures or possible primer-dimers using the site of finnzymes http://www.finnzymes.com/java_applets/multiple_primer_analyzer.html. To verify amplification in *P. butyracea*, the 24 designed primer pairs were first tested separately on 4 individuals from Benin with the following PCR conditions in a volume of 10 µL: 0.25 U Taq polymerase (TopTaq DNA Polymerase, QIAGEN, Venlo, Netherlands) and the associated buffer following the manufacturer recommendation, 1mM $MgCl_2$, 0.3 mM of each dNPT, 0.2 µM of each primer, 1 to 5 ng of template DNA. Amplifications were performed as follows: 94° C (4 min), followed by 40 cycles of 94°C (30 s), 56°C (45 s), 72°C (1 min), and a final extension at 72°C for 10 min.

In total, seven primer pairs failed to amplify, while the 17 remaining were used to quantify levels of polymorphism on eight individuals chosen across the species distributional range (i.e. Benin, Ivory Coast, Cameroon and Gabon). PCRs were conducted individually using the modified M13 protocol of Schuelke (2000), which incorporates three primers: (1) an unlabeled reverse primer, (2) a tailed forward primer (with one of the four unique sequence added to the 5′ end, Table 1), and (3) a third primer composed of this same unique universal sequence but with a fluorescent dye attached to the 5′ end (Table 1). PCR conditions were identical to those mentioned before, but the tailed forward primer was used at one-third the amount of the reverse and fluorescently labeled primers, and the last 10 cycles of the PCR were done at an annealing temperature of 53°C.

Of the 17 loci tested, four were monomorphic and two suffered weak amplification. The remaining 11 loci were used for preliminary genetic analyses a population comprising 30 individuals from Benin (Igbo Aladja, 8°44′59.712″N, 2°13′44.909″E), and on a group including 18 individuals originating from three countries of West Africa rainforests (Guinea, eight individuals; Ivory Coast, five individuals and Ghana, five individuals). They were amplified in two multiplexed reactions (Table 1) using the QIAGEN Multiplex kit. PCRs were carried out in a volume of 15 µL as follows: Multiplex PCR Master Mix following the

manufacturer recommendations, 0.07 µM of each Q-tailed primer, 0.2 µM of each reverse primer, and 0.1 µM of each fluorescent Q1 – Q4. Multiplex PCR programs consisted of 95°C for 15 min, followed by 30 cycles each of 94°C (30 s), 57°C (90 s), 72°C (90 s), followed by 10 cycles each of 94°C (30 s), 53°C (45 s), 72°C (45 s), and a final extension at 60°C for 30 min. PCR products were run on an ABI3730 sequencer (Applied Biosystems, Lennik, Netherlands).

Table 1: Characterization of 11 microsatellite markers isolated from *P. butyracea*. Forward and reverse primer sequences, labeled primer, repeat motif, annealing temperature (T_a), range of allele sizes (bp), number of alleles (*N*a), and GenBank accession number are shown for each primer pair.

Locus	Primer sequence (5' – 3')*	Labeled primer*	Repeat motif	T_a (°C)	Allele size (bp)	N_a	GenBank Accession No.
Pent1†	F: Q1-CCCCTTTGCTCTTGAGTGAG R: CCATTCAGCCATTCCCAG	Q1-6-FAM	$(CT)_{12}$	56	119-125	4	HE663069
Pent10‡	F: Q2-TCTTCCTCTTGTGTTTCGGC R: AAATTGTTGGAGCTATGGTGA	Q2-NED	$(AAC)_{22}$	56	-	-	HE663070
Pent11‡	F: Q3-GAATGCATGGGAAGCCATT R:CCTTTGTAATGCAGTTGATTTCC	Q3-VIC	$(AG)_{14}$	56	-	-	HE663071
Pent12†	F: Q4-GGTAGATTGTCAAGTTAAAGCAAAGA R: GGTGACCCACAAAAGGAACA	Q4-PET	$(CAT)_{11}$	56	151-165	4	HE663072
Pent13‡	F: Q1-CACATCATCTTTGTTAGTAATTGTTTT R: GAAATGCTATGCATGTCTAGTTGC	Q1-6-FAM	$(AC)_{10}$	56	166-170	3	HE663073
Pent14†	F: Q2-AAAAAGAGAGCGGAGATTTTGA R: GGAGGTGCCTGTCAGTGAGT	Q2-NED	$(AG)_{11}$	56	161-184	8	HE663074
Pent16†	F: Q4-AACTTTGCCACATCAAAGGC R: CCAAGTGCATTTGTACTTCCTTC	Q4-PET	$(GA)_{12}$	56	209-223	6	HE663075
Pent17†	F: Q1-GGAAAACCACACGCAAGAAG	Q1-6-FAM	$(GAG)_{10}$	56	211-217	3	HE663076

	R: GGAAATAGAGGAAACGGACCA						
Pent18‡	F: Q2-CTTCCTCTTCCTTGTTAGAAGCC	Q2-NED	$(TTG)_{11}$	56	228-257	6	HE663077
	R: AACTTTCAGGGCTTCATCTTCT						
Pent22†	F: Q2-GCCAAGAGGAGATTTGAGGA	Q2-NED	$(TTG)_{13}$	56	283-300	5	HE663078
	R: TTAAGCATGGTGAGGTTCGG						
Pent24‡	F: Q4-GTGAAAGAACTAGTTGAACTTGCTTAAA	Q4-PET	$(GTT)_{12}$	56	337-345	3	HE663079
	R: TGTATTCTTTACAACTACCTTTCTTTTTGT						

*Q1 = TGTAAAACGACGGCCAGT (Schuelke 2000); Q2 = AGGAGTGCAGCAAGCAT; Q3 = CACTGCTTAGAGCGATGC; Q4 = CTAGTTATTGCTCAGCGGT (Q2 – Q4, after Culley *et al.* 2008).

† Locus included in multiplex 1.

‡ Locus included in multiplex 2.

Results were analyzed using Peak Scanner Software Version 1.0 (Applied Biosystems). The number of alleles per locus, observed and expected heterozygosities and tests for deviation from the Hardy–Weinberg equilibrium (HWE) were computed using SPAGeDi1.3 (Hardy and Vekemans 2002). Null allele frequencies were estimated using the software INEst under the population inbreeding model (Chybicki and Burczyk 2009). Markers were tested for genotypic linkage disequilibrium using Genepop version 4.0.10 (Rousset 2010) using default parameters. These tests were performed only on the population from Benin because the sample from West African rainforests does not originate from a single population. All of 11 loci were polymorphic, but Pent11 and Pent10 amplified weakly in multiplexed reactions and were discarded from the analyses.

Results

Nuclear genome size and karyotype of *Pentadesma butyracea*

The separate analysis of nuclei of *S. globulifera* and *P. butyracea* yielded a single flow cytometry peak corresponding to the phase G of the cell cycle. When two species were analyzed simultaneously, we obtained two large peaks (see Fig. 1) indicating that the nuclear DNA content was higher in *P. butyracea* than in *S. globulifera*, itself higher than in *M. domestica*.

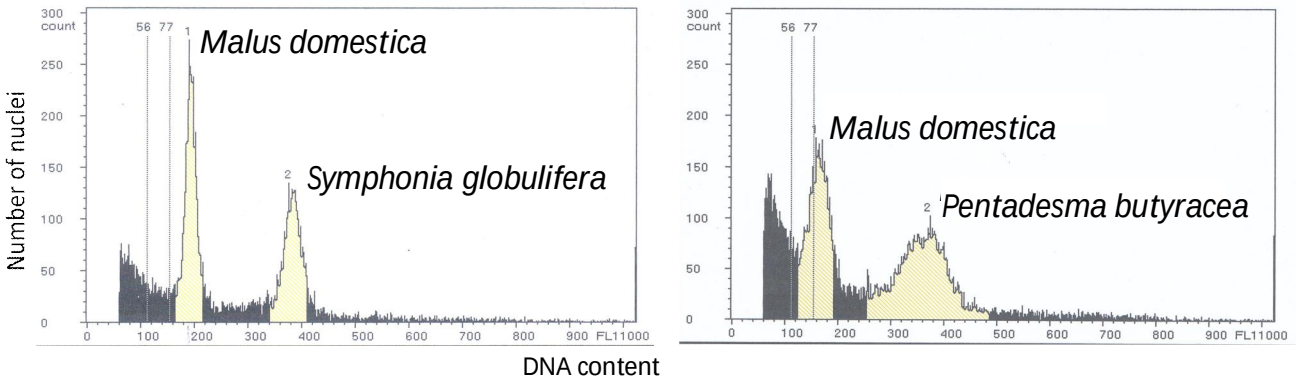

Fig. 1: Histogram of relative nuclear DNA content.

By comparison with *M. domestica*, genome sizes were estimate to 1723 Mbp for *P. butyracea* and 1522 Mbp for *S. globulifera* (Table 2). These values are higher than those found in other tree species such as *Theobroma cacao* (415 Mbp, Figueira *et al.* 1992), *Prunus domestica* (883Mbp, Arumuganathan and Earle 1991) and *Quercus robur* (184 Mbp, Zoldo et al. 1998) but lower than those found in the grass *Zea mays* (2292-2716 Mbp, Arumuganathan and Earle 1991).

Table 2: Genome characteristics of each plant species

	Relative fluorescence intensities of stained nuclei against those of of *Malus domestica*		Genome size (Mbp)
Malus domestica	192.84	161.55	770
Symphonia globulifera	381.39		1,523*
Pentadesma butyracea		359.44	1,713*

*genome size inferred from the of *Malus domestica* and the relative nuclei flurecence intensities

The karyotype of *P. butyracea* shows that the species has 56 chromosomes (see Fig.2). *P. butyracea* has a similar but somewhat larger genome size (ca. 13% or 200 Mbp larger) than its diploid relative *S. globulifera*. Hence, *P. butyracea* is most likely also diploid (2n=56), consistent with the fact that most microsatellite markers displayed at most two alleles in each individual. Nevertheless, the presence of four allele peaks observed in some microsatellite loci, for instance Pent24 (see Fig. 3), might result from a partial duplication of the genome or from an ancient polyploidisation event followed by genome divergence and ultimate diploidisation of the majority of the genome. Such ancient polyploidisation was also suggested in the related genus *Garcinia* (Osman and Milan 2006) and would explain the high number of chromosomes.

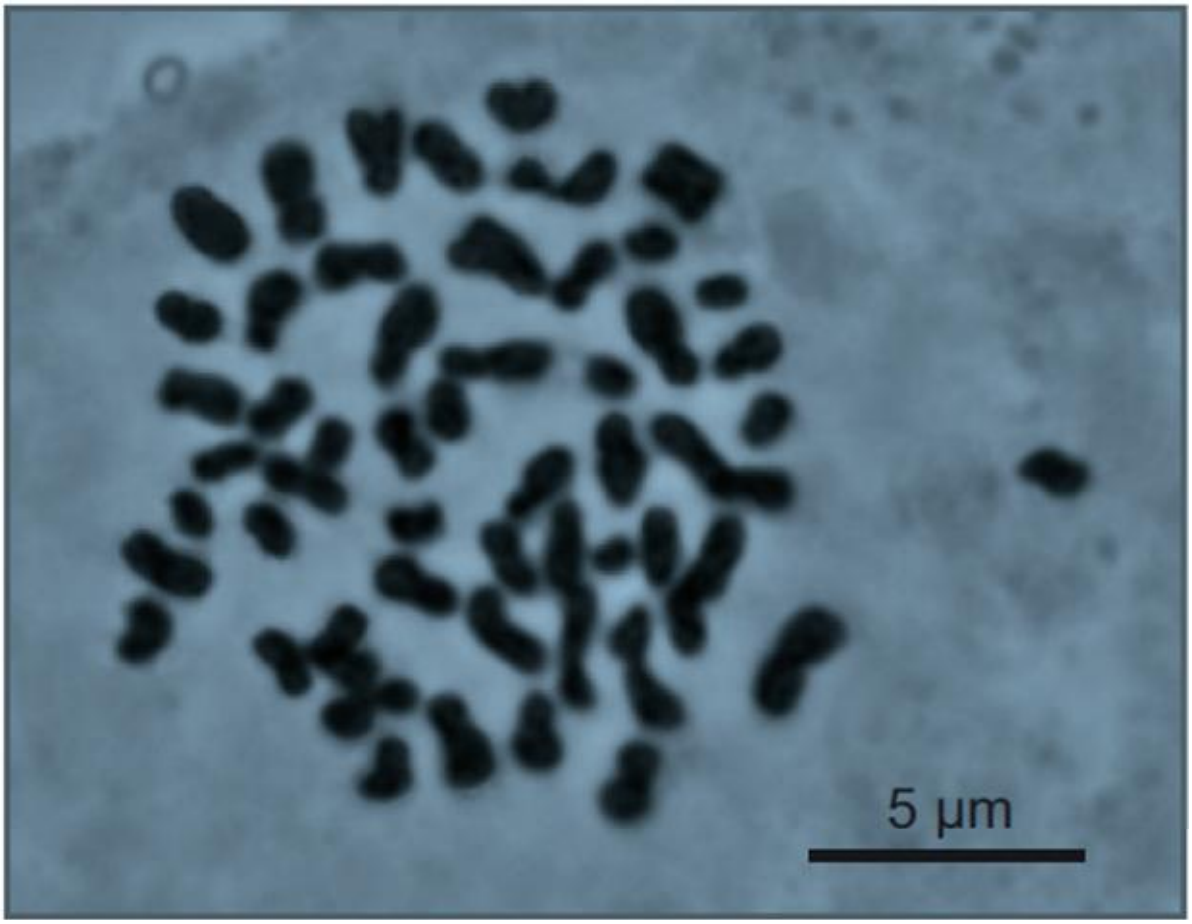

Fig.2: Karyotype of *Pentadesma butyracea*, 2n = 56

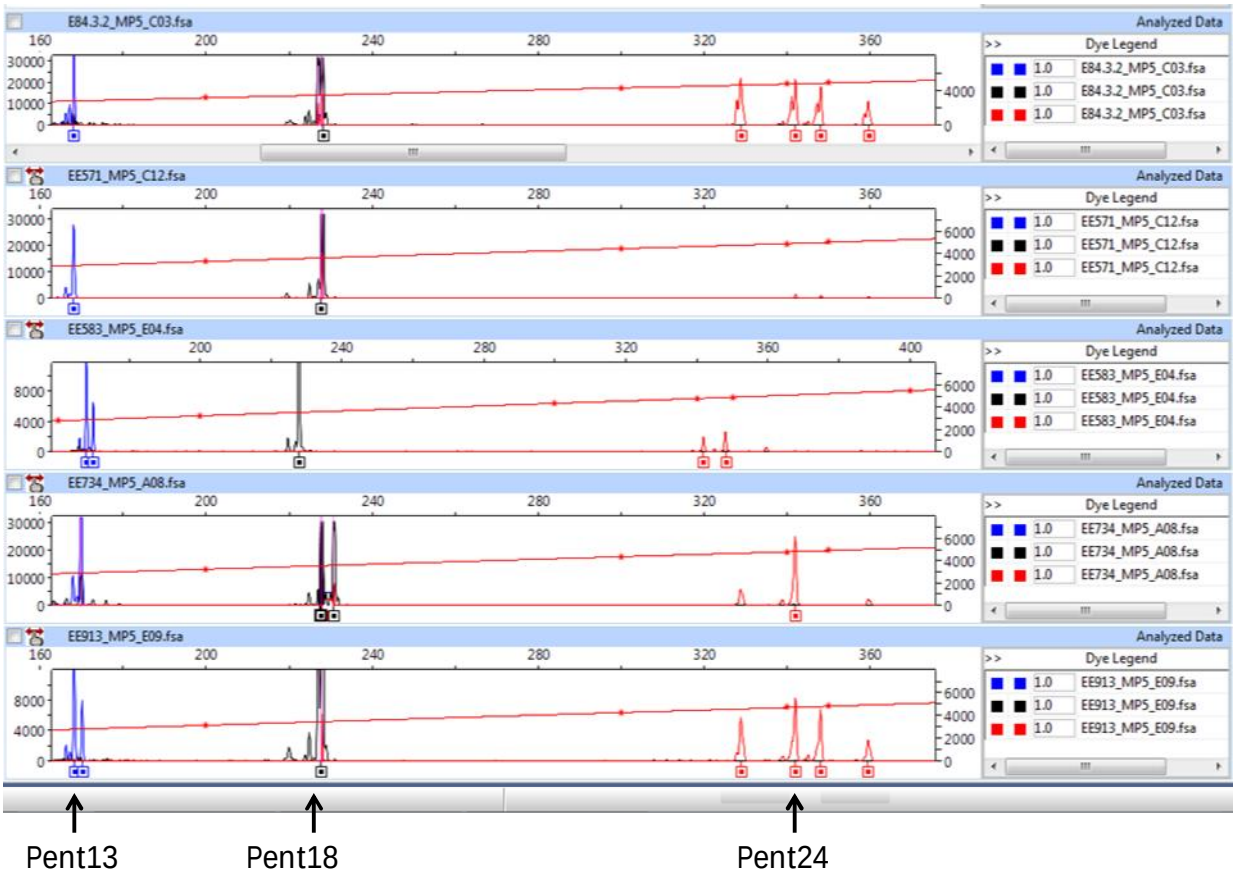

Fig. 3: Plot view of allele size in Peak Scanner, showing alleles number per locus (three loci are shown here: Pent13, Pent18, Pent24)

Characterization of microsatellite loci

The number of alleles per locus ranged from three to eight. This number was lower in the population from Benin (*N*a = 2.7; min = 2; max = 4) than in West African rainforests (*N*a = 4.1; min = 2; max = 8). Similarly, the expected heterozygosity was moderate in Benin (H_E = 0.38), ranging from 0.06 to 0.68, and higher in West African rainforests (H_E = 0.59), ranging from 0.34 to 0.82 (Table 3). Tests for deviation from HWE suggest that the nine amplified loci matched Hardy-Weinberg equilibrium. Estimated null allele frequencies were null or very low, except for locus Pent16 (0.17) but the standard error was nearly as large as the estimated value (Table 3). Tests of linkage disequilibrium were significant between locus Pent16 and both the loci Pent14 and Pent22 (p = 0.0002 and p = 0.001, respectively, considering a probability threshold adjusted for multiple comparisons using a Bonferroni correction). However no linkage disequilibrium was found between Pent14 and Pent22 (p = 0.16). Either these loci were physically linked or the linkage disequilibrium resulted from a population bottleneck (Lefèvre *et al.* 2012), a plausible hypothesis in Benin where natural populations

were of small size, experiencing substantial genetic drift in comparison with rainforests populations from West or Central Africa.

Eight loci successfully amplified *P. grandifolia* (n = 11 sampled individuals) and *P. reyndersii* (n = 4 sampled individuals) (Table 3). In *P. grandiflora*, most of the loci were polymorphic within the range of expected allele sizes, demonstrating the transferability of these microsatellite markers across the genus *Pentadesma*.

Conclusion

Our result show that *Pentadesma butyracea* is diploid (2n =56); however the larger genome size more than in *Symphonia globulifera* and the presence of more than two alleles in a few loci suggest gene duplications in concerned loci or occurrence of an ancient polyploidisation event. By using a new procedure that associates multiplex microsatellite enrichment and next-generation sequencing for the development of microsatellite-enriched libraries, we successfully genotyped nine polymorphic microsatellite loci using two multiplex reactions in the vulnerable food tree *P. butyracea*. These markers are now used to further investigate levels of genetic variation and spatial genetic structure in *P. butyracea* across its natural range in Central and West Africa.

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Table 3: Characterization of nine microsatellite loci from *Pentadesma butyracea* in a population from Benin and a mixed sample of individuals from West African rainforests. Sample size (*N*), number of alleles (N_a), observed heterozygosity (H_O), expected heterozygosity (H_E) are shown for each microsatellite locus. The fixation index (*F*), the associated tests of significance deviation from Hardy–Weinberg equilibrium (HWE, ns = not significant), and the estimation of null alleles frequency (*F.null*) and their standard error (*se*) are also given for the population from Benin. N_{Ag}, and N_{Ar} correspond to the number of alleles found in the congeneric species *P. grandifolia* (11 individuals from 7 sites in Nigeria, Cameroon and Gabon) and *P. reyndersii* (4 individuals from 4 sites in Rwanda).

Locus	Igbo Aladja (Benin)							West African rainforests						
	N	*N*a	*A*	H_O	H_E	*F* (HWE)	*F.null* ± *se*	*N*	*N*a	*A*	H_O	H_E	N_{Ag}	N_{Ar}
Pent1	30	3	2.67	0.07	0.06	-0.00^{ns}	0.00±0.00	6	2	2.00	0.00	0.49	3	3
Pent12	29	3	3.00	0.50	0.52	0.04^{ns}	0.01±0.03	18	4	2.55	0.17	0.34	7	4
Pent13	30	2	2.00	0.37	0.49	0.25^{ns}	0.08±0.06	18	3	2.97	0.33	0.66	4	1
Pent14	30	2	2.00	0.60	0.45	-0.32^{ns}	0.00±0.00	17	8	5.46	0.28	0.82	7	1
Pent16	27	2	2.00	0.27	0.53	0.49^{ns}	0.17±0.14	17	6	4.26	0.39	0.77	0	1
Pent17	30	3	2.97	0.17	0.16	-0.05^{ns}	0.00±0.00	17	2	1.98	0.11	0.43	3	1
Pent18	30	3	2.83	0.13	0.12	-0.04^{ns}	0.00±0.00	18	5	3.82	0.22	0.68	9	2
Pent22	28	4	3.99	0.63	0.68	0.07^{ns}	0.00±0.00	17	4	2.94	0.39	0.47	6	0
Pent24	25	2	2.00	0.10	0.36	0.72^{ns}	0.00±0.00	16	3	2.37	0.33	0.63	4	2
Average	-	2.7	-	0.31	0.38	0.16^{ns}	-	-	4.1	-	0.24	0.59	3.7	1.5

CHAPITRE III.-

LA STRUCTURE PHYLOGÉOGRAPHIQUE DE L'ARBRE À BEURRE, *PENTADESMA BUTYRACEA* SABINE (CLUSIACEAE), MONTRE L'EXISTENCE D'UN ÉVÈNEMENT DE FONDATION SÉVÈRE SUITE À LA MISE EN PLACE DU DAHOMEY GAP.

Manuscript not submitted

La structure phylogéographique de l'arbre à beurre *Pentadesma butyracea* Sabine (Clusiaceae) montre l'existence d'un évènement de fondation sévère suite à la mise en place du Dahomey Gap.

La phylogéographie s'investit à déterminer les lignées génétiques et leur distribution spatiale pour inférer des informations sur l'histoire démographique des populations (Avise 2009) et suppose que les populations spatialement structurées sont génétiquement liées en fonction des barrières aux échanges de gènes entre populations.

Cette étude cherche à détecter l'origine et comprendre l'histoire des populations de *Pentadesma butyracea* au Bénin sachant que cet arbre s'y trouve en position azonale (dans le couloir sec du Dahomey Gap, DG) en contraste avec les forêts denses humides du domaine guinéo-congolais (Haute-Guinée HG en Afrique de l'Ouest et Basse-Guinée BG en Afrique Centrale). Elle vise ensuite à identifier les signes des évènements passés de fragmentation et recolonisation en rapport avec les crises climatiques de l'Holocène. L'échantillonnage comprend 101 individus : 19 (HG), 48 (DG) et 34 (BG). Trois types de marqueurs moléculaires explorant divers aspects de l'évolution ont été utilisés : des marqueurs universels chloroplastiques ADNc (*psb*A-*trn*H et *trn*C-*ycf*6 séquencés chez 64 individus) et nucléaires ITS (*ITS2 S*2F /*ITS2 S*3R séquencés chez 36 individus), puis des marqueurs microsatellites nucléaires, nSSR développés sur l'espèce et génotypés chez 77 individus.

Tous les marqueurs indiquent une différentiation substantielle entre les trois régions : G_{ST} = 0.32, N_{ST} = 0.60 (ADNc); G_{ST} = 0.81, N_{ST} = 0.93 (ITS); F_{ST} = 0.33, R_{ST} = 0.65 (nSSR), attestant un signal phylogéographique ($N_{ST} > G_{ST}$ et $R_{ST} > F_{ST}$). Les séquences chloroplastiques et nucléaires montrent une profonde divergence génétique entre deux lignées. Ce motif correspond à une barrière à la migration qui remonterait aux changements climatiques du Quaternaire qui auraient engendrés la fragmentation des forêts denses humides de l'Afrique tropicale. Les forêts sèches du DG présentent une faible diversité (un seul haplotype ADNc et ITS ; hétérozygotie attendue H_E = 0.45, richesse allélique A = 2.35 pour les microsatellites) par rapport aux forêts humides du HG (huit haplotypes ADNc et un haplotype ITS ; H_E = 0.57, A = 3.02) ou aux forêts du BG (12 haplotypes ADNc et 3 haplotypes ITS ; H_E = 0.59, A = 3.09). Les marqueurs ADNc et nSSR ont identifié des variants génétiques similaires ou très proches dans le Dahomey Gap et dans les Monts Nimba (situés en zone de contact forêts-savanes) par rapport à ceux des forêts humides (HG, BG), suggérant une discontinuité génétique liée aux facteurs environnementaux. En conclusion, les populations du Benin seraient issues des forêts denses humides du Haut-Guinéen, et constituent peut-être des populations résiduelles lorsque la forêt pluviale africaine a connu son extension maximale durant l'Holocène humide avant de se rétracter en formant le DG. La faible diversité génétique de ces populations s'expliquerait par des évènements de fondation et/ou de la dérive génétique à cause de leur faible taille.

Phylogeographic structure of the African butter tree *Pentadesma butyracea* Sabine (Clusiaceae) supports the existence of a severe foundation event following the formation of the Dahomey Gap.

Ewédjè E.B.K, Duminil J., Ahanchédé A. and Hardy O.J.

Abstract

African rainforests have developed discontinuous blocks both in space and in their floristic composition mainly due to successive climatic crises which have impacted their biological diversity at both the interspecific and intraspecific levels. The present phylogeographic study aims to establish the relationships between populations of a tree species, *Pentadesma butyracea* Sabine (Clusiaceae), occurring both in Guineo-Congolian evergreen forests from West (Upper Guinea) and Central (Lower Guinea) Africa and in gallery forests situated in drier climate zones between the two rainforest blocks (notably in Benin, north of the Dahomey Gap). To this end, the pattern of population differentiation was assessed using plastid (pDNA) and ribosomal (ITS) DNA sequences as well as nuclear microsatellite loci (nSSR). Both plastid DNA sequences (22 haplotypes defined by 59 polymorphic sites within 1523bp of two intergenic regions, *psb*A-*trn*H and *trn*C-*ycf*6) and ITS sequences (4 haplotypes defined by 7 polymorphic sites within 493 bp of the *ITS2* region) congruently showed the existence of two allopatric and divergent lineages separating Upper Guinea (including Benin) and Lower Guinea. All the markers indicate substantial differentiation among Upper Guinea, Benin and Lower Guinea (pDNA: G_{ST} = 0.32, N_{ST} = 0.60; ITS: G_{ST} = 0.81, N_{ST} = 0.93; nSSR: F_{ST} = 0.33, R_{ST} = 0.65) with significant phylogeographic structuring (N_{ST} > G_{ST} and R_{ST} > F_{ST}) that can be interpreted as a persistent barrier to gene flow between Upper and Lower Guinea. All natural populations from dryer forests (Benin and Eastern Ghana) were characterized by a much lower diversity (single haplotype at pDNA and ITS, H_E = 0.45 at nSSR) than the ones in the rainforest of Upper Guinea (eight haplotypes at pDNA and a one haplotype at ITS, H_E = 0.57 at nSSR) or Lower Guinea (12 haplotypes at pDNA and 3 haplotypes at ITS, and H_E = 0.59 at nSSR). In conclusion, the populations from Benin probably derive from Upper Guinean rainforest populations and had undergone a severe foundation event and/or much higher genetic drift due to small population sizes. They may represent remnant populations resulting from the temporary expansion of the rainforest into the Dahomey Gap during the humid Holocene period. The management of these populations, currently threatened by overexploitation and habitat loss, requires special attention.

Keyword: genetic diversity, *Pentadesma butyracea*, population structure, phylogeography, Dahomey Gap, African rainforest history

Introduction

The African tropical rainforest (dating to Cretaceous times, Morley 2000, Davis *et al.* 2005) represents one of the largest extents of Africa's biome considered as a biodiversity hotspot (Myers *et al.* 2000). Since the late Miocene (last six million years), it was subjected to ongoing alterations in forest cover in response to drastic climate change (warm periods up to c. three million years followed by successive glacial and interglacial cycles; Maley 1996, Morley 2000, Bonnefille 2007). These changes have impacted the biological diversity of forests and their intraspecific diversity.

During the cool and dry glacial periods of the Pleistocene, African rainforests located in the Guineo-Congolian region are assumed to have retracted, forming at least three discontinuous biogeographic regions while mountain forests and savannas expanded (White 1979). The isolation of these forest blocks would explain their current floristic difference that lead to the recognition of the following phytogeographic domains: the Upper Guinea domain includes forests of West Africa stretching from southern Guinea to eastern Ghana; the Lower Guinea domain comprises Atlantic Central Africa from the west coast of Nigeria to Congo; and the Congolian domain includes most of the Congo basin until the Albertine Rift in the East of the Democratic Republic of the Congo.

More recently, during the early part of the Holocene, warmer and wetter climatic conditions favored the expansion of evergreen forests whereby the three rainforest blocks became contiguous during the humid Holocene period (c. 8500 to 4500 BP; Maley 1991). At the late Holocene in West Africa, drier climatic conditions between c 4500 and 3400 cal. year BP led to a rapid deterioration of the rainforest and subsequent spread of Sudano-Guinean savannas creating a forest-savanna mosaic with a high number of pioneer tree taxa (Salzmann and Hoelzmann 2005). A broad savanna corridor reaching the coast of southern Ghana, Togo and Benin interrupted the zonal West African rainforest between c. 0° and 3°E longitude. This so-called Dahomey Gap (DG) is currently characterized by a decline in annual precipitation (1000-1200 mm compared to >2000 mm in the rainforest areas of Nigeria and Ivory Coast). DG is viewed as a physical barrier to gene flow between Upper Guinean and Lower Guinean rainforest blocks. A few patches of rainforests surviving in wet places in this dry corridor are however considered as refuge for several rainforest species (Leal 2004, Adomou 2005, Chaïr *et al.* 2011). This is the case for *Octoknema borealis, Anthonotha macrophylla, Distemonanthus benthamianus, Mansonia altissima, Rinorea brachypetala, Celtis mildbraedii, Amphimas pterocarpoides, Discoglypremna caloneura* which can be found in the South of latitude 7°N (DG *sensu stricto*), as well as for *Aubrevillea kerstingii, Khaya*

grandifoliola and *Pentadesma butyracea* (Adomou 2005) which can be found at more northern latitudes (up to 11°N for *Pentadesma butyracea*), in an area that we will consider as the DG *sensu lato* for convenience. As populations of these species have experienced stochastic demographic and genetic factors in these remnant areas and are currently threatened by human activities, understanding their demographic history, their reaction to past climate changes and the dynamics of their diversity is essential in evolutionary biology and conservation genetics (Frankham *et al.* 2002).

Molecular phylogenetic approaches help to understand the main forces that shaped diversity within and among species. Nuclear and organelle DNA markers have already demonstrated that within-species genetic diversity is spatially structured in Lower Guinean trees, highlighting the impact of contemporary physical barriers to gene flow and past forest fragmentations (Born *et al.* 2012, Dauby et al. 2010, Daïnou et al. 2010, Debout et al 2010, Dumunil et al. 2010, Duminil *et al.* unpublished, Koffi *et al.* 2011). However, there is a lack of studies focusing on Upper Guinean forest tree species. Combining information from different markers allow inferring different aspects of evolution. Nuclear DNA sequences are suitable for the analysis of phylogenetic structure among species or among divergent populations (e.g. Olsen 2002). Nuclear microsatellite markers often allow detecting genetic discontinuities and to infer historical barriers to gene flow and recent history of populations by identifying groups of individuals of common recent ancestry. In plant species, chloroplast DNA (pDNA) is maternally inherited in the majority of angiosperms and used to explore the structure of gene genealogies resulting from seed dispersal, hence colonization history (e.g. Cavers *et al.* 2003, Petit *et al.* 2003; Heuertz *et al.* 2004), in spite of its slow rate of sequence evolution (Soltis *et al.* 1997, Schaal and Olsen 2000, Meister *et al.* 2005). It is expected that differentiation at plastid markers level be higher than at nuclear ones, due to the more limited dispersal capacity of seeds versus pollen and lower effective population size of the haploid chloroplast genome (Omondi *et al.* 2010).

In order to understand the role of the Dahomey Gap on within-species genetic differentiation, we used as biological model *Pentadesma butyracea* Sabine (Clusiaceae), the single food tree species among the rainforest plants surviving in this dry corridor. It is naturally distributed in Guineo-Congolian evergreen forests from Guinea-Bissau and Sierra Leone to the Democratic Republic of Congo (Bamps 1971) where annual rainfall ranges from 1,300 to 3,000 mm (locally 5,000 mm in Cameroon). In DG, *P. butyracea* is naturally distributed in gallery forests within savannahs of the Sudanian climatic zone where it benefits from a higher relative humidity and lower temperatures, the accumulation of water at the

hills' feet and the presence of water in streams all the year (Zepernick and Timler 1984). In this region, *P. butyracea* is threatened due to seeds' overexploitation and unsustainable agriculture and gardening in Benin and Togo (Dah-Dovonon 2002). Due to its large distribution area and its long life cycle, *P. butyracea* can be considered as a biological model to detect signs of past population fragmentation that occurred following a number of Quaternary climatic crises.

The present phylogeographic study aims to establish the relationships between populations of *Pentadesma butyracea* occurring both in Guineo-Congolian evergreen forests from West (Upper Guinea) and Central (Lower Guinea) Africa and in gallery forests situated in DG (notably in Benin). We used plastid (pDNA) and ribosomal (ITS) DNA sequences as well as nuclear microsatellite loci to address the two following questions:

- Are current hypotheses on the history of the African rainforest during the Pleistocene and Holocene supported by the phylogeographic patterns of *P. butyracea*?
- Do populations occurring in DG originate from Upper Guinean and/or Lower Guinean rainforests, and have they suffered from a bottleneck event?

Material and Methods

Study species

P. butyracea Sabine (Clusiaceae), known as African yellow butter tree, is one of the 1610 species from the 37 genera of its family. It belongs to the tribe Symphonieae in the subfamily Clusioideae (Steven 2007). Genus *Pentadesma* harbours four species all African : (1) *P. butyracea* Sabine widely distributed from Sierra Leone to Gabon; (2) *P. grandifolia* E.G.Baker, found in Cameroon and Gabon; (3) *P. lebrunii* Staner, known in Democratic Republic of Congo and Burundi; and (4) *P. reyndersii* Spirlet endemic in Rwanda. According to van Meer (1965), *P. reyndersii* is a synonym of *P. grandifolia*. The genus is present in Africa for very long since fossil pollen attributed to *P. butyracea* type has been reported as far back as the Upper Eocene period in Nigeria (Muller 1981).

P. butyracea occurs both in Guineo-Congolian evergreen forests from West (Upper Guinea) and Central (Lower Guinea) Africa and in gallery forests situated in drier climate between the two rainforest blocks (Dahomey Gap). Although it is currently absent from southern Benin (DG *sensu stricto*), its pollen was found in South Benin in sediments dating from the Middle Holocene period (Tossou 2002). In Benin, *P. butyracea* shows two eco-geographical groups according to morphological characteristics. Trees located in the more humid southern part of

the species distribution range are taller producing larger fruits and seeds than trees located in the North (Ewedje *et al.* 2012).

P. butyracea is mainly outcrossed, its pollen being dispersed by nectar-feeding animals including sunbirds and bees (Ewédjè unpublished manuscript). Its fruits are heavy (263 to 1705 g) and known to be dispersed by elephants in Lower Guinea, but no specific study has been conducted on seed dispersal in this species. However, we saw seeds picked from immature fruits broken by rodents.

Sampling and DNA extraction

A total of 101 adult trees were sampled: 48 from Dahomey Gap from (45 originated from 19 sites in Benin and 3 from two sites in eastern of Ghana), 19 individuals from the Upper Guinea (seven sites); and 34 from the Lower Guinea (17 sites) (Appendix 1). Leaf and /or cambium of each sample were dried and conserved in silica gel for DNA extraction. Total DNA was isolated from 10 mg of dried leaf or cambium following the CTAB protocol (Doyle & Doyle 1987) or with the NucleoSpin 96 plant kit from Macherey–Nagel (Düren, Germany).

DNA sequencing

Polymerase chain reactions (PCRs) of the *trn*C-*petN1*R and *psb*A-*trn*H inter-genic spacers were carried out using universal pDNA primers (Kress *et al.* 2005). The internal transcribed spacer (ITS) region of nuclear ribosomal DNA was amplified using three primer pairs *ITS2 S*2F / *ITS2 S*3R; ITS *C* / ITS *A* and ITS *B* / ITS *D* (White *et al.* 1990) but only the ITS2 fragment could be recovered. PCR reactions (total volume of 25 µl) included 2 µL of template DNA (10-100 ng), 0.1 µL of Taq polymerase (Qiagen), 2 µL PCR buffer, 1 µL MgCl2 (25 mM), 0.5 µL dNTP (10 µM), 0.25 µL of each primer (10 µM) and 18.9 µL of H2O. Cycling profiles for PCR reactions included an initial step of 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, and a final elongation step at 72°C for 5 min. Sequencing reactions were performed using standard protocols and sequences were resolved on a 3100 Genetic Analyser from Applied Biosystems. Sequence trace files were assembled and edited using Codon Code Aligner software (version 3.0.3, CodonCode Corporation, http://www.codoncode.com/aligner/download.htm). In total, 64 individuals were successfully sequenced for each pDNA inter-genic spacer while 36 individuals were successfully sequenced for ITS2 (Table 1, less sequencing effort was done for ITS2 because it proved monomorphic in Upper Guinea and the Dahomey Gap). After alignment, the two plastid

regions (*psb*A-*trn*H and *trn*C-*petN*1R) were concatenated because they are linked on a single circular DNA. Insertions or deletions were coded as single mutations.

nSSR genotyping

We performed PCR of nuclear microsatellites for all 77 samples using eleven loci (namely Pent1, Pent10, Pent11, Pent12, Pent13, Pent14, Pent16, Pent17, Pent18, Pent22, Pent24). They were amplified in two multiplexed reactions using the QIAGEN Multiplex kit in a final 15 µL reaction volume following Ewédjè et al. (in press). As only seven primer pairs (Appendix 1) consistently amplified across the geographic range, probably because of the presence of null alleles, further data analyses are limited to these loci.

Table 1: *Pentadesma butyracea* sampling populations including number of individuals used for each marker and assignment to genetic clusters determined by the software STRUCTURE

Regions	N_{SSR} [A]	N_{ITS} [B]	N_{pDNA} [C]	**Cluster1**	**Cluster2**	**Cluster3**	***T2*(IAM)**	***T2*(SMM)**
Dahomey Gap	37	10	33	100%	-	-	0.869^{ns}	-1.261*
Upper Guinea	15	10	9	6.7%	93.3%	-	0.485^{ns}	-2.713*
Lower Guinea	25	16	22	-	-	100%	-1.023^{ns}	-7.931**

Number of individuals included in analyses of microsatellites genotypes (N_{SSR}), ITS2 DNA sequences (N_{ITS}) and plastid DNA sequences (N_{pDNA}). Clusters indicate the percentages of individuals from each region assigned to a genetic cluster at a probability > 0.50 according to microsatellite genotypes. Tests of demographic stability (heterozygosity excess/deficit) according to the *T2* statistic of Cornuet and Luikart (1996) under infinite allele model and probability, *T2*(IAM), or the stepwise mutation model, *T2*(SMM), and probability of heterozygosity excess/deficit (ns, not significant, $**$, $P \leq 0.01$; $*$, $P \leq 0.05$).

Data analyses

Sequences analysis and diversity.

Plastid and ITS haplotypes were defined using the program DnaSP 5.0 (Rozas *et al.* 2010). We reconstructed a phylogenetic network of haplotypes with a maximum parsimony method based on a median-joining algorithm using the software Network 4.510 (Bandelt *et al.* 1999).

Genetic diversity was partitioned within and among the three phytogeographic regions (Upper Guinea, Lower Guinea, Dahomey Gap) which are hereafter called "populations". Within-population diversity was quantified using the unbiased estimate of gene diversity H_S which is based on haplotype frequencies and using v_S which takes into account the genetic distances between haplotypes, computed as the number of mutational steps distinguishing two sequences.

We used the program SPAGeDi to compute $G_{ST} = (1 - H_S) / H_T$ and $N_{ST} = (1 - v_S / v_T)$ as measures of population differentiation, where H_T and v_T estimate overall diversity (Pons and Petit 1996). A phylogeographic signal occurs when distinct haplotypes sampled within population are more related on average than distinct haplotypes sampled at large distance, in which case $N_{ST} > G_{ST}$. We tested if N_{ST} was significantly higher than G_{ST} using a randomization procedure permuting haplotype assignments in the genetic distance matrix between haplotypes.

SSR diversity and Bayesian cluster analysis.

We defined groups of individuals within which Hardy-Weinberg equilibrium and linkage equilibrium tend to occur through a Bayesian clustering analysis. To this end the software STRUCTURE version 2.3.3 (Pritchard *et al.* 2000) was run 10 times for a number of clusters *K* ranging from 1 to 9. The following parameters were chosen: burn-in length of 10000 with a number of MCMC repetitions after burnin of 10000, admixture model, and allele frequencies correlated among clusters. Because the likelihood for the data increased steadily with increasing *K*, the most likely number of clusters was inferred using the method of Evanno *et al.* (2005). These first analyses provided an optimal value of $K = 2$, i.e. two genetic clusters. Similar analyses with STRUCTURE were run within each cluster to check if sub-structuring was occurring.

After defining the global pattern of population structure with the above methods, differentiation statistics (F_{ST}, R_{ST}) were computed between inferred clusters using SPAGeDi

version 1.3 (Hardy and Vekemans 2002). R_{ST} is an analogue of F_{ST} based on allele size. The null hypothesis of no differentiation i.e. $F_{ST} = R_{ST} = 0$ was tested for overall F_{ST} and R_{ST} values using 999 permutations of individuals among clusters in SPAGeDi. The presence of a phylogeographic structure, i.e., whether alleles within populations were more related than alleles in the overall sample, was tested by comparing R_{ST} with its value after permuting allele sizes among allelic types within each locus ("permuted R_{ST}") using SPAGeDi. A significant one-sided test establishes the alternative hypothesis of R_{ST} > "permuted R_{ST}", meaning that stepwise mutations contributed to population differentiation, which is interpreted as a phylogeographic structure (Hardy *et al.* 2003).

Demographic history of populations

Population demographic change events can leave a signature on the genome of a species. In particular, allele frequencies can be affected because a reduction in effective population size (population bottleneck) leads to accidental loss of rare alleles, whereas population growth tends to produce new alleles (Dick and Heuertz 2008). When rare alleles are lost, the frequencies of the remaining alleles are more evenly distributed than predicted under drift-mutation equilibrium. This results in a gene diversity value higher than expected under drift-mutation equilibrium with the same number of alleles. In addition to natural disturbance effects, *P. butyracea* populations might have suffered significant fragmentation due to human impacts in DG. We tested for the presence of population bottleneck signals at the level of each nSSR cluster following the procedure of Cornuet and Luikart (1996), implemented in the program BOTTLENECK v1.2 (Piry *et al.* 1999). The infinite alleles model (IAM) and stepwise mutation model (SMM) were applied. This approach compares observed and expected gene diversities based on the observed number of alleles under mutation-drift equilibrium. To test for deviations from equilibrium demographic processes in genetic clusters, we computed the *T2* statistic of Cornuet and Luikart (1996), which represents an average over loci of standardized deviates for heterozygosity. Its significance was tested with the Wilcoxon signed ranks test (Piry *et al.* 1999). Positive values of T2 reflect a gene diversity excess possibly caused by recent founder events.

Results

DNA sequences variation

Ribosomal (ITS2) DNA polymorphism

ITS primer pairs ITS *C* / ITS *A* and ITS *B* / ITS *D* failed to amplify while the ITS2 region could be amplified (493 base pairs) and presented seven polymorphic sites defining four haplotypes H1-H4 (Fig. 1A and Appendix 2). Mutations consisted of single nucleotide substitutions including 4 transitions (A-G) and 2 transversions (G-T) in sequences from Lower Guinea. Sequences from four individuals of the closely related species *P. grandifolia* (which was a priori considered as an outgroup) lead to two other haplotypes (H5, H6). The haplotypes network shows the existence of two allopatric lineages separated by four mutation events: a West African lineage (Upper Guinea and Dahomey Gap), that harbours a single haplotype H1, and the Lower Guinea lineage (Fig.1A). This disjunction can correspond to a long-standing barrier to gene flow between the two phytogeographic regions. Global genetic diversity was $H_E = 0.56$ and genetic differentiation between the three phytogeographic regions was strong: $G_{ST} = 0.81$ and $N_{ST} = 0.93$ (p-value < 0.01) indicating a phylogeographic structure.

Plastid DNA polymorphism

Aligned DNA sequences reached 633 bp for *psb*A-*trn*H and 890 bp for *trn*C-*pet*N1R. After their concatenation i.e. linkage, 59 polymorphic sites resolved 22 haplotypes. Polymorphisms consisted of single nucleotide substitutions (N = 43), duplication of a single sequence composed of 4 – 6 – 8 and 15 bp in Lower Guinea (Cameroon and Gabon), single nucleotide indels (N = 10) and indels of six or eight bp (N = 2). The parsimony network distinguished a West African lineage (Upper Guinea + Dahomey Gap) containing nine haplotypes (H1-H9) and a Lower Guinean lineage made up of 13 haplotypes (H13-H22; Fig. 1B). These allopatric lineages are separated by four mutations including three substitutions and one indel. Drier forests of Dahomey Gap [Benin (N = 33 individuals) and East of Ghana (N = 2)] were all monomorphic exhibiting a single haplotype (H1; Fig. 1B). By contrast, moist forests displayed much more polymorphism. One of the two samples of Mount Nimba situated at the margin of rainforests near savannahs of Ivory Coast displayed the closest haplotype (H3) from H1 (see haplotypes network). Within Upper Guinea, haplotype H2 was shared between Ghana and Guinea as did haplotype H5 between mounts Nimba and Diecke in Guinea. The site Ziama from Guinea harboured the largest number of private haplotypes (H7, H8, H9). In

Lower Guinea, Cameroon and Gabon harboured 4 and 10 haplotypes respectively, sharing a single haplotype H10 between Korup and the National Park of Minkebe. Within Gabon, the site Gamba harboured three private haplotypes (H18, H19, H20) while H16 and H22 were shared between three and two populations, respectively.

Global genetic diversity was H_E = 0.94 (Nei 1978) whereas genetic differentiation between the three regions was fairly strong: G_{ST} = 0.32 and N_{ST} = 0.60 (p-value < 0.01). The test for phylogeographic structure was significant in the overall data set and between Benin and Lower Guinea [$N_{ST} > N_{ST}$ (permuted), $P < 0.001$].

It is worth noting that sequences from four individuals of the closely related species *P. grandifolia* (which was *a priori* considered as an outgroup) lead to two other haplotypes (H23, H24) closely related to the ones found for *P. butyracea* in Lower Guinea.

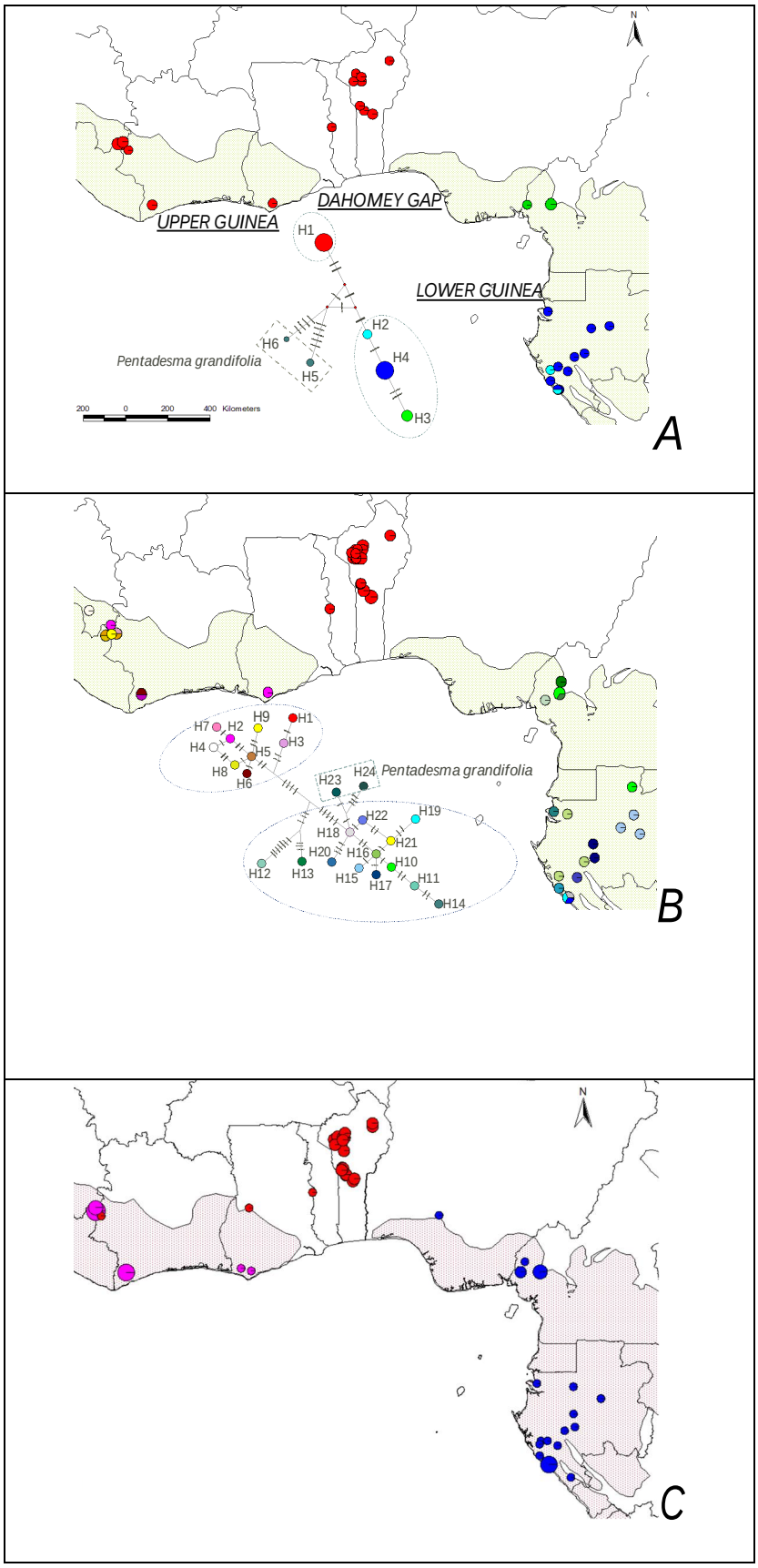


Fig. 1. Repartition of the genetic diversity and haplotype networks within *Pentadesma butyracea*. **(A)** ITS DNA sequences; **(B)** pDNA sequences; **(C)** nSSR inferred genetic cluster (results for K = 3 according to the software Structure). Circle size is representative of the number of individuals having each haplotype /genotype.

Nuclear microsatellites

SSR diversity

Four of the 11 loci tested did not successfully amplify in all samples and were discarded from the analyses. Over the seven remaining loci, the mean number of alleles per locus was 7.63 (range 6-13) whereas allelic richness was 3.72 **(**2.3-5.0) for a total of 56 alleles (Appendix 3). Expected heterozygosity (H_E) per locus ranged from 0.40 (Pent17) to 0.85 (Pent14) (mean H_E = 0.70).

Bayesian clustering analyses and detection of phylogeographic structure

According to the Evanno et al. method (2005) the best supported number of genetic clusters was K = 2 (Appendix 4). The resulting genetic discontinuity occurred between West African populations (including Dahomey Gap) and Central African ones. Additional analyses revealed two sub-clusters within each cluster. Genetic discontinuity was found in West Africa between Upper Guinea and the Dahomey Gap; except for a single genotype from Mount Nimba, at the margin of the rainforest, that was assigned to the Dahomey Gap sub-cluster. In Lower Guinea, the first sub-cluster included all Cameroonian genotypes and 13 genotypes from Gabon while the second was composed of 5 other genotypes distant from each other in Gabon. Because the later sub-clusters did not seem to form geographically coherent entities, they were not considered as revealing a reliable genetic discontinuity. Hence, we finally considered three genetic clusters, congruent with the DNA lineages detected: cluster 1 (Dahomey Gap) included all individuals from the dry forests of Benin and East Ghana as well as one individual situated at the margin of the West African moist forests (Mount Nimba in Ivory Coast), cluster 2 included all the other individuals from the rainforests of Upper Guinea, and cluster 3 included all individuals from Lower Guinea (Fig. 1C).

Table 2: Within-region genetic diversity of the seven nuclear SSR loci developed for *P. butyracea*

Locus	Repeated motifs	Lower Guinea (n = 25)			Upper Guinea (n = 18)			Dahomey Gap (n = 34)			Overall regions		
		*N*a	*A*	H_E	*N*a	*A*	H_E	*N*a	*A*	H_E	*N*a	*A*	H_E
Pent12	(CAT)n	5	2.82	0.58	4	2.14	0.34	4	2.51	0.54	6	5.76	0.72
Pent13	(AC)n	6	3.35	0.60	3	3.06	0.66	5	3.05	0.66	6	5.98	0.70
Pent14	(AG)n	6	3.32	0.64	8	4.49	0.80	5	3.09	0.68	12	11.28	0.85
Pent16	(GA)n	4	2.80	0.62	6	3.73	0.74	3	2.27	0.48	8	7.83	0.78
Pent17	(GAG)n	2	1.80	0.28	2	1.91	0.37	2	1.31	0.08	4	3.99	0.40
Pent18	(TTG)n	11	3.58	0.61	5	3.36	0.68	3	1.85	0.28	13	11.93	0.73
Pent22	(TTG)n	6	3.93	0.79	4	2.48	0.40	3	2.35	0.43	7	6.00	0.62
Average		6	3.09	0.59	5	3.02	0.57	4	2.35	0.45	8	7.54	0.69

H_E: expected heterozygosity after Nei (1978); *N*a: number of alleles per locus; *A*: allelic richness per locus

SSR diversity (Table 2) indicates that populations in the Dahomey Gap had a much lower diversity (H_E = 0.45, allelic richness A = 2.35) than the ones in the wet regions of Upper (H_E = 0.57, A = 3.02) or Lower Guinea (H_E = 0.59, A = 3.09). Except for genotypes of sites from the margin of West African moist forests (Mount Nimba in Ivory Coast) that were similar to those of Dahomey Gap, all remaining alleles were specifics to their genetic clusters.

The three phytogeographic regions show strong genetic differentiation: F_{ST} = 0.33 ± 0.04 and R_{ST} = 0.65 ± 0.12. According to permutation test, global R_{ST} was significantly higher than the global F_{ST} ($p < 0.01$) revealing a phylogeographic structure. The following differentiation pattern occurred between pairs of regions: F_{ST} = 0.32, R_{ST} = 0.36, no phylogeographic signal ($p = 0.65$) between Upper Guinea and Dahomey Gap; F_{ST} = 0.26, R_{ST} = 0.48; marginal phylogeographic signal ($p = 0.12$) between Upper and Lower Guinea; F_{ST} = 0.42, R_{ST} = 0.68 and a marginal phylogeographic signal ($p = 0.07$) between Dahomey Gap and Lower Guinea. Hence, the phylogeographic signal is essentially found between West and Central Africa, a result fully consistent with plastid and nuclear DNA sequences.

Population bottlenecks

Different results were obtained according to the model of molecular evolution used (IAM or SMM; Table 1). Under the IAM assumption, all tests of demographic signal were non-significant. Under the SMM assumption, a signal of population expansion was detected in all clusters but was considerably stronger in Lower Guinea (Table 1).

Discussions

The various molecular markers used revealed a congruent pattern of differentiation in *Pentadesma butyracea* that can be summarized as follows. A deep genetic divide with a phylogeographic signal separates West Africa from Central Africa (Lower Guinea), whereas within West Africa, populations from the drier areas of the Dahomey Gap are differentiated (but without phylogeographic signal) from the populations of the rainforest area (Upper Guinea) and display a much reduced genetic diversity.

Differentiation among biogeographic regions

In each rainforest block investigated (Upper and Lower Guinea), *P. butyracea* bears exclusively specific ITS and pDNA haplotypes. The fixation indices G_{ST} or F_{ST} indicate that the percentage of total genetic variation due to the isolation between these regions reached 65% at ITS, 38% at pDNA and 26% at nSSR. When accounting for the distance between

haplotypes or alleles, N_{ST} or R_{ST} indices reached 93% at ITS, 54% at pDNA and 48% at nSSR. The lower values at pDNA compared to nuclear markers was unexpected due to the more limited dispersal capability of seeds versus pollen. This may result from a high mutation rate in the *psb*A-*trn*H inter-genic region (a region also very polymorphic in the closely related species *Symphonia globulifera,* Dick and Heuertz 2008) which reduces G_{ST} and/or to the concerted mode of evolution of ITS which may decrease the diversity within each gene pool.

The phylogeographic signal between Upper and Lower Guinea indicates a prolonged separation, supporting a long-standing fragmentation of forest cover along an east-west axis. A clear divergence between haplotypes and/or nuclear variants from Upper Guinea and those from Lower Guinea occurs in other widespread tropical African trees, such as *Erythrophleum ivorense* (Duminil *et al.* unpublished manuscript), *Santiria trimera* (Koffi *et al.* 2010) or *Coffea canephora* (Gomez *et al.* 2009). Current hypotheses on the history of the African rainforest during the Quaternary generally assume that the Guineo-Congolian rainforest was fragmented during the cool and dry "glacial" periods and re-expanded during the interglacial periods where it could form a continuous forest block, such as during the humid Holocene period (e.g. Dupont *et al.* 2000). The high degree of divergence between Upper and Lower Guinea suggests, however, that a discontinuity such as the Dahomey Gap was present for most of the (recent) Pleistocene, so that the humid Holocene period may not be representative of most interglacial periods.

We detected two sites (Ziama in Guinea and Gamba in Gabon) harbouring three endemic pDNA haplotypes within hypothetical forest refuge areas during the last glacial maximum according to Maley (1996). This pattern is expected if *P. butyracea* populations have persisted long enough in those putative forest refuges to generate unique haplotypes that have not been dispersed during phases of forest expansion.

Table 3: Differentiation between pairs of the three regions according to genetic markers. P-values refer to tests of phylogeographic signals, i.e. whether $N_{ST} > G_{ST}$ or $R_{ST} > F_{ST}$.

Pair of regions		**ITS2**			**pDNA**			**nSSR**		
Region i	Region j	G_{ST}	N_{ST}	p-value	G_{ST}	N_{ST}	p-value	F_{ST}	R_{ST}	p-value
Lower Guinea	Dahomey Gap	0.66	0.94	0.06	0.45	0.66	0.18	0.42	0.68	0.076
Lower Guinea	Upper Guinea	0.65	0.93	0.05	0.38	0.54	0.001	0.26	0.48	0.12
Dahomey Gap	Upper Guinea	-	-	-	0.40	0.59	0.008	0.32	0.36	0.654
Overall		0.81	0.93	0.01	0.32	0.60	0.001	0.33	0.65	0.001

It is worth noting that *P. butyracea* displays also a morphological differentiation between West and Central Africa. First, leaf from Lower Guinea are tough showing more prominent nerves than leaf from Upper Guinea (Ewédjè E.B.K, pers. obs.). Second, fruits are smaller in Lower Guinea, typically measuring 15 cm x 10 cm and containing up to 10 seeds (White and Abernethy 1996), than in Upper Guinea and in Dahomey Gap where fruits are typically large measuring 29 cm x 17 cm and contain up to 32 seeds in (Aubreville 1959, Ewedje *et al.* 2012). The deep molecular differentiation associated with morphological differentiation may in fact call for a reconsideration of the taxonomic status of *P. butyracea*, especially given that pDNA sequences from Lower Guinea were more closely related to the ones of another species occurring in Lower Guinea (*P. grandifolia*) than to the sequences found in Upper Guinea. Under the hypothesis that *P. butyracea* forms in fact two species, the divergence between Upper Guinean and Lower-Guinean populations might in fact predate the Pleistocene, like the majority of congeneric species from Guineo-Congolian forests (Plana 2004). Adequate data for estimating accurately divergence time (in particular calibration points) are currently lacking but this deserves future studies.

Origin of populations from Dahomey Gap

Both nuclear and chloroplast genealogical lineages indicate that populations from Dahomey Gap (Benin, Togo and part of Ghana) are more closely related to Upper Guinean populations than Lower Guinean ones. Moreover, the delimitation of Dahomey Gap's genetic cluster which coincides with the distribution of its pDNA haplotype is well correlated with the West African forest-savanna boundary, suggesting a genetic discontinuity related to environmental factors. It would be interesting to collect and/or get samples from remaining dry forests of Ghana and Ivory Coast to check if the pattern observed from Mount Nimba (corresponding to a contact zone between savannahs and West African rainforests) would be maintained. In conclusion, in the Dahomey Gap, where *P. butyracea* occurs only in locations where there is more humidity (gallery forests, foot of hills), its populations most likely derive from the Upper Guinean rainforests.

Despite the high mutation rate of nuclear microsatellites and the absence of a real physical barrier between Dahomey Gap and Upper Guinea, the Dahomey Gap showed a much reduced diversity probably due to substantial genetic drift resulting from small population size and/or founder effects in the remnant patches of forests. In addition, low water availability within the Dahomey Gap seems to be followed by reproductive isolation through a shift in flowering

phenology: individuals growing in evergreen moist forests flower from February to April and July to September (Aubreville 1959, Keay *et al.* 1964, White and Abernethy 1996) while those in the Dahomey Gap flower from September to November (Ewédjè E.B.K unpublished). This flowering shift might strongly limit gene flow between DG and forested regions, despite the fact that the main pollinators (sunbird (White and Albernethy 1996, Borrow and Demey 2001) and bees) occur over the whole distribution area of *P. butyracea*. Genetic drift and the isolation of populations from DG thus well explain their reduced diversity. If these populations originate from Lower Guinean forests, we might also expect to detect the genetic signature of a bottleneck event but our analyses do not allow to conclude. This might result from a lack of power of the method used (Chikhi *et al.* 2010).

As populations from Dahomey Gap are little differentiated from Upper Guinean ones, the hypothesis that they are remnant populations dating from the humid Holocene period when the Guineo-Congolian forest covered the DG *sensu stricto* seem plausible. Given that *P. butyracea* occurs in the north of Benin between 8 and 11° of latitude N, above the proposed limit of the maximal extension of rainforest during the humid Holocene period (latitude of Dangbo 6°36'N, Tossou 2002), the two following hypotheses may explain its actual distribution:

The first hypothesis assumes that humid forests actually extended further towards the north during the humid Holocene period (Demenocal *et al.* 2000). This is supported by a reconstruction of paleovegetation for Africa (Anhuff *et al.* 2000) suggesting that 8000 yrs BP, moist evergreen forests from West Africa reached the latitude of 11°N (Fig. 2). When forest retraction occurred in the middle Holocene as a result of drier climatic conditions (between ca 4500 and 3400 years BP), some remnants populations found refuge within more humid azonal locations of the Dahomey Gap where they experienced genetic drift.

The second hypothesis assumes that the rainforests did not reach such high latitude but that the maximal extension of the forests areas was followed by a colonization of the species from Eastern Ghana along gallery forests through mountainous chains, eventually reaching the Atacora Chains in North-West Benin where the species benefits regularly from a higher relative humidity and lower temperatures, the accumulation of water at the hills' feet and the presence of water in streams all year long. This colonization process drove founder events.

Our data can not discriminate among these hypotheses but additional population data from DG might allow us to conclude.

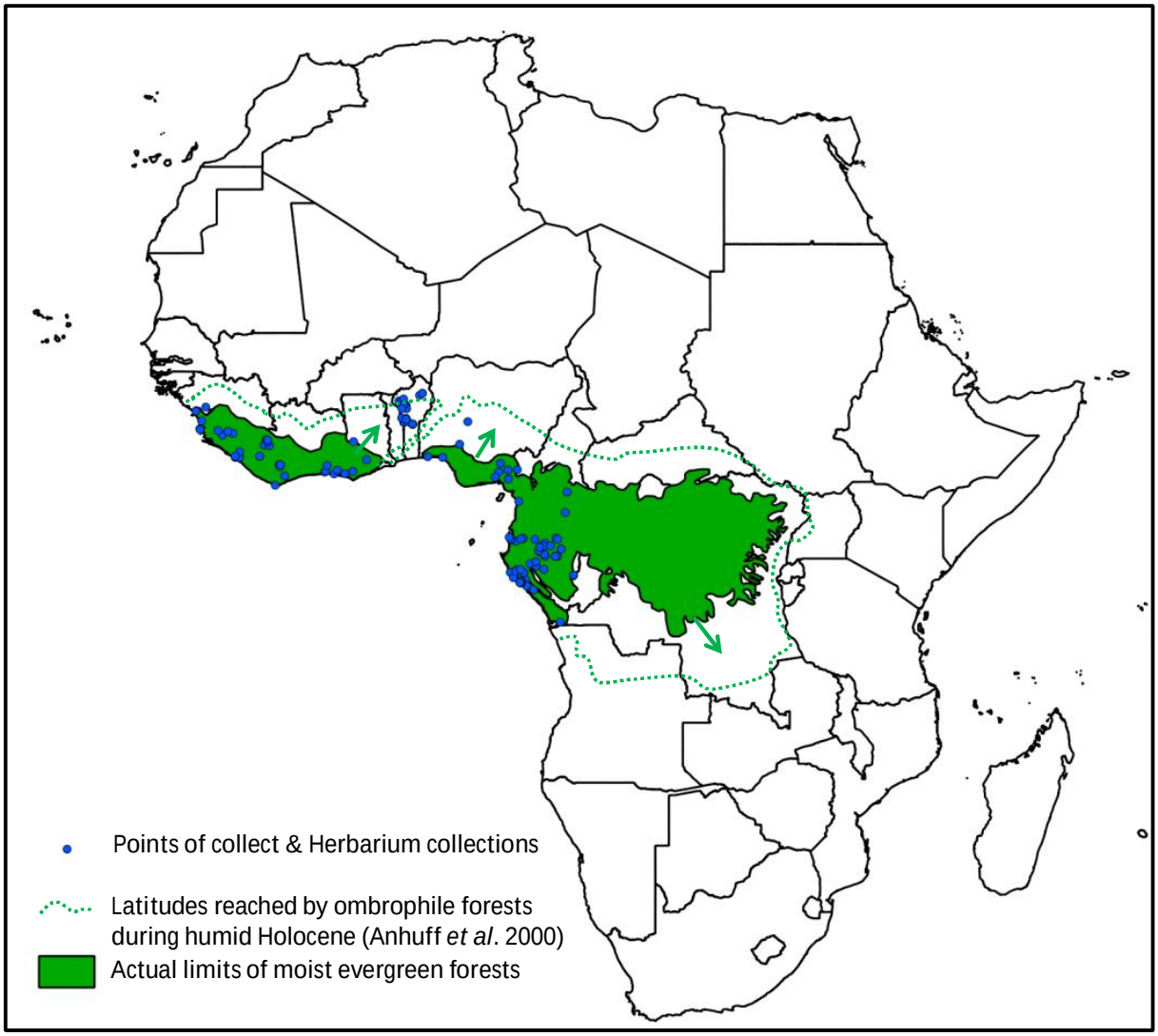


Fig.2. Actual and past limits of African moist evergreen forests, and distribution of known collection points of *Pentadesma butyracea*.

Conclusion

This study illustrates two major factors that shaped diversity and spatial genetic structure of *P. butyracea* and it can be related to past climate changes: (1) a deep and ancient fragmentation of African rainforest populations that matched the major distribution range shifts experienced by Upper and Lower phytogeographic regions and which may have caused a speciation if additional data support our hypothesis that *P. butyracea* constitutes different species in Lower and Upper Guinea, and (2) a recent diversification dependent on ecological gradient within West Africa which occurred in the Dahomey Gap, possibly due to the closure of the Dahomey Gap during the humid Holocene period followed by an adaptation of the remaining populations to drier conditions. Both induced gene flow barriers between all genetic clusters identified. In West Africa, *P. butyracea* populations displayed low genetic diversity particularly in dry semi-deciduous forests from DG where the species was subjected to bottleneck and/or founder events. In this region where the species is exploited for butter production, special attention should be paid to its genetic resources.

Appendix 1: *Pentadesma butyracea* sampling sites

Site name	N_{SSR} [A]	N_{ITS} [B]	N_{pDNA} [C]	Country	Regions	K3 [D]	Longitude	Latitude
Diecke	5	4	1	Guinea	UG	2	-8.28	7.54
Ziama	3	3	4	Guinea	UG	2	-8.29	7.68
Dodo	4	2	1	Ivory Coast	UG	2	-7.04	5.04
Mont Nimba	1	1	3	Ivory Coast	UG	1	-8.26	7.34
Ankassa	1	-	-	Ghana	UG	2	-2.65	5.21
Nueng South	1	1	1	Ghana	UG	2	-1.93	5.11
Techiman	1	-	-	Ghana	DG	1	-2.02	7.56
Sabu R valley	2	1	2	Ghana	DG	1	0.57	8.29
Bassila	2	1	2	Benin	DG	1	1.65	8.99
Bensekou	2	-	1	Benin	DG	1	2.80	10.94
Bongou	2	-	2	Benin	DG	1	1.62	10.40
Boribansifa	2	1	2	Benin	DG	1	1.29	10.54
Igbo Aladja	2	-	4	Benin	DG	1	2.22	8.74
Kikele	2	-	-	Benin	DG	1	1.95	9.01
Koda	2	1	-	Benin	DG	1	2.28	8.84
Kouatena	-	1	-	Benin	DG		1.82	10.21
Kouba	2	-	1	Benin	DG	1	1.61	10.49
Nioro	2	-	-	Benin	DG	1	1.49	9.29
Penessoulou	2	-	3	Benin	DG	1	1.82	9.26
Peperkou	2	-	4	Benin	DG	1	1.45	10.43
Perma	-	-	3	Benin	DG		1.82	10.21
Setou	2	1	-	Benin	DG	1	1.81	9.20
Tandafa	2	-	3	Benin	DG	1	1.63	10.68
Tassigourou	2	-	-	Benin	DG	1	1.85	9.97
Tchiapeta	2	1	3	Benin	DG	1	1.64	10.22
Tchoundegou	2	1	-	Benin	DG	1	1.51	9.97
Yagua	2	1	2	Benin	DG	1	3.00	11.08
Oka	1	-	-	Nigeria	LG	3	5.72	7.37
Korup NP	2	-	-	Cameroun	LG	3	8.86	5.07
Korup Plot	3	3	3	Cameroun	LG	3	8.84	5.06
Park of Korup	-	1	-	Cameroun	LG		8.87	5.05
Mamfe	1	-	-	Cameroun	LG	3	9.24	5.48
Lokando	-	-	1	Cameroun	LG		9.28	4.83
Fontem	-	-	2	Cameroun	LG		9.93	5.50
Moukalaba Park	1	1	1	Gabon	LG	3	10.57	-1.99
Minkebe Park	-	-	1	Gabon	LG		12.80	1.49
Lopé	-	1	-	Gabon	LG		11.57	-0.17
Milolé	-	-	2	Gabon	LG		13.12	-0.31
Sogademin	-	-	1	Gabon	LG		10.18	0.44
Waka Park	1	1	1	Gabon	LG	3	11.29	-1.23
NKomi	1	-	-	Gabon	LG	3	9.89	-1.80
Iguela	1	1	1	Gabon	LG	3	9.83	-1.93

Waka	1	1	1	Gabon	LG	3	10.86	-1.38
Haut-Abanga	1	-	-	Gabon	LG	3	11.22	0.41
Mandji	1	1	1	Gabon	LG	3	10.16	-1.80
CFAD of Rimbunan	2	-	-	Gabon	LG	3	11.22	-0.69
Ivindo	1	1	1	Gabon	LG	3	12.35	-0.07
Tchibanga	1	-	-	Gabon	LG	3	11.12	-3.28
Cap Esterias	1	1	1	Gabon	LG	3	9.33	0.54
NR848	-	-	1	Gabon	LG		11.29	-1.23
Gamba	5	3	3	Gabon	LG	3	10.22	-2.76
Luango	1	1	1	Gabon	LG	3	9.64	-2.40

[A] Number of individuals genotyped SSR-based analyses; [B] Number of individuals sequenced at ITS2 DNA sequences; [C] Number of individuals sequenced at plastid DNA sequences; [D] Genetic cluster assignment for K= 3. UP, Upper Guinea; DG, Dahomey Gap; LG, Lower Guinea

Appendix 2: Polymorphic sites and ITS2 haplotypes on the basis of DNA sequences

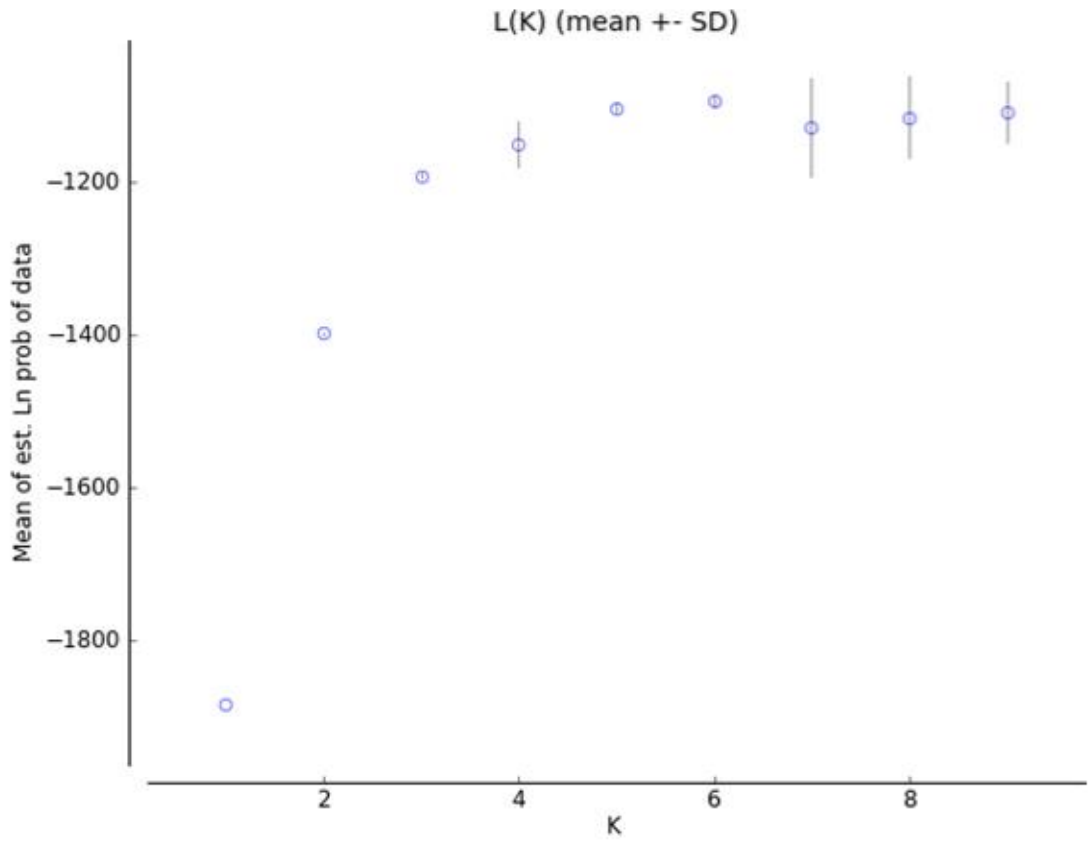
Polymorphic sites		**203**	**218**	**230**	**241**	**326**	**347**	**383**
Haplotypes	**N [A]**							
H1	19	T	A	G	A	G	A	A
H2	4	G	G	T	G	A	G	G
H3	11	G	G	T	G	A	A	A
H4	2	G	G	T	G	G	A	A

[A]N, number of sequences

Appendix 3: SSR diversity values and genetic differentiation between the three regions (UG, DG, LG) of *P. butyracea*

Locus	**Repeated motifs**	***N*a**	***A***	**Size**	***H*o/*H*e**	H_E **(Nei 1978**	***h* (Pons & Petit1996)**	F_{IT}	F_{IS}	F_{ST}	R_{ST}
Pent12	(CAT)n	6	3.63	148-179	0.67	0.72	0.79	0.43	0.05	0.39	0.001
Pent13	(AC)n	6	3.56	162-172	0.74	0.70	0.73	0.28	0.18	0.12	-0.011
Pent14	(AG)n	12	5.09	157-186	0.63	0.86	0.94	0.42	0.20	0.27	0.738
Pent16	(GA)n	8	4.39	209-223	0.39	0.78	0.92	0.65	0.47	0.34	0.644
Pent17	(GAG)n	4	2.30	202-217	0.16	0.40	0.59	0.87	0.69	0.57	0.365
Pent18	(TTG)n	13	4.02	215-281	0.42	0.74	0.89	0.64	0.35	0.45	0.679
Pent22	(TTG)n	7	3.05	274-300	0.71	0.62	0.76	0.41	0.20	0.25	-0.018
		7.63	3.72±0.90	-	0.53±0.21	0.69±0.15	0.81±0.12	0.51±0.06	0.27±0.06	0.33±0.04	0.65±0.12

H_E: expected heterozygosity after Nei (1978); *N*a: number of alleles per locus; *A*: allelic richness per locus; F_{IT}, individual's inbreeding coefficient across all populations; F_{IS} kinship coefficient



Appendix 4: Log-likelihood of the microsatellite genotypes of 77 *P. butyracea* individuals grouped into *K* clusters, obtained through 10 runs of the STRUCTURE software.

CHAPITRE IV.-

VARIABILITÉ MORPHOLOGIQUE DE L'ARBRE À SUIF, *PENTADESMA BUTYRACEA* SABINE (CLUSIACEAE), AU BENIN

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(NOTES ON NEGLECTED AND UNDERUTILIZED CROPS) **59**: 625-633

Variabilité morphologique de l'arbre à suif, *Pentadesma butyracea* Sabine (Clusiaceae), au Benin

Au regard des menaces qui pèsent sur cette espèce au Bénin, sa diversité génétique devra être fortement réduite dans l'avenir si des mesures de conservation appropriées ne sont pas prises. Cependant les patrons de la variabilité morphologique et génétique au sein des populations naturelles sont très peu connus. Notre objectif ici vise à caractériser la variabilité morphologique des arbres au Bénin et à vérifier si les caractères relatifs à la graine, au fruit et au tronc varient avec la position géographique et les gradients climatiques. Des mesures ont été effectuées sur un total de 14 caractères quantitatifs et 13 traits qualitatifs sur 348 arbres, 796 fruits et 4900 graines provenant de 12 populations, et nous avons établi la corrélation entre ces caractères et l'indice d'aridité De Martonne. Les caractères mesurés sont fortement variables au sein des populations ($p < 0.0001$). La variabilité détectée entre populations est faible (5-25% pour les caractères des arbres, 20-50% pour ceux des fruits et 31- 44% pour ceux des graines). Les analyses multivariées ont permis de distinguer deux groupes éco-morphologiques : les arbres situés dans les zones plus humides (au sud de l'aire de distribution de l'espèce) sont les plus gros, produisant des fruits et graines de grandes dimensions par rapport aux arbres situés en milieu sec (au nord).

Pour distinguer la contribution de la variation génétique entre populations par rapport à la plasticité phénotypique liée aux conditions environnementales, cette étude doit être complétée par une analyse de la diversité génétique (objet du prochain chapitre) et/ou en réalisant un suivi de diverses provenances dans un verger de conservation de l'espèce.

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Morphological variability of the tallow tree, *Pentadesma butyracea* Sabine (Clusiaceae), in Benin

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Abstract

Seeds of *Pentadesma butyracea* (butter tree) are exploited by rural populations in West Africa to produce butter used in cooking for food and as an ingredient in cosmetics and medicinal preparations. To improve our knowledge of this under-studied species, the morphological variation of the trees, fruits and seeds in 12 natural populations of Benin distributed over a gradient of climate aridity is described with 14 quantitative and 13 qualitative descriptors. Most traits, except seed shape categories, show significant differences among populations. Multivariate analyses distinguish two eco-geographical groups: trees located in the more humid southern part of the species distribution are taller and have larger fruits and seeds than trees located in the north. To disentangle the relative roles of genetic variation and phenotypic plasticity to this pattern, further phenotypic studies should be performed in common garden experiments.

Keywords: aridity, butter, ecogeographical groups, morphological variability, *Pentadesma butyracea*, tallow tree

Introduction

Most plant species in natural populations present an intraspecific genetic variability that enables adaptation to fluctuating environmental conditions which is generally reflected in morphological variability (Cornelius 1994). This genetic variation within species is the basis for the selection by humans to domesticate species or to favour varieties more suited to their needs. Preserving this genetic variation is therefore an essential component of conservation and sustainable management programs of useful species (Brown and Hardner 2000, El Kassaby 2000, Yagoubi and Chriki 2000).

Pentadesma butyracea Sabine (Clusiaceae) is a forest tree widely distributed in Africa from Guinea-Bissau to the West of the Democratic Republic of Congo. It is known as “tallow tree” or “butter tree” in English, “arbre à suif” or “arbre à beurre” in French, “Lami”, “Krinda” or “Tama”, “Lorokiéré”, “Ouotéra”, “Djréhé” in Ivory Coast, “Abotoasebie” in Ghana, “Kpangnan, “Akoto” or “Sesseido” in Benin and “Agnuhé“ in Gabon. In West Africa, its seeds are transformed to make a butter (“kanya”) used in cooking for food and as an ingredient in cosmetics and medicinal preparations (Avocevou 2011, Natta *et al.* 2010). It plays a non-negligible role in the rural economy of Benin, Togo and Ivory Coast. In Central Africa, notably in Gabon, the sweet mesocarp of mature fruits is used as fruit juice (White and Albernethy 1996).

In Benin, this plant species is threatened because (1) seeds are overexploitated, (2) its habitat is destroyed for agricultural expansion, and (3) seasonal fires let by farmers and hunters damage the trees. Unfortunately, *ex situ* conservation is difficult because seeds are recalcitrant: they die when frozen (Ewedje, pers. obs.). Genetic diversity of the species might therefore be strongly reduced in the future if no appropriate conservation measures are taken. Information about *P. butyracea* uses, butter biochemical composition, socio-economic value, regeneration, spatial distribution and ecology is available (Avocèvou-Ayisso 2011, Natta *et al.* 2010, Tchobo *et al.* 2007, Natta 2003, Sinsin and Sinadouwirou 2003). However, little is known about the sustainable use of the species and about the patterns of genetic and morphological variation within natural populations. In Ivory Coast, Ouattara (1999) observed that bigger fruits and heavier seeds develop in populations where pluviometry is regular and more abundant and/or where soils are deeper and more fertile. Our objective here is to characterize the morphological variability of *P. butyracea* trees in Benin, and to test if traits related to the seed, fruit and trunk vary according to geographical location and climatic gradients.

Material and methods

Taxonomy and ecology

The genus *Pentadesma* belongs to the tribe Symphonieae, the subfamily Moronobeoideae and family Clusiaceae. It includes four African species: (1) *P. butyracea* Sabine with a large geographical distribution; (2) *P. grandifolia* E.G.Baker only present in Nigeria, Cameroon and Gabon; (3) *P. lebrunii* Staner only known from Democratic Republic of Congo and Burundi, and (4) *P. reyndersii* Spirlet endemic of Rwanda. All species of the genus produce an edible fat. *P. butyracea* is a long-lived tree that often reaches 20-35 m in height and 80-100 cm diameter at breast height with bole straight (without buttresses) and horizontal branches (Ouattara 1999, White and Abernethy 1996). Its latex is yellow or orange-yellow. The bark cracks longitudinally and transversely, leaf are glabrous and grouped at the top of branches. Inflorescences are terminal, carrying bisexual and large flowers. Each flower has five petals and numerous stamens arranged in five staminal phalanges alternating with 5 gland-discs. Fruits are berries with a yellow mesocarp and seeds have a pyramidal shape. *P. butyracea* is naturally distributed from Guinea-Bissau, Sierra Leone, Ivory Coast and Togo to the Democratic Republic of Congo (Bamps 1971) and is extending eastwards into Tanzania and Uganda, where it is found under cultivation (Sama and Sacandé 2007). In Benin, the species is not known to be cultivated. Natural populations mostly occur in Guineo-Congolian evergreen forests where annual rainfall ranges from 1300 to 3000 mm (locally 5000 mm in Cameroon). However, they can also be found in dryer areas in gallery forests, for example in Benin and Ghana. In very moist sites such as in swamp forests, it develops stilt roots (Sama and Sacandé 2007, Hawthorne and Jongkind 2006).

Study area

In Benin, *P. butyracea* occurs in the Sudanian phytogeographical zone as defined by White (1983, see Fig. 1). Climate is characterized by one rainy season (March-May to October) and nearly seven months of dry season. Annual rainfall ranges from 900 mm to 1400 mm (Fig 1), daily relative humidity and temperature vary between 18 and 99%, and 18 and 42°C, respectively, and yearly average potential evapotranspiration (ETP) is 1550 mm (ASECNA, Agence pour la Sécurité de la Navigation Aérienne en Afrique et à Madagascar 2010). *P. butyracea* is restricted to gallery forests, savannah woodlands and at the feet of hills, where soil humidity is sufficient.

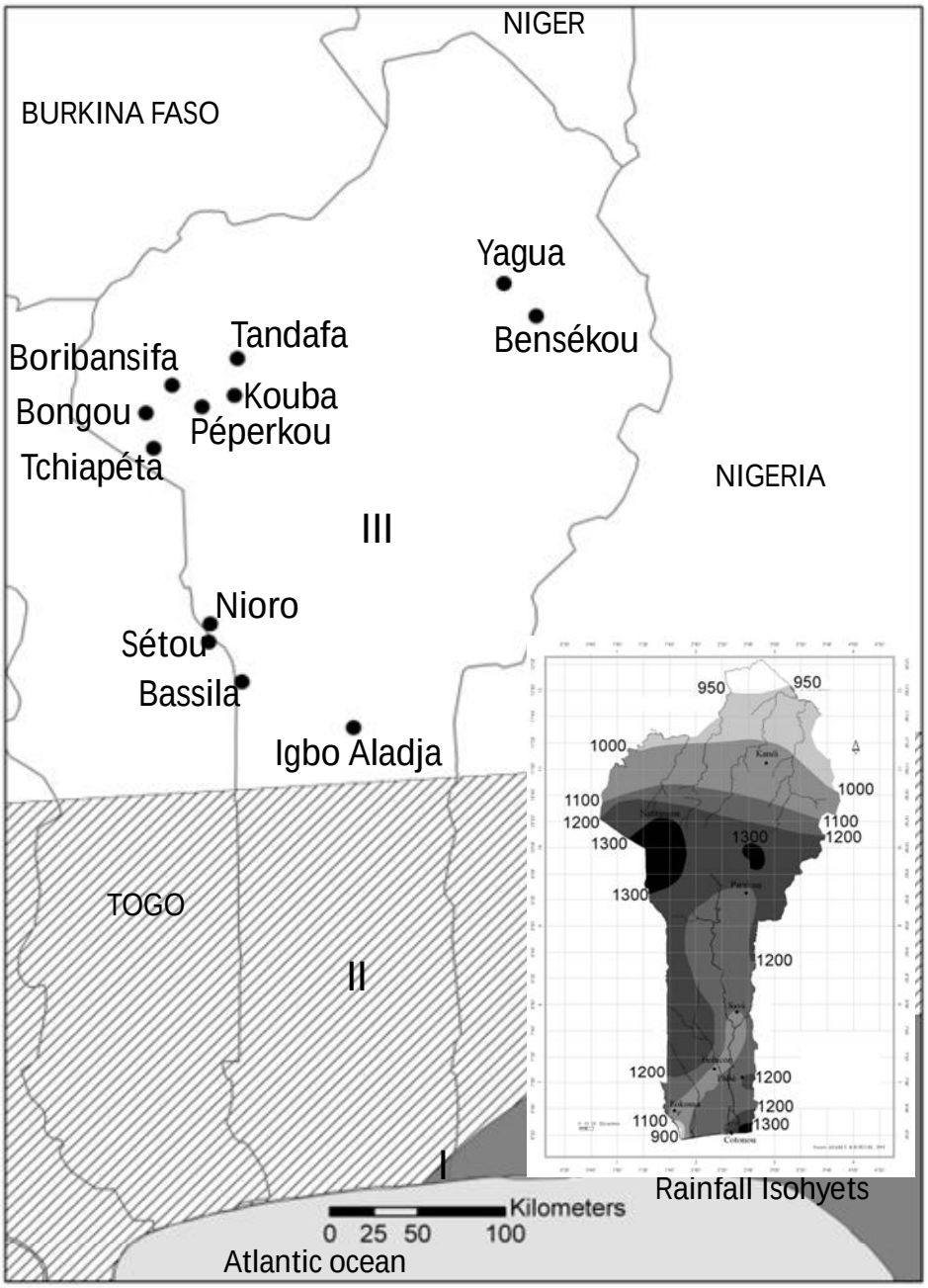

Figure 1: Location of the 12 *Pentadesma butyracea* populations sampled in Benin. Phytogeographical zones are adapted from White (1983): I = Guineo-Congolian, II = Guineo/Sudanian transition, III = Sudanian. The insert shows the distribution of rainfall (isohyets in mm, Akoegninou (2004))

Geology is determined in the west by very hard schists and quartzites (the Atacora hills), and in the east by Cretaceous clay and sandstone (Kandi plateau). Soils are tropical ferruginous with a breastplate of sandstone, or lateritic on a sandy subsoil.

Sampling and morphological characterization

A total of 348 fruiting *P. butyracea* trees were randomly sampled in 12 natural populations distant by 20 to 300 km, and at an elevation varying from 220 to 530 m (Table 1 and Fig. 1). Two different agro-ecological zones were targeted based on *P. butyracea* distribution range: (1) Centre-East and North-West of the Sudanian zone and (2) North and North-East of the Sudanian zone (Dagbénonbakin *et al.* 2003). Population sizes (number of adult individuals) ranged from 8 to 144 individuals. Within each population, trees included in the study were separated by 5 to 200 m.

For each selected tree, two to four fresh mature fruits were collected. A set of 27 morphological variables relative to the tree, fruits and seeds were recorded (Fig. 2). Materials used for dendrometric measures included circumferential ribbon (for diameter at breast height), clinometer suunto (for tree height), pentadecameter (for crown diameter). Caliper was used to measure fruit and seed sizes. According to their shape, the 796 fruits and 4900 seeds collected were categorized into six and seven shape categories respectively: fruits were ellipsoid, globular, oblong, obovoid, ovoid, or twisted, whereas seeds were ellipsoid, globular, oblong, obovoid, ovoid, pyramidal, or reniform (Fig. 3). The descriptors are nearly similar but modified from those used for shea, litchi and bush mango fruits (IPGRI 2006, IPGRI 2002, Leakey *et al.* 2000, see details in Appendix 1). Leaf characters were not measured because accessing leaf was often difficult due to tree height. Compound variables have also been computed: crown thickness and several ratios of tree, fruit and seed measures providing shape information.

Table 1: Study sites and sampling of *P. butyracea* trees: geographical coordinates of the studied populations (UTM, zone 31N), elevation recorded by GPS (Elev.), habitat: dry semi deciduous forest surrounded by savannahs (DDF), gallery forest (GF), mean annual rainfall, number of adult trees in the population (N), number of these trees that were sampled and included in the study (N. S.)

Population	Lat.	Long.	Elev. (m)	Habitat	Rainfall (mm)	N.	N. S.
Bassila	994051	352286	376	DDF	1360	78	45
Igbo Aladja	967239	415216	257	GF	1199	90	29
Nioro	1027648	334795	431	DDF	1381	11	10
Sétou	1017132	333860	267	DDF	1381	144	115
Bongou	1150260	299291	267	GF	1062	19	12
Péperkou	1160673	332713	393	GF	1269	37	27
Kouba	1165435	350697	498	GF	1269	90	20
Tandafa	1181752	350529	366	GF	1269	43	34
Tchiapéta	1129815	303458	388	GF	1269	10	6
Boribansifa	1166512	313953	506	GF	1112	8	7*
Bensékou	1206072	518022	224	GF	994	22	9
Yagua	1225048	499778	272	GF	994	43	34

*seeds could not be studied in population Boribansifa

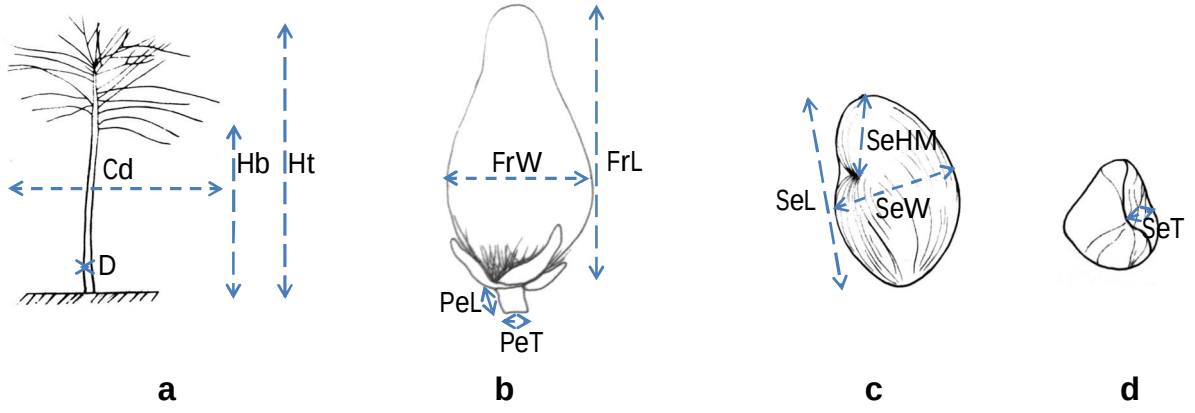

Figure 2: Morphological traits measured on *Pentadesma butyracea*

(a) tree: crown diameter (Cd), diameter at breast height (D), bole height (Hb), total height (Ht)

(b) fruit: fruit length (FrL), fruit width (FrW), pedicel's length (PeL), pedicel's thickness (PeT), number of seeds (SeN)

(c) and (d) seed: seed's length (SeL), seed's width (SeW), distance between hilum and micropyle (SeHM), seed thickness (SeT)

Data analyses

The mean and variance of each morphological trait was calculated within each population and for all the samples. The variability of each trait within and between populations was evaluated using Variance Component Analysis (Dagnelie 2006). The differences among populations were assessed using ANOVA's and Tukey's tests. When variances were not homogeneous among populations according to F-ratio tests, non-parametric Kruskal-Wallis tests were performed. Mean trait values per population were regressed on latitude, longitude and De Martonne's Index of aridity (1926): $I = \frac{P}{T+10}$, where P is the mean annual rainfall (mm) and T the mean annual temperature (°C). The *I* index is especially weak when the aridity is strong. These analyses were run in R2.12.0 (Venables and Smith 2010).

Groups of individuals with similar morphological characteristics were defined using a hierarchical cluster analysis realized with the Community Analysis Package 2.15 (Hendeson and Seaby, 2002). In this analysis, the unit is the tree. For quantitative traits, means of the fruit and seed traits were used while, for qualitative traits, the percentage of occurrence for each fruit and seed shape category was used. Ratios of traits were also considered to define shape parameters. In multivariate analyses, for each ratio of traits included, only one of the traits involved in the ratio was also included to avoid redundancy. The (ratios of) traits included in multivariate analyses were: Ht, Ht/D, Ht-Hb, Ht-Hb/Cd, Br/Ht, FrL, FrL/FrW, PeW, PeL, PeL/PeT, SeN, SeL/SeW, SeL/SeHM, SeT, FrL/SeL, and the proportions of each fruit and seed shape categories. Prior to analysis, each trait was standardized. Cluster analysis was run using Euclidian distances and Ward's method. A principal component analysis (PCA) performed with Canoco (ter Braak and Smilauer 1998) identified the main morphological gradients. The PCA factor scores were correlated with the climatic index of De Martonne (1926).

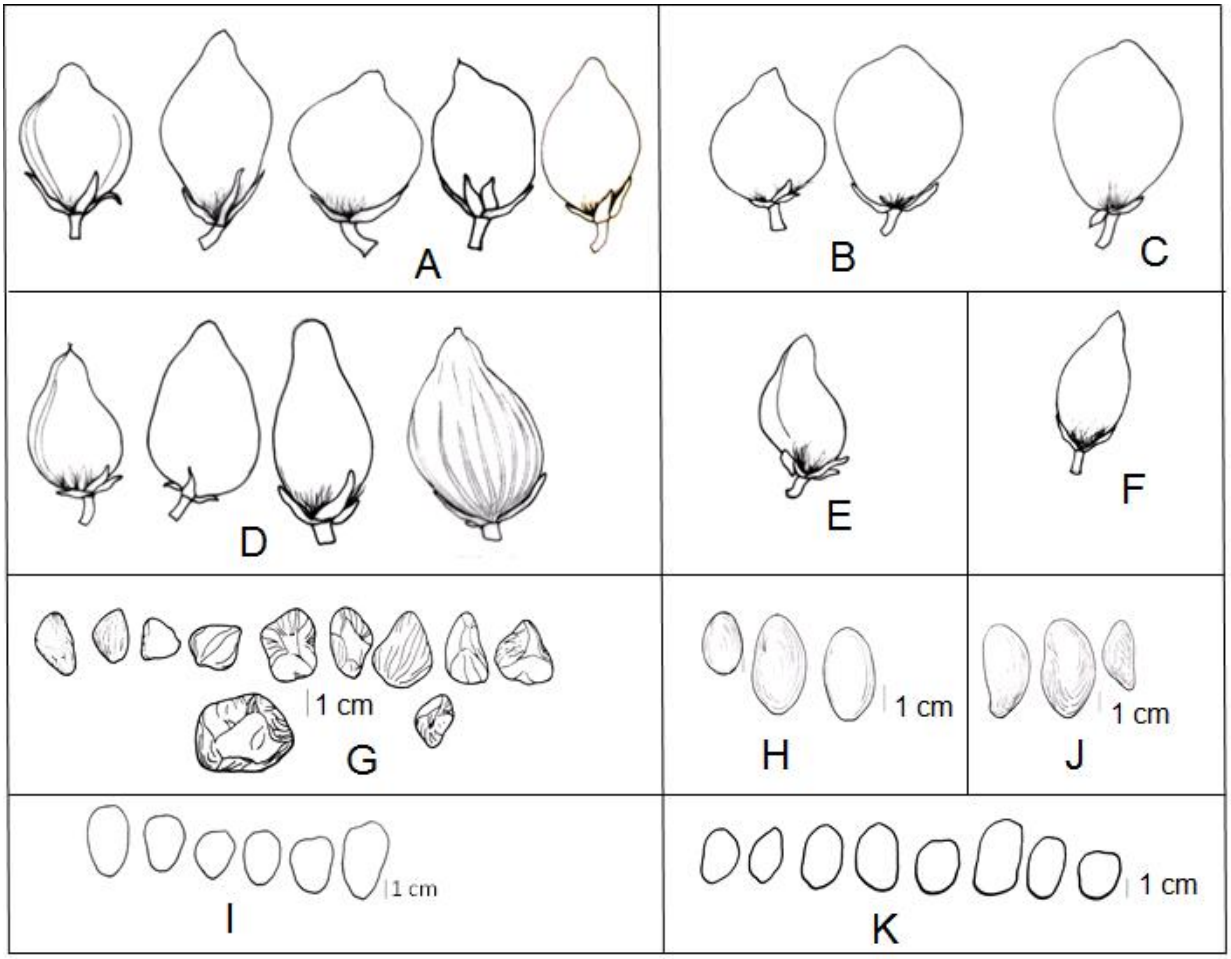

Figure 3: Morphological variability of fruits and seeds of *Pentadesma butyracea* (results from 12 populations of Benin).
Fruit shapes: ellipsoid (A), globular (B), obovoid (C), ovoid (D), ellipsoid twisted (E), oblong (F)
Seed shapes: pyramidal (G), ellipsoid (H), obovoid (I), reniform (J), oblong (K)

Results

Variability of morphological traits

Values and analyses for quantitative morphological traits at population level are provided in Online Resources 1 & 2. Traits were highly variable within populations and most morphological variability was expressed within populations rather than between populations. Nevertheless, the variance among populations was highly significant ($p < 0.0001$, ANOVA or Kruskal-Wallis test). Variance among populations ranged from 5 to 25% of total variance for traits related to tree characteristics, from 20 to 50% for fruit traits and from 31 to 44% for seed traits. Main phenotypic differences among populations can be summarized as follows:

- Populations in the north of the country presented smaller fruits and seeds than the four populations in the south, and their fruits contained a lower number of seeds (1-3). Northern populations counted a small number of trees.

- Number of branches, total height and height/diameter ratio were higher for trees in the southern populations than for those in the northern populations.
- Diameter at breast height was significantly different ($p < 0.01$) only between the populations Kouba ($D = 49.9 \pm 14.9$ cm) and Boribansifa (23.7 ± 11.0 cm).

Values and analyses for qualitative morphological traits are provided in Online Resources 3 & 4. Apart from twisted fruits that were only observed in the Tchiapeta population, the four main fruit shapes (ellipsoid, globular, obovoid, ovoid) were abundant in several populations. However, relative proportions of these four shapes varied significantly among populations (Kruskal-Wallis test, p-value <0.001), with more than 50% of overall variance expressed among populations. By contrast, for seeds, most populations presented all types of seed shapes in similar proportions.

At tree level, a cluster analysis of morphological traits identified two groups at the 80% dissimilarity level. The first group or trees, that will be referred to as the “South Sudanian group” includes 173 individuals, of which 92% came from the four populations in the south of the study area. The second group that will be referred to as the “North Sudanian group”, consisted of 165 individuals of which 85% belong to populations from the North-west and North-east.

Correlations between ecological conditions and morphological traits

A significant negative correlation was observed between latitude and mean trait values over all trees within a population for fruit length (Pearson $r = 0.34$; $p < 0.05$), canopy thickness Ht-Hb, ($r = 0.38$; $p < 0.05$) and number of branches ($r = 0.45$; $p < 0.05$). Similar results were obtained when latitude was replaced by De Martonne's Index of aridity ($r = 0.39$ and 0.42 for fruit length and canopy height, respectively). Hence, tree height and fruit size decrease with latitude and degree of aridity. However, a large number of tests were run and these correlations became non-significant after Bonferroni correction.

The PCA performed on individual trees is presented in Fig. 4. Although the ordination does not show any clear discontinuity, trees from the same population tend to be clustered in a same part of the ordination plan. Trees from populations located in the south were positively and highly correlated with tree height and seed size, contrary to those located in the north. This axis was positively correlated with the climate index of De Martonne (Pearson's correlation $R = 0.73$). The second axis was negatively correlated to the length over width ratios of pedicel and fruit, and positively correlated to fruit length and the ratio of fruit and seed length. It separated individuals from Igbo Aladja (open circles on Fig. 4) in the south with big trees and longer fruits from those of Yagua (star symbols on Fig. 4) in the north-east with low tree diameter and high length over width ratios of pedicel and fruit.

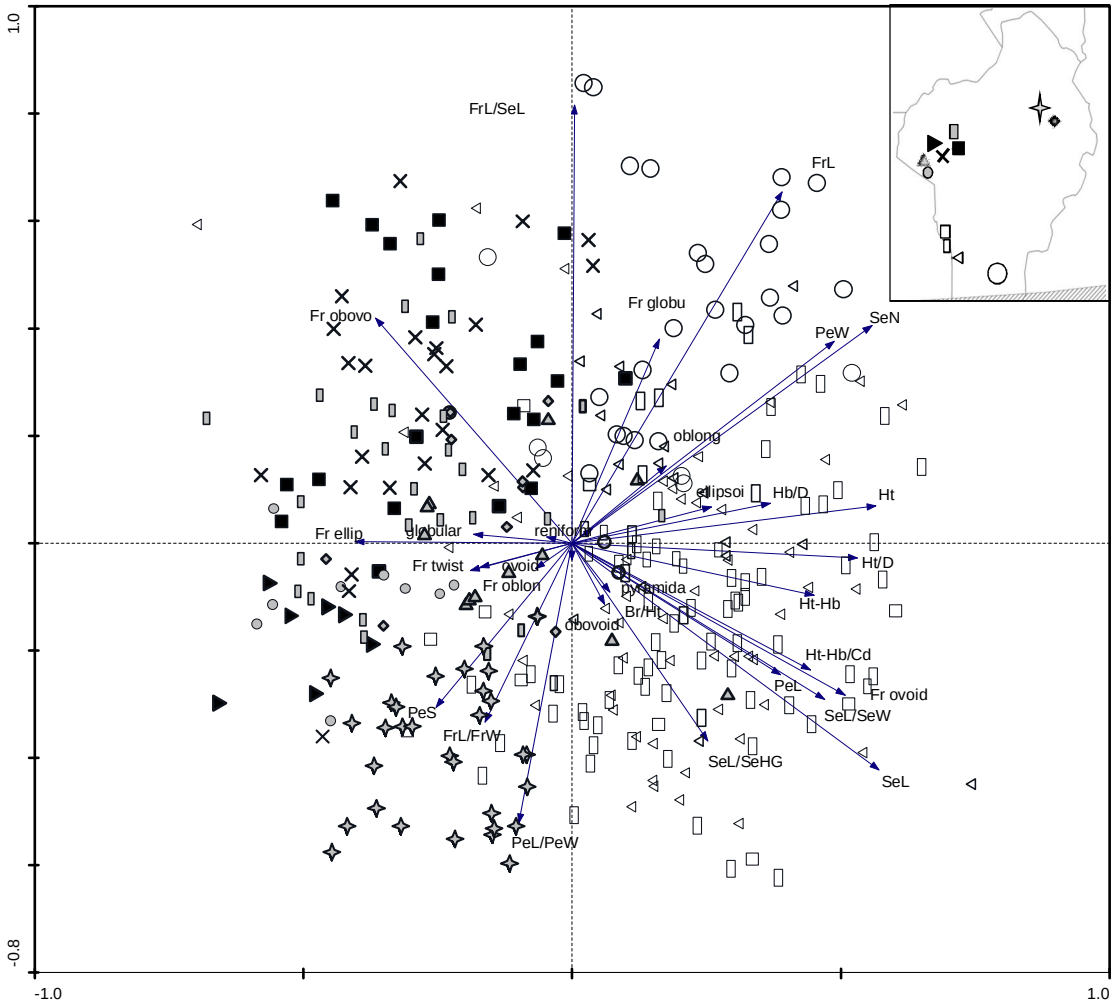

Figure 4: PCA ordination of *Pentadesma butyracea* individuals from 12 populations in Benin according to their morphological traits. Full symbols indicate trees from northern populations while hollow symbols indicate trees from southern populations (see map on the insert). Axis 1 and axis 2 represent respectively 32.6% and 26.6% of total variance of 27 trait values measured on 348 trees. Vectors indicate the ordination of traits or trait ratios (see Fig. 2 and Online Resource 1 of the Electronic supplementary material for the definitions of traits).

Discussion

Our study confirms the existence of a large morpological variability in *Pentadesma butyracea* in Benin for tree, fruit and seed traits. Trees in the north are smaller, produce less and smaller fruits and seeds than trees in the south. It is very likely that these differences are due to the better increase in rainfall when one goes from the north to the south of the country. Comparable results showing distinct morphological groups in Benin have been obtained with the tree *Tamarindus indica* L. by Fandohan *et al.* (2011): fruits, seed sizes and seed weights were positively correlated with climatic index and negatively with insolation and maximum temperature. In Shea trees (*Vitellaria paradoxa* C.F. Gaertn.), a positive correlation was found between leaf or fruit sizes and rainfall (Sanou *et al.* 2006).

When compared to measures reported in literature for *P. butyracea*, our results in Benin showed smaller values or size-related tree, fruit and seed traits than what was recorded in the Guineo-Congolian evergreen forest zone (Sama and Sacandé 2007, Hawthorne and Jongkind 2006, White and Abernethy 1996, Aubreville 1959). This confirms that there is an important variability of these characters within the natural range of *P. butyracea* in Africa. Such differences could be explained by climatic differences: the drier conditions in Benin (Sudanian zone) compared to the the Guineo-Congo evergreen forest zone. According to Zepernick and Timler (1984), the presence of this plant species in the Sudanian zone of Benin is explained by a higher relative humidity and lower temperatures than in the lowland, the accumulation of water at the hills' feet and the presence of water in streams all the year.

Plants growing in different environments often grow at different rates, and will be of different sizes and developmental stages at a given age. Our results show that large fruits and seeds were produced by larger trees, within each population as well as between populations where a latitudinal gradient occurs. Taller trees have an advantage over shorter ones: they intercept more light for photosynthesis and might therefore be able to produce large seeds and fruits. However, the allometry relationship between fruit and seed size might also be attributed to differences in breeding system among the two regions. Indeed, *Pentadesma* trees in the north belong to small and isolated populations (mean ± sd = 34 ± 26 trees) while populations in the south are larger (80 ± 54 trees). *P. butyracea* produces hermaphrodite flowers but self-pollination seems to be prevented (Ewedje, unpublished results), possibly by mechanisms of self-incompatibility, or by spatial and/or temporal maturity separation of sexes within flowers (herkogamy and/or dichogamy). In small isolated populations, self-incompatibility reactions in self-pollinated flowers and/or resources limitation could reduce fertilization success and

consequently the number of seeds per fruit (Diallo *et al.* 2008). Opposed to this, higher levels of outcrossing might promote significantly larger seed and fruit sizes (Mitchell-Olds and Waller 1985, Jordano 1993) in the southern populations.

Trees in the population Boribansifa have the lowest mean values for tree height as well as for fruit and seed sizes. This could be explained by the presence of lateritic breastplate in this area. Even trees with large trunk diameters were dwarfed and produced small fruits. This is why seed traits could not be studied in this population because there were not enough fruits produced. This suggests that edaphic conditions affect phenotypic variation of this tree species, as was described for many other plant species (Sultan 2000, Schlichting and Pigliucci 1998).

Since phenotypic traits were recorded in natural populations, it is not possible to assess whether the differences between populations and in particular between climatic regions result from genetic differences or phenotypic plasticity i.e., the ability of a genotype to generate different phenotypes depending on environment differences where trees were growing. Morphological records on trees growing in common garden conditions would be necessary to quantify the part due to the genetic differentiation from that due to environmental variation. Nevertheless, our results have already implications for the conservation and further improvement of *Pentadesma butyracea* because at least two eco-geographical regions should be considered in programs aiming at conserving the genetic resources or aiming at improving the resource for butter production.

Conclusion and perspectives

Pentadesma butyracea populations in Benin can be subdivided into two eco-geographical groups according to their morphological characteristics. Trees in the north are smaller and produce smaller fruits containing a lower number of seeds than trees in the south. To distinguish the relative roles of genetic variation between populations and phenotypic plasticity related to environmental conditions, this morphological study should be completed with an analysis of the intra-specific genetic variation within and between populations, and further phenotypic studies should be conducted in common garden experiments. This would provide insights to define a conservation strategy for natural *Pentadesma butyracea* populations and would be very valuable to develop breeding programs in order to increase kanya butter production in Benin.

Acknowledgments

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Appendix 1: Sites and plant descriptors measured in *P. butyracea*

	Plant descriptors
Tree	[†]Tree height, [†]trunk circumference, [†]trunk surface, [†]crown diameter, [†]tree vigour (burned or unburned tree), [†]branching density (number of branches), [†]branching pattern
Fruits	[†]Fruit shape including shoulder and tip (fruit shapes), [†]fruit segment (fruit texture), [†]cracking/splitting of fruit skin, [†]mature fruit colour, [†]distribution of colour on fruit surface, [†]fruit length (per fuit), [†]fruit width (at the widest point),
Seeds	[†]Seed length, [†]seed thickness and [†]seed width (for each seed), [†]seed coat colour

[†]similar traits with existing descriptors (IPGRI 2006, IPGRI 2002, Leakey *et al.* 2000), [0]modified morphological traits

Online Resource 1
Morphological variability of the tallow tree, *Pentadesma butyracea* Sabine (Clusiaceae), in Benin

Mean values ± standard deviations of tree traits in each *P. butyracea* population and variance partitioning. All ANOVA or Kruskal-Wallis tests were significant (p-value < 0.001), letters (a, b, c, d) indicate homogeneous groups (α = 0.05 after Bonferroni correction).
Br: number of branches, D: diameter at breast height, Hb: bole height, Ht: total height, Cd: crown diameter

Population	Br	D	Hb	Ht	Cd
Bassila	25 ± 10 b	34.8 ± 10.3 ab	9.5 ± 3.7 a	17.1 ± 3.8 d	9.8 ± 2.7 b
Igbo Aladja	28 ± 10 b	43.6 ± 14.3 ab	13.0 ± 5.3 b	19.0 ± 5.8 d	11.3 ± 3.0 b
Nioro	25 ± 13 b	41.0 ± 24.6 ab	7.3 ± 2.5 a	13.5 ± 4.7 ab	8.0 ± 2.1 a
Sétou	30 ± 15 b	39.6 ± 16.3 ab	11.7 ± 5.3 b	18.8 ± 5.7 d	10.9 ± 3.3 b
Bongou	26 ± 8 b	36.7 ± 12.6 ab	9.5 ± 2.9 ab	13.9 ± 3.4 b	10.2 ± 2.5 b
Péperkou	13 ± 4 a	34.8 ± 9.6 ab	7.7 ± 2.1 a	13.0 ± 3.0 ab	12.1 ± 3.6 b
Kouba	13 ± 6 a	44.9 ± 14.9 b	10.0 ± 2.1 ab	14.5 ± 2.8 b	10.7 ± 3.2 b
Tandafa	17 ± 7 a	39.7 ± 14.8 ab	10.0 ± 2.8 ab	16.0 ± 3.3 b	11.1 ± 3.7 b
Tchiapéta	13 ± 7 a	34.2 ± 11.6 ab	8.6 ± 3.8 ab	14.5 ± 4.4 b	13.0 ± 4.2 b
Boribansifa	10 ± 4 a	23.7 ± 11.0 a	5.2 ± 2.8 a	7.3 ± 3.6 a	6.3 ± 2.2 a
Bensékou	23 ± 9 b	31.8 ± 17.6 ab	10.1 ± 2.2 ab	14.9 ± 2.5 b	8.4 ± 3.3 a
Yagua	17 ± 8 a	35.8 ± 12.1 ab	8.3 ± 2.5 a	13.7 ± 3.0 ab	7.6 ± 1.8 a
All populations: mean ± sd (range)	23 ± 12 (2 - 80)	37.0 ± 13.8 (7.6 - 88.9)	9.8 ± 4.0 (1.3-2.4)	16.1 ± 4.7 (3.9 - 29)	10.1 ± 3.3 (3 - 20)
Var. between pop.	36.75	9.64	2.18	5.84	1.94
Total variance	144	190.44	16	22.09	10.89
% var. between pop.	24	5.06	13.62	24.93	17.58

Online Resource 2

Mean values ± standard deviations of fruits and seed traits in each *P. butyracea* population and variance partitioning. All ANOVA or Kruskal-Wallis tests were significant (p-value < 0.001), letters (a, b, c, d) indicate homogeneous groups (α = 0.05 after Bonferroni correction).
PeL: pedicel's length, PeT: pedicel's thickness, FrL: fruit length, FrW: fruit width, SeN: number of seeds, SeL: seed length, SeW: seed width, SeT: seed thickness, SeHM: distance between hilum and micropyle.

Variables	PeL	PeT	FrL	FrW	SeN	SeL	SeW	SeT	SeHM
Bassila	3.5 ± 0.4 d	1.1 ± 0.2 bc	13.6 ± 3.0 b	9.1 ± 2.2 bc	11 ± 4 c	3.8 ± 0.3bc	2.8 ± 0.2 ab	2.44 ± 0.2 c	2.4 ± 0.3 ab
Igbo Aladja	2.8 ± 0.5 b	1.2 ± 0.2 bc	14.1 ± 2.9 b	10.3 ± 1.7 c	10 ± 5 bc	3.8 ± 0.4bc	2.9 ± 0.2 bc	2.34 ± 0.2 bc	2.6 ± 0.3 bc
Nioro	3.8 ± 0.7 d	1.3 ± 0.4 c	13.8 ± 2.5 b	9.3 ± 2.1 bc	5 ± 3 ab	3.9 ± 0.7bc	2.9 ± 0.3 bc	2.30 ± 0.2 abc	2.6 ± 0.5 abc
Sétou	3.5 ± 0.3 d	1.2 ± 0.2 bc	13.3 ± 3.4 b	8.7 ± 1.8 bc	10 ± 4 bc	4.1 ± 0.5c	3.0 ± 0.3 c	2.43 ± 0.3 c	2.7 ± 0.3 c
Bongou	3.2 ± 0.5 b	1.2 ± 0.1 bc	12.9 ± 2.0 b	9.0 ± 1.5 bc	8 ± 3 b	3.5 ± 0.3ab	2.8 ± 0.2 ab	2.19 ± 0.3 ab	2.4 ± 0.2 ab
Péperkou	3.1 ± 0.4 b	1.1 ± 0.2 bc	13.9 ± 2.2 b	9.8 ± 1.7 bc	7 ± 3 b	3.5 ± 0.5 ab	2.8 ± 0.3 ab	2.20 ± 0.2 abc	2.5 ± 0.3 abc
Kouba	2.8 ± 0.3 b	1.1 ± 0.2 bc	13.2 ± 3.0 b	9.4 ± 2.3 bc	7 ± 3 b	3.1 ± 0.3a	2.5 ± 0.2 a	2.01 ± 0.2 a	2.2 ± 0.3 a
Tandafa	3.1 ± 0.4 b	1.1 ± 0.1 bc	13.1 ± 3.5 b	8.8 ± 2.3 bc	8 ± 4 b	3.6 ± 0.2 abc	2.9 ± 0.1 bc	2.23 ± 0.2 abc	2.5 ± 0.2 abc
Tchiapéta	2.8 ± 0.4 b	1.1 ± 0.1 bc	12.5 ± 0.4 b	7.4 ± 0.4 b	2 ± 0 a	3.8 ± 0.0 bc	2.5 ± 0.3 a	2.10 ± 0.3 ab	2.1 ± 0.0 a
Boribansifa	1.2 ± 0.2 a	0.5 ± 0.1 a	10.0 ± 1.2 ab	6.5 ± 1.3 a	3 ± 1 a	-	-	-	-
Bensékou	2.4 ± 0.4 b	1.0 ± 0.2 b	12.6 ± 1.9 b	9.2 ± 1.5 bc	8 ± 4 bc	3.5 ± 0.3 ab	2.7 ± 0.1 ab	2.11 ± 0.1 ab	2.4 ± 0.2 ab
Yagua	3.3 ± 0.2 b	1.1 ± 0.1 bc	8.3 ± 0.7 a	4.6 ± 0.8 a	3 ± 1 a	3.5 ± 0.3 ab	2.8 ± 0.2 ab	2.16 ± 0.3 ab	2.3 ± 0.3 ab
All populations: mean ± sd (range)	3.3 ± 0.6 (1.0 – 5.4)	1.1 ± 0.2 (0.4 – 2.3)	12.9 ± 3.2 (6.0 – 29.0)	8.6 ± 2.3 (2.8 – 17.0)	8 ± 4 (1-31)	3.8 ± 0.5 (2.5 – 5.5)	2.9 ± 0.3 (2.0 – 3.8)	2.3 ± 0.3 (1.6 -3.3)	2.5 ± 0.4 (1.7 – 3.7)
Var. between pop.	0.2	0.01	2.75	2.27	7.69	0.14	0.04	0.03	0.05
Total variance	0.36	0.04	10.24	5.29	16	0.25	0.09	0.09	0.16
% var. between pop.	51.36	19.9	25.62	37.88	33.75	43.99	36.12	32.13	30.99

Online Resource 3

Percentages of occurrence of each fruit shape per population and standard deviations (individual trees), and variance partitioning. Populations differ significantly (p-value < 0.001) for all traits (Kruskal-Wallis tests).

Variables	ellipsoid	globular	oblong	obovoid	ovoid	twisted
Bassila	0	14.9 ± 11.5	0	6.0 ± 5.2	78.4 ± 13.1	0
Bensekou	0	6.1 ± 7.6	0	23.6 ± 7.6	70.8 ± 6.7	0
Bongou	0	0	0	0	100 ± 0	0
Igbo Aladja	0	24.0 ± 11.1	0	17.6 ± 13.6	57.8 ± 15.3	0
Kouba	0.8 ± 1.8	19.3 ± 13	0	15.9 ± 13.8	62.0 ± 15.7	0
Nioro	19.4 ± 11.3	0	0	0	80.5 ± 11.3	0
Peperkou	47.5 ± 9.6	0	0	20 ± 8.2	22.5 ± 5	5 ± 5.8
Setou	6.1 ± 5.5	3.1 ± 3.2	0	0	90. 8 ± 6.5	0
Tandafa	72 ± 9.1	13.4 ± 13.5	1 ± 2.2	4.6 ± 3.6	9 ± 12.4	0
Tchiapeta	0	0	0	0	0	100 ± 0
Yagua	25 ± 12	0	0	0	75 ± 12	0
Var. between pop.	229.69	79.78	0.02	85.29	478.36	119.42
Total variance	254.69	147.38	0.12	130.19	591.36	119.93
% var. between pop.	90	54	20	65	80	99

Online Resource 4

Percentages of occurrence of each seed shape per population and standard deviations (individual trees). Significant differences among populations: *p < 0.05, **p < 0.01 (Kruskal-Wallis tests)

Variables	ellipsoid	globular**	oblong*	obovoid	ovoid	pyramidal	reniform*
Bassila	19.7 ± 18.8	2.64 ± 7	15.9 ± 22.7	24.8 ± 22.4	13.0 ± 19.9	19.0 ± 18	4.8 ± 10.4
Bensekou	16.9 ± 15.4	6.4 ± 9.3	12.0 ± 9.21	21.6 ± 10.8	19.4 ± 12.3	16.7 ± 19.2	6.8 ± 9.2
Bongou	21.2 ± 27	7.4 ± 11.9	3.6 ± 8.6	23.7 ± 19.7	16.4 ± 13.5	22.0 ± 18.4	5.6 ± 10.7
Igbo Aladja	15.6 ± 11.8	3.0 ± 4	14.9 ± 7.7	18.6 ± 11.8	17.5 ± 12.9	25.2 ± 16	5.0 ± 5.9
Kouba	12.7 ± 8.9	11.1 ± 11.6	13.7 ± 8.4	17.2 ± 10.8	17.9 ± 12.6	19.6 ± 15.1	7.7 ± 9.6
Nioro	21.8 ± 34.1	11.5 ± 11.7	14.7 ± 11.7	12.1 ± 17.1	13.8 ± 11.8	25.4 ± 17.1	0.6 ± 1.8
Peperkou	24.9 ± 5.4	0.6 ± 1.2	14.4 ± 10.9	24.3 ± 6.5	11.2 ± 11.9	16.4 ± 6.7	8.1 ± 5.5
Setou	16.8 ± 17	3.8 ± 9	17.9 ± 19.2	19.4 ± 21.2	13.4 ± 16	23.8 ± 20.5	4.8 ± 10.9
Tandafa	17.8 ± 14.5	5 ± 11.2	3.7 ± 5	19.7 ± 6.8	18.3 ± 11.4	24.8 ± 27.2	10.7 ± 11.4
Tchiapeta	75 ± 35.3	0	0	0	0	25 ± 35.3	0
Yagua	17.9 ± 20.9	1.8 ± 6	6.4 ± 11.6	22.7 ± 14.5	21.5 ± 16.8	23.3 ± 20.6	6.4 ± 11.6
Var. between pop.	27.39	7.29	11.23	0	0	11.11	0
Total variance	365.39	85.69	286.23	339.07	247.48	348.89	96.96
% var. between pop.	7	8	4	0	0	3.6	0

CHAPITRE V.-

DIVERSITÉ GÉNÉTIQUE DE *PENTADESMA BUTYRACEA* SABINE (CLUSIACEAE) AU BÉNIN

Manuscript not submitted

Diversité génétique de *Pentadesma butyracea* Sabine (Clusiaceae) au Bénin

Dans le but de définir les bases de conservation et de gestion durable des ressources génétiques de *Pentadesma butyracea*, l'organisation de la diversité génétique de l'espèce a été étudiée au sein de 16 populations (comprenant 9 à 144 individus) représentant l'aire de distribution de l'espèce au Bénin. Pour ce faire, huit loci microsatellites nucléaires ont été analysés.

Toutes les populations présentent des indices de diversité génétiques similaires et de valeur faible. L'hétérozygotie attendue est d'une part positivement corrélée avec la taille des populations (R^2 = 0,22 ; F = 3,89 ; p-value = 0,06) et d'autre part négativement et significativement corrélée avec la longitude (R^2 = 0,36 ; F = 7.79 ; p-value = 0.01). Pour un total de 51 allèles trouvés, le nombre moyen d'allèles par locus est estimé à *Na* = 2,42 ± 0,82, la richesse allélique moyenne est *A* = 2,04 ± 0,21 et l'hétérozygotie moyenne attendue par locus est H_E = 0,34 ± 0,07. La population Kouba, située à l'Ouest est la plus diversifiée (*Na* = 4,8 ; *A* = 2,6 ; H_E = 0,48) tandis que celle de Yagua à l'Est de Kandi est la moins diversifiée (*Na* = 1,2 ; *A* =1,7 ; H_E = 0,13).

Les analyses bayésiennes de détection des discontinuités génétiques réalisées à l'aide du logiciel STRUCTURE ont montré l'existence de deux unités génétiques montrant une différentiation faible le long d'un gradient Ouest-Est (F_{ST} = 0.05 ; R_{ST} = 0.08 ; R_{ST} non significativement différent de F_{ST}). Ces résultats indiquent une faible contribution des mutations à la différentiation par rapport à la dérive génétique (Hardy et al. 2003). Ceci suggère une différentiation récente entre les deux pools de gènes soumis à des évènements de goulots d'étranglement démographiques. Selon des résultats phylogéographiques antérieurs, ces populations résulteraient d'une population ancestrale provenant des forêts denses humides de l'Afrique de l'Ouest (Chapitre 3).

Les deux pools génétiques contrastent avec les deux groupes éco-morphologiques identifiées au Bénin selon un gradient Nord-Sud, ce qui suggère que la variabilité morphologique observée découlerait soit d'une plasticité phénotypique (tout au moins les variables quantitatives), soit d'une pression de sélection (non détectée par les marqueurs génétiques utilisés) contre la baisse de la pluviométrie.

En vue de la conservation de *Pentadesma butyracea* au Bénin, nous recommandons l'enrichissement des habitats existants et/ou la création de vergers, à partir de semences provenant des deux unités génétiques détectées.

Genetic diversity of *Pentadesma butyracea* Sabine (Clusiaceae) in Benin

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Abstract

Tallow tree (*Pentadesma butyracea* Sabine) is an Africa rainforest species and an important food tree species in dried forests of West Africa. Recent interests in socio-economic value, biochemical characterization of butters and implementation of conservation strategies requires a good understanding of biology and genetics of the species. Using eight informative microsatellite loci, we: (a) assessed the nuclear genetic diversity of sixteen natural populations, and (b) investigated temporal effective-size fluctuations in *Pentadesma butyracea* natural populations, with a view to identify the role of past demographic events in the genetic structure of the studied species. The eight loci were variable; moderate levels of genetic diversity (A = 2.04 ± 0.21; H_E = 0.39 ± 0.24) and feeble differentiation among populations (F_{ST} = 0.13 and R_{ST} = 0.15) were found within and among populations in wild stands, indicating extensive gene flow. Analysis of population structure provided evidence for the presence of two groups of populations displaying a few contrast due to an extensive gene flow.

Keywords: *Pentadesma butyracea*, genetic variation, gene flow, nuclear microsatellite loci, population structure.

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Introduction

African tropical rainforests are subjected to both continuous land degradation and climate change that lead to significant loss in biodiversity. Degradation is more pronounced in West Africa where it reached 1.5% annually in some countries from 1990 to 2000 (FAO 2001). These deforestation threats are also very marked in drier areas, especially in the Dahomey Gap between Ghana and Nigeria where savannahs replaced moist forests, due to a decline in annual precipitation from more than 2000 mm in the rainforest areas of Nigeria and Ivory Coast to about 1200-1000 mm in the Dahomey Gap, where remnant patches of African rainforests survived in more humid habitats (gallery forests, foot of hills). In Benin, some of these patches harbour many specific moist forest plants species such as *Octoknema borealis, Anthonotha macrophylla, Distemonanthus benthamianus, Mansonia altissima, Pentadesma butyracea,* etc. Some of these remnant areas of rainforest probably originate from the humid Holocene period (c. 8400 and 4500 years BP) during which the rainforest reached its maximal extension covering the Dahomey Gap (Tossou 2002, Salzmann and Hoelzmann 2005). Since then, they have been subject to the effects of stochastic and determinist factors resulting in a significant decrease in the number of plant species. Consequently, inbreeding and loss of genetic diversity followed by reduced adaptability, survival and reproduction may result from habitat fragmentation (Frankham *et al.* 2002).

Among the species found in these forest patches, the case of *Pentadesma butyracea* is troubling. While the species mostly occurs in African moist forests where it has limited use, it is also growing in drier forests where it plays an important socio-economic role especially in Benin and Togo where women produce and market a yellow butter extracted from the seeds' kernel which is used as a food and medicinal ingredient (Tchobo *et al.* 2007). Overexploitation is so high that seed harvesting can reduce seeds available for recruitment by as much as 70% (Avocèvou *et al.* 2009). So, this plant species is considered as one of the ten most threatened food tree species in Benin and Togo (Dah-Dovonon 2002; Poidy 2002) due to habitat fragmentation and reduced recruitment due to overexploitation. Currently, there is a lack of sylvicultural knowledge for its restoration and preservation in sub-Saharan Africa (Eyog-Matig *et al.* 2002). This has generated renewed scientific interest in the species particularly in Benin and Cameroon.

To be sustainable, any breeding program should be based on the prior assessment of genetic diversity, both at phenotypic and molecular levels. Indeed, the assessment of pattern of genetic diversity, as well as investigations of the relationships between gene flow, demographic connectedness and historical partitions within species (Avise 1996), constitute a

prerequisite to any management of genetic resources. It is known that butter from various origins is characterized by different proportions of fatty acid in Benin (Tchobo *et al.* 2007). Moreover, morphological traits indicate the existence of two eco-geographical groups following a latitudinal gradient (Ewedje *et al.* 2012). If these variations have a genetic basis, there is a potential for genetic improvement.

In spite of the biological and socio-economic value of *P. butyracea*, no data is available on the extent of its genetic variability across its entire natural range in general and particularly in Benin. Moreover, due to the rapid deforestation of its natural habitat in Benin, i.e. the conversion of gallery forests in favor of unsustainable agriculture and gardening, the knowledge of its genetic diversity (recognized as factors ensuring the long-term survival of species) and its population structure is crucial to formulate relevant conservation and management strategies (SGRP 2000), for example to define efficient sampling strategies for *ex situ* conservation or to prioritize populations that could benefit from an *in situ* conservation program. To improve our knowledge on the genetic diversity of *P. butyracea* in Benin, nuclear microsatellites were genotyped in 16 populations to answer the following questions: (1) What is the level of genetic variation in Benin and does it vary among populations? (2) Is the pattern of genetic structure of natural populations concordant with eco-geographical groups detected from phenotypic variation?

Material and Methods

Study species

P. butyracea Sabine (Clusiaceae) known as yellow butter tree is one of 1610 species from the 37 genera of the family. Within Clusiaceae, *Pentadesma* belongs to the tribe Symphonieae, one of three tribes of the subfamily Clusioideae (Steven 2007) which has, however, been found to be paraphyletic based on *rbc*L sequences (Gustafsson *et al.* 2002). The genus harbors four species. *P. butyracea* is a pantropical tree species occurring both in Guineo-Congolian evergreen forests from West (Upper Guinea) and Central (Lower Guinea) Africa and in gallery forests situated in drier climate between the two rainforest blocks, between c. 0° and 3°E longitude. In Benin, *P. butyracea* showed two eco-geographical groups: trees located in the more humid southern part of the species distribution are taller producing larger fruits and seeds than trees located in the north (Ewedje *et al.* 2012). *P. butyracea* is mainly outcrossed (tm ranged from 0.88 to 0.95), nectar-feeding animals included Sunbirds and bees pollinating large flowers (Ewédjè unpublished).

Nuclear microsatellite study

Population sampling. A total of 402 adults were sampled in 16 populations representing the overall distribution range of the species in Benin (Table 1) where the species occurs in two of the seven agro-ecological zones known in Benin: (1) Centre-East and North-West of the Sudanian zone and (2) North and North-East of the Sudanian zone (Dagbénonbakin et al. 2003). Leaf material and/or cambium tissue of all adult trees were dried and conserved in silica gel before DNA extraction.

Microsatellites Analysis. We performed polymerase chain reaction (PCR) using eleven microsatellites loci (namely Pent1, Pent10, Pent11, Pent12, Pent13, Pent14, Pent16, Pent17, Pent18, Pent22, Pent24) that were amplified in two multiplexed reactions using the QIAGEN Multiplex kit in a final 15 µL reaction volume following Ewédjè et al. (in press, see Chapter 2). However, only eight primer pairs (Table 2) consistently amplified across the geographic range.

Genetic diversity and inbreeding. For each locus, we recorded the number of alleles along with their sizes and computed observed and expected heterozygosity (Nei's 1978) and tests for deviation from the Hardy-Weinberg equilibrium (HWE) using SPAGeDi version 1.3 (Hardy and Vekemans 2002). Allelic richness was estimated using Fstat version 2.9.3.2 (Goudet 2002).

Bayesian cluster analysis. The genotypes were analyzed with the software STRUCTURE version 2.3.3 (Pritchard *et al.* 2000) to identify groups of individuals within which Hardy-Weinberg equilibrium and linkage equilibrium tend to occur. Due to inaccurate assignment that can result from heterogeneous population sizes, three populations that were heavily sampled (>90 individuals) were subsampled to keep 20 or 22 individuals, keeping a total of 217 genotypes. STRUCTURE was run 10 times for a number of clusters *K* ranging from 1 to 10. The following parameters were chosen: burn-in length of 10000 with a number of MCMC repetitions after Burnin of 10000, 10 iterations, admixture model, and allele frequencies were correlated among clusters. Because the likelihood increased steadily with increasing *K*, the most likely number of clusters was inferred with the method of Evanno *et al.* (2005).

Table 1: Sampling locations (populations), estimates of genetic diversity and inbreeding coefficient and assignment to genetic clusters determined by STRUCTURE. N_S, total number of adult individuals within population; N_{nSSR}, population size for nSSR-based analyses; N_{BAY}, sample size for Bayesian cluster analysis; d_{ij} max., maximal distance between two individuals within population; *N*a, number of alleles; *A*, allelic richness; H_E and H_O (expected and observed heterozygosity estimated after INEst). F_{IS}, inbreeding coefficient estimate after INEst, $F_{IS'}$ is estimated from INEst with account of null allele.

n°	Population	N_S	N_{nSSR}	N_{BAY}	d_{ij} max. (m)	Cluster	% of individuals assigned (> 50%)	*N*a	*A*	H_E	H_O	F_{IS}	$F_{IS'}$
1	Igbo Aladja	90	83	22	600	2	100.0	3.2 (2.25)	2.07 (2.12)	0.41 (0.36)	0.34 (0.35)	0.16 (0.04)	0 (0)
2	Kouba	91	64	20	500	1	85.0	4.8 (2.87)	2.57 (2.87)	0.48 (0.41)	0.36 (0.35)	0.25*** (0.16*)	0.07 (0)
3	Setou	144	103	23	250	1	86.9	3.5 (2.25)	2.02 (2.12)	0.41 (0.30)	0.34 (0.32)	0.16 (-0.06)	0 (0)
4	Nioro	11	11	11	50	1	72.7	1.87	1.79	0.33	0.49	-0.47	0
5	Bassila	86	11	11		1	72.7	2.25	2.07	0.34	0.27	0.19*	0
6	Kikele	18	9	9	160	2	88.9	2.00	1.86	0.30	0.29	0.03	0
7	Boribansifa	9	8	8	800	1	75.0	2.00	1.93	0.38	0.40	-0.06	0
8	Bongou	19	9	9	1200	1	55.5	2.12	1.96	0.30	0.33	-0.09	0
9	Koda	18	17	17	50	2	88.2	2.25	1.99	0.34	0.39	-0.15	0
10	Penessoulou	27	10	10	200	1	100.0	2.25	2.08	0.35	0.29	0.18	0
11	Tandafa	21	15	15	800	1	93.3	2.50	2.00	0.36	0.31	0.14	0
12	Tassigourou	19	14	14	250	2	50.0	2.00	1.95	0.36	0.44	-0.23	0
13	Tchiapeta	12	8	8	150	1	87.5	2.25	2.20	0.43	0.40	0.06	0
14	Tchoundegou	15	12	12	500	1	58.3	2.62	2.19	0.39	0.33	0.14	0
15	Yagua	43	11	11	140	2	90.9	1.25	1.70	0.13	0.12	0.01	0
16	Bensekou	23	17	17	200	2	94.1	1.87	2.35	0.28	0.22	0.22*	0

() estimates based on resampling (N_{BAY}) in the three larger populations

Table 2: Single-locus and mulitlocus estimates of genetic diversity and pairwise genetic differentiation. H_E, expected heterozygosity; F_{IT}, individual's inbreeding coefficient across all populations; F_{IS}, individual's inbreeding coefficient within population; F_{ST}, pairwise genetic differentiation; R_{ST}, equivalent of F_{ST} based on allele size.

Locus	H_E **(Nei, 1978)**	F_{IT}	F_{IS}	F_{ST}	R_{ST}
Pent1	0.06	0.12	0.10	0.05	0.04
Pent12	0.61	0.10	0.02	0.08	0.09
Pent13	0.56	0.34	0.32	0.03	0.04
Pent14	0.67	0.07	0.01	0.07	0.00
Pent16	0.47	-0.02	-0.03	0.01	0.03
Pent17	0.09	0.26	0.19	0.03	0.04
Pent18	0.15	0.22	0.22	0.00	0.03
Pent22	0.43	0.26	0.19	0.09	0.14
Average (±SD)	-	0.15 ±0.05	0.10 ± 0.06	0.05 ± 0.01	0.08 ± 0.03

Differentiation and spatial genetic structure

After defining the global pattern of population structure with the above methods, differentiation statistics (F_{ST}, R_{ST}) were computed between (pairs of) sampling locations or inferred clusters using SPAGeDi. R_{ST} is analogue to F_{ST} based on allele size; it is expected to be larger than F_{ST} if stepwise mutations have contributed to differentiation. Then null hypothesis of no population differentiation was tested for overall F_{ST} and R_{ST} using 999 permutations of individuals among locations or clusters in SPAGeDi. The presence of phylogeographic structure, i.e., whether alleles within populations were more related than alleles in the overall sample, was tested by comparing R_{ST} with its value after permuting allele sizes within loci ("permuted R_{ST}") using SPAGeDi. A significant one-sided test establishes the alternative hypothesis of R_{ST} > "permuted R_{ST}", meaning that stepwise mutations contributed to population differentiation pattern which can be interpreted as phylogeographic structure (Hardy *et al.* 2004).

The spatial genetic structure among populations was assessed using standard F-statistics. Differentiation between pairs of populations is considered as a function of their geographic distance using the ratios F_{ST} / (1 - F_{ST}). Isolation by distance was inspected through the regression of those ratios on the logarithm of the geographic distance (Rousset 1997).

Results

SSR diversity and inbreeding

Detailed information on allele sizes ranges and null allele frequencies per locus within each population are given in Appendix. Null alleles were detected in each population for some of the loci but it was more frequent in the locus Pent13 than the seven other ones. Estimates of genetic diversity and inbreeding coefficient of sampling locations are shown in Table 1. The mean number (±SD) of alleles per locus within population was 2.42 ± 0.82 (ranging from 1.2 in Yagua to 4.8 in Kouba) and allelic richness was 2.04 ± 0.21 (range 1.7-2.57 in the same populations respectively) for a total of 51 alleles (Table 1). Expected heterozygosity (H_E) per locus ranged from 0.13 in Yagua to 0.48 in Kouba (mean H_E = 0.34 ± 0.07) showing that the population Kouba was more diverse than the others. The largest population Setou with 103 individuals genotyped displayed a lower diversity (H_E = 0.41) than Kouba with 64 individuals successfully genotyped. Statistical analysis of diversity showed a marginal and positive correlation between expected heterozygosity and population size on the one hand (p-value = 0.06; F = 3.89; R^2 = 0.22), and a significant negative correlation between expected heterozygosity and longitude on the other hand (p-value = 0.01; F = 7.79; R^2 = 0.36). The global inbreeding coefficient was significantly positive in three populations: Bensekou (F_{IS} = 0.22), Bassila (F_{IS} = 0.19) and Kouba (F_{IS} = 0.25). We checked whether departure from HWE at a given locus might be explained by the presence of null alleles by using the software INEst (Chybicki and Burczyk, 2009), which jointly estimates inbreeding and null allele frequencies to account for deviation from HWE. Under the population inbreeding model, null allele frequency estimates were significantly different from zero at all loci that deviated from HWE. Particularly, Kouba displayed 3 loci with null allele frequencies above 0.1 (Appendix). However, corrected estimates obtained by INEst remained positive and superior to zero (F_{IS}' = 0.07) only in Kouba, suggesting that this population was not panmictic.

Identification of genetic clusters and spatial genetic structure

The maximal delta K estimates of Evanno *et al.* (2005) corresponds to K = 2 (Fig. 1). The two genetic clusters detected form a longitudinal gradient placing the populations situated towards East (Yagua, Bensekou, Igbo Aladja, Koda) and those in middle (Kikele, Tassigourou) within one group whilst the remained populations fell within a second group. The position of the population Tchiapeta in West was ambiguous, because the percentage of assignment to each cluster was close to 0.5 for three individuals. This suggests a genetic discontinuity between Western from Eastern populations. However, at each locus, all populations shared some common alleles (heterogeneity is clearly visible in Fig. 2) and differentiation statistics indicate moderate genetic differentiation between populations (F_{ST} = 0.13 and R_{ST} = 0.15). Considering each cluster, genetic diversity was A = 2.62, H_E = 0.36 and H_O = 0.29 in East while its value was A = 3.48, H_E = 0.39 and H_O = 0.35 in West. Hence, the genetic clusters display a low genetic differentiation (F_{ST} = 0.05 ± 0.01 and R_{ST} = 0.08 ± 0.03) and not phylogeographic signal (R_{ST} not significantly larger than F_{ST}).

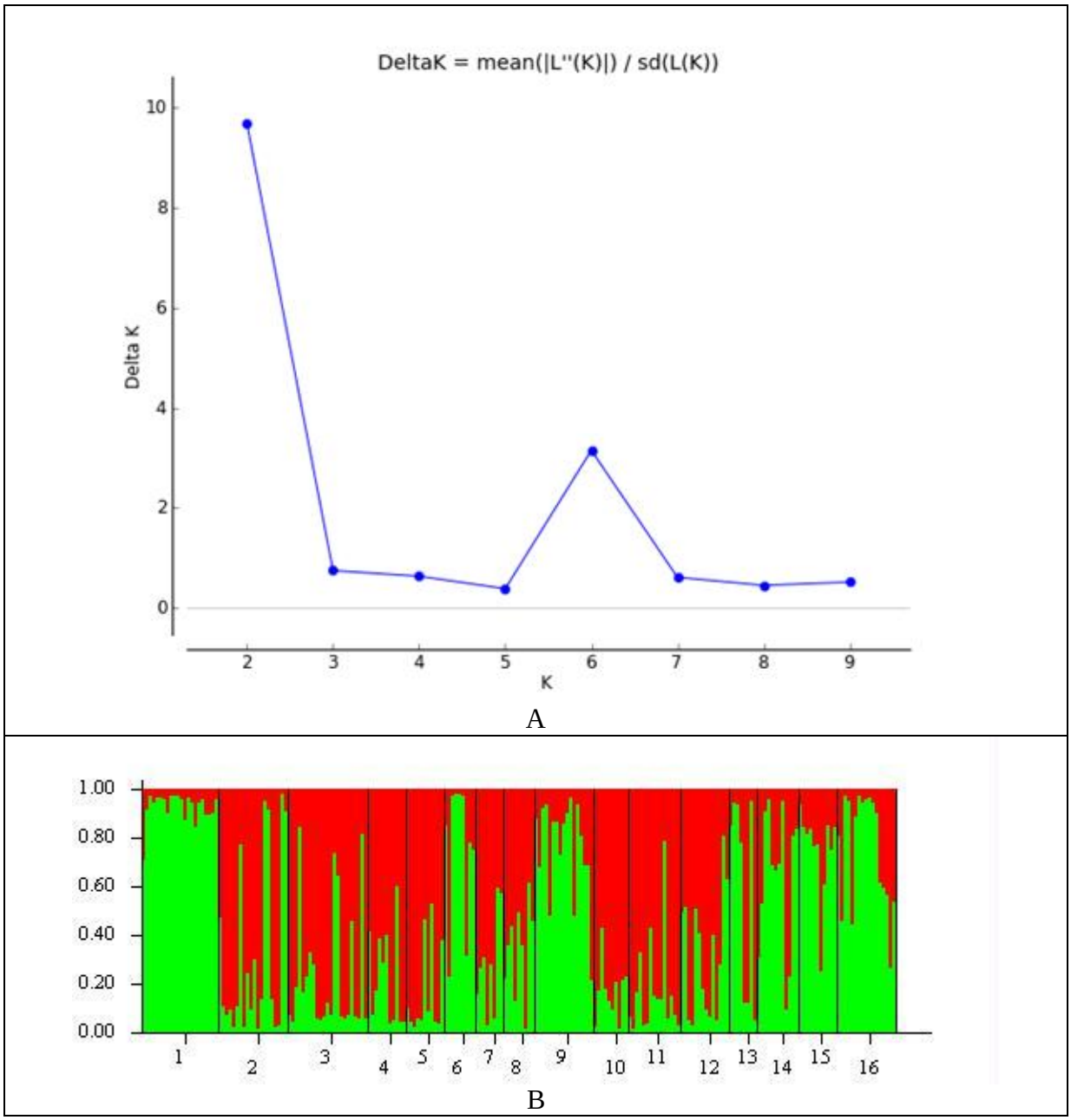

Figure 1: **A-** Most-likely number of clusters (K) following the method of Evanno et al. (2005) using the microsatellite data on 16 *P. butyracea* populations, obtained through 10 runs per K value of the STRUCTURE algorithm.

B- Histogram of genetic structure among 217 individuals re-sampled within populations. The red color represents the genetic group-east while the blue represents the western group. The numbers denote the populations sampled: 1. Igbo Aladja, 2. Kouba, 3. Setou, 4. Nioro, 5. Bassila, 6. Kikele, 7. Boribansifa, 8. Bongou, 9. Koda, 10. Penessoulou, 11. Tandafa, 12. Tassigourou, 13. Tchiapeta, 14. Tchoundegou, 15. Yagua, 16. Bensekou.

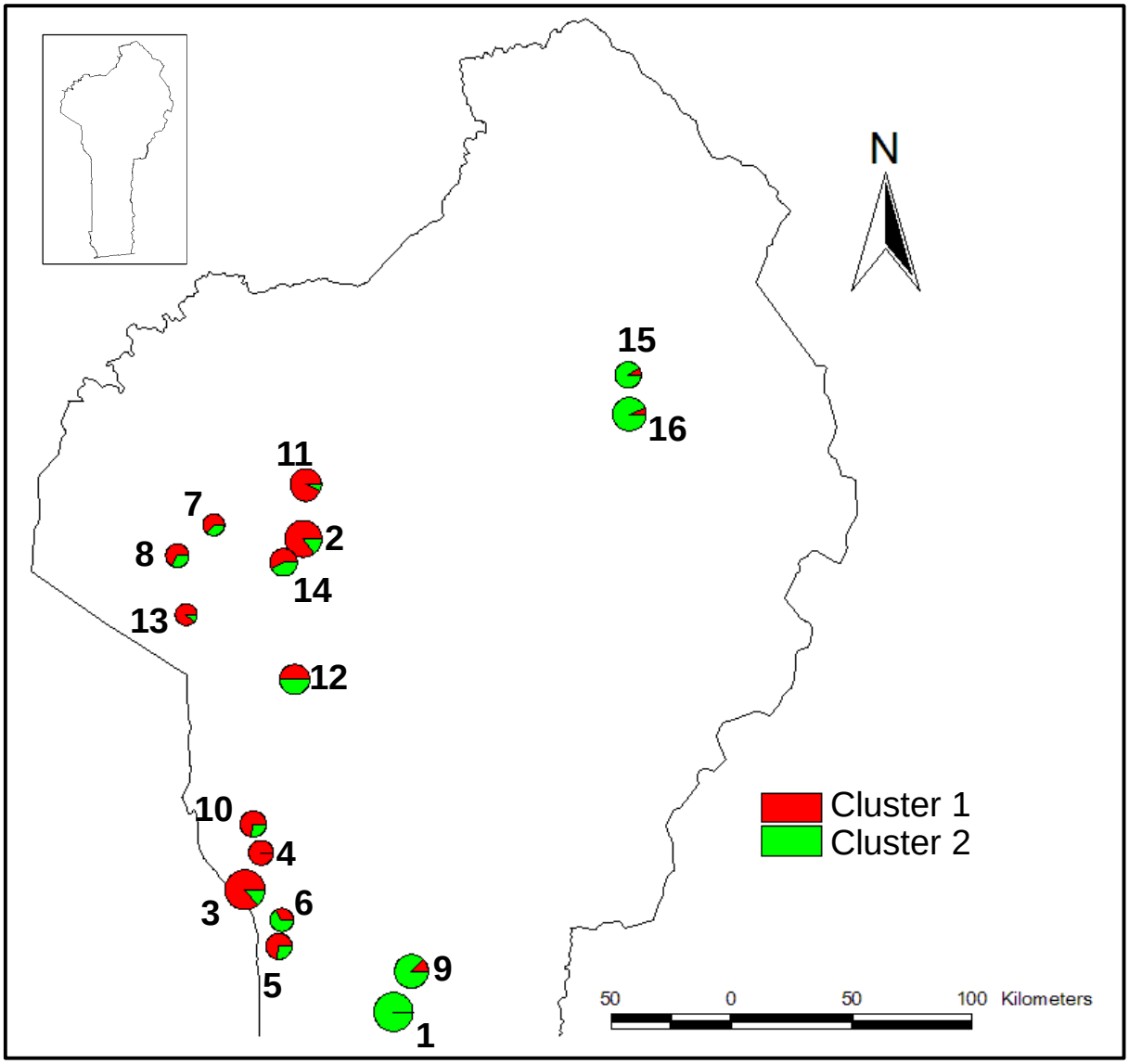

Figure 2: Spatial repartition of nSSR inferred genetic cluster (results for $K = 2$ according to the software Structure). Circle size is representative of the number of individuals having each genotype. The numbers denote the populations sampled: 1. Igbo Aladja, 2. Kouba, 3. Setou, 4. Nioro, 5. Bassila, 6. Kikele, 7. Boribansifa, 8. Bongou, 9. Koda, 10. Penessoulou, 11. Tandafa, 12. Tassigourou, 13. Tchiapeta, 14. Tchoundegou, 15. Yagua, 16. Bensekou.

Ratios $F_{ST}/(1 - F_{ST})$ computed over population pairs were positively and significantly correlated with geographical distance (F-statistic = 15.41, p = 0.0001, Fig. 3) suggesting isolation by distance. Analysis of the plot showed that differentiation was higher between both clusters, moderate within cluster1 and lower in cluster2.

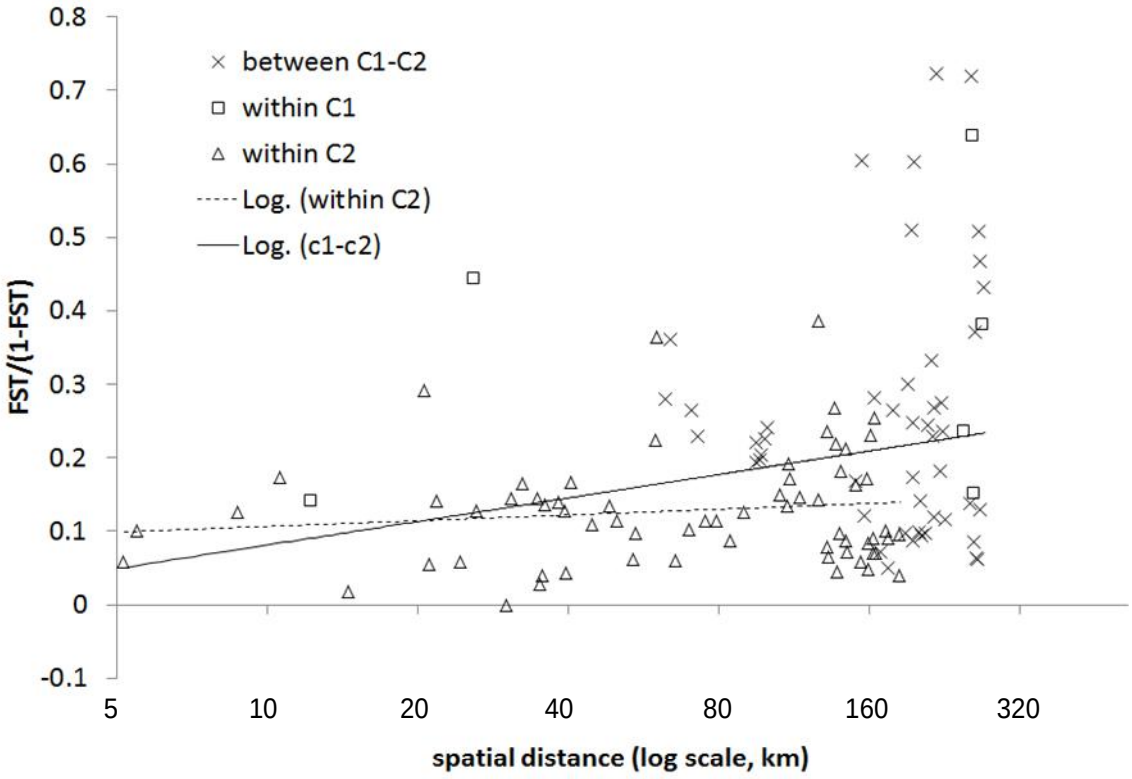

Figure 3: Ratio F_{ST} / (1- F_{ST}) computed for pairs of populations as a function of the distance. Bold (dash) line indicates increasing of ratio according to linear distance, solid line indicates linear regression.

Discussions

Genetic diversity

All populations showed similar levels of genetic diversity apart from those with very small size. The levels of heterozygosity detected (mean ± SD, H_E = 0.39 ± 0.24 after INEst and H_E ± SE = 0.33 ± 0.07 after SPAGeDi) were feeble for nuclear microsatellite data. These estimates were lower than those detected in other tropical tree species such as *Symphonia globulifera* (*N*a = 7 to 20; H_E = 0.71-0.85, Aldrich *et al.* 1998), *Coffea canephora* (*N*a = 3 to 20; H_E = 0.00-0.85, Gomez *et al.* 2009), *Milicia excelsa* (*N*a = 4 to 20; H_E = 0.31-0.85; Bizoux *et al.* 2009), *Allanblackia floribunda* (*N*a = 4.5 to 5.38; H_E = 0.49-0.81, Atangana *et al.* 2010), *Acacia senegal* var. *kerensis* (*N*a = 4.4 to 5.3; H_E = 0.53-0.86, Omondi *et al.* 2010), *Santiria trimera* (Na = 1 to 10; H_E = 0.03-0.72, Koffi 2010) and *Erythrophleum* spp (*N*a = 3.5 to 8.7, H_E = 0.61-0.77, Duminil *et al.* 2011), *Ceiba pentandra* (H_E = 0.85, Brondani *et al.* 2003), *Carapa guianensis* (H_E = 0.61, Dayanandan *et al.* 1999). However, it depends on the effective size of gene pool at long-term and on the mutation rate (and on mutation mode). The low diversity found in populations of Benin contrast with the fairly high diversity found in populations of *P. butyracea* from the rainforest of West Africa (H_E = 0.57) or Central Africa (H_E = 0.59; Ewédjè unpublished, Chap. 3). It most probably results from the founder events at the origin of the populations of Benin which derives from West African rainforest populations, possibly during the humid Holocene period, and/or from the high genetic drift as the populations in Benin are typically of small size (Table 1).

Smaller populations tend to have less diversity. As they are subject to severe anthropogenic landscape change and habitat fragmentation, they are at risk of loss of genetic diversity that is critical to their long-term survival. However in spite of fragmentation, populations situated in West side benefit from better ecological conditions displaying higher diversity than those from East. This supports the assumption that the phytogeographic district of Bassila and the Atacora Chain turn out to be ecologically and biogeographically outstanding floristic areas harboring the Guineo-Congolian endemic species *Aubrevillea kerstingii* and the Benin's endemic species *Thunbergia atacorensis* (Adomou 2005).

Apart from Kouba, all remained populations showed no significant deviation from HWE when accounting for null alleles. A previous study supports that *Pentadesma butyracea* is a predominantly outcrossing species. However, a higher selfing rate (c. 12%) was found in

Kouba (Ewedje unpublished data). Hence, the heterozygosity deficiency in Kouba probably results from inbreeding through selfing.

Population differentiation and structure

In spite of the existence of two admixed gene pools, the level of differentiation among populations was low (F_{ST} = 0.05), even taking allele sizes into account (R_{ST} = 0.08), suggesting few physical barriers to gene flow and/or a too recent common demographic history to allow a significant differentiation. As R_{ST} is close to $F_{ST,}$ there is no or a low relative contribution of mutations to differentiation compared to genetic drift (Hardy *et al.* 2003), indicating that differentiation is not deep in time. This is also supported by a previous chloroplast DNA variability based-analysis showing that all populations studied here were monomorphic. This suggests that the populations from Benin have a recent history resulting from West African rainforests. It is worth noting that the hydrographic network in Benin is defined by rivers mostly oriented according to North-South axis. As gallery forests along rivers might constitute main dispersal routes, one might formulate two hypotheses to explain the origin of the genetic clusters. First, the interfluves might constitute barriers to gene flow causing a low connectivity between Eastern and western populations. Second, the eastern and western genetic clusters might originate from different colonization routes following the main river basins, possibly after the maximal extension of the rainforest during the humid Holocene period.

Isolation by distance among populations was detected at this regional scale as found in fine scale within parental populations, suggesting limited seed and pollen dispersal distances. From the partition of genetic differentiation (Fig. 3), the lower level of isolation by distance detected in West (close to 0.1) than in East suggests that the fundamental differentiation between populations in Benin would result from divergence between both gene pools. Populations from East might be subjected to an important bottleneck event and increasing differentiation, as suggested by the low genetic diversity which was found to decrease significantly with longitude (H_E = 0.39 and A = 3.48 in West and H_E = 0.36 and A = 2.62 in East).

Whatever the origin of the two genetic clusters, their East-West distribution contrasts sharply with a study of morphological variation of adult trees in the same populations showing a North-South gradient (Ewedje *et al.* 2012). Morphological traits being measured on adults in situ, it was not possible to assess whether the differentiation had a genetic basis. Our results with microsatellites would rather support the hypothesis that the morphological variability

was due to phenotypic plasticity (except qualitative traits such as fruit shape), but one cannot exclude that morphological traits were under selection pressures following the reduction of rainfall towards the North, a pattern not detectable with microsatellites that are probably neutral.

Conclusion

The moderate genetic diversity and low genetic differentiation in *Pentadesma butyracea* in Benin indicate a high occurrence of gene flow at long distances and/or a recent common demographic history of the populations, despite the existence of substantial genetic drift given the limited population sizes, providing valuable information for conservation and improvement purposes. In the context of *ex-situ* conservation, our results support reforestation and/or orchard plantation from seedlings originated from the two genetic groups detected.

Acknowledgments

Financial support was jointly provided by the Belgian Technical Cooperation BTC for the grant of a PhD scholarship and the Belgian Fund for Scientific Research (FRS-FNRS).

Appendix 1: Locus characteristics; F.null indicates frequency of null allele within each population.

Locus (repeat motif)	Pent1 (CT)n		Pent12 (CAT)n		Pent13 (AC)n		Pent14 (AG)n		Pent16 (GA)n		Pent17 (GAG)n		Pent18 (TTG)n		Pent22 (TTG)n	
Populations	Size	F.null	Size	F.null	Size	F.null	Size	F.null	Size	F.null	Size	F.null	Size	F.null	Size	F.null
Igbo Aladja	119-125	0.000	151-165	0.016	164-170	0.013	171-184	0.000	215-221	0.315	211-217	0.076	228-257	0.190	283-300	0.000
Kouba	116-127	0.140	145-165	0.092	164-170	0.191	161-184	0.000	215-223	0.000	202-220	0.046	225-231	0.087	283-303	0.196
Setou	116-123	0.117	151-165	0.000	162-172	0.007	171-186	0.000	151-165	0.000	214	0.000	225-257	0.179	287-303	0.000
Nioro	123-125	0.000	151-165	0.000	168-170	0.000	171-184	0.067	217-219	0.000	214	0.000	228	0.000	287-303	0.000
Bassila	123	0.000	151-165	0.000	123	0.34	171-184	0.000	217-219	0.000	214	0.000	228-231	0.000	287-297	0.000
Bongou	123	0.000	151-165	0.000	168-170	0.000	171-192	0.000	217-223	0.000	202-214	0.000	228-231	0.000	287-300	0.071
Boribansifa	123	0.000	151-165	0.000	166-170	0.000	171-184	0.000	217-219	0.000	214	0.000	228-231	0.000	287-297	0.000
Kikele	123	0.000	151-159	0.000	170-184	0.084	161-171	0.030	217-219	0.000	214	0.000	228-231	0.000	287	0.000

Koda	123	0.000	151-165	0.014	168-170	0.000	171-184	0.000	217-219	0.000	214	0.000	228-231	0.000	287-300	0.080
Penessoulou	123	0.000	151-165	0.000	164-170	0.000	171-184	0.030	217-219	0.000	214	0.000	228-231	0.000	287-297	0.000
Tandafa	123-125	0.000	151-165	0.000	168-170	0.065	171-184	0.010	217-223	0.104	202-214	0.000	228-231	0.000	287-300	0.000
Tassigourou	123	0.000	151-165	0.000	168-170	0.000	171-184	0.000	217-219	0.000	214	0.000	228-231	0.000	287-297	0.022
Tchiapeta	123-125	0.000	151-165	0.230	168-170	0.000	171-182	0.008	217-223	0.000	202-214	0.000	228	0.000	287-297	0.104
Tchoundegou	123	0.000	151-165	0.000	164-172	0.126	171-184	0.000	217-223	0.189	202-214	0.074	228-231	0.000	287-297	0.000
Yagua	123	0.000	151	0.000	168-170	0.027	179	0.000	217	0.000	214	0.000	228	0.000	287-297	0.000
Bensekou	123	0.000	151-165	0.000	168-170	0.000	171-179	0.000	217-219	0.000	214	0.000	225-231	0.000	287-297	0.000

CHAPITRE VI.-

BIOLOGIE REPRODUCTIVE DE *PENTADESMA BUTYRACEA* SABINE (CLUSIACEAE) AU BENIN

Manuscript submitted in *Annals of Botany*

Biologie reproductive de *Pentadesma butyracea* Sabine (Clusiaceae) au Benin

Pour asseoir les bases nécessaires à la conservation de *Pentadesma butyracea*, nous avons examiné les principaux paramètres liés à sa reproduction sexuée (profil phénologique, caractères floraux associés au système de reproduction et nombre de grains de pollen par ovule, pollinisateurs, productivité, germination et potentiel de conservation *ex situ* des graines). Les données ont été collectées en 2008 et 2009 sur 77 arbres adultes provenant de trois populations naturelles du Bénin.

Pentadesma butyracea entre en floraison une fois par an pendant les mois de septembre à décembre au début de la saison sèche. L'entrée en floraison présente un faible décalage (0-14 jours) entre populations alors que le décalage est plus important (0-18 jours) entre arbres au sein de la population. Cependant l'indice de synchronisme demeure élevé ((Z = 0,74 ± 0.12)) à cause de la longue période de floraison (à peu près deux mois par arbre) qui favorise le chevauchement de certains stades phénologiques de la fleur et par conséquent l'échange de pollen entre arbres. Le ratio pollen:ovule est estimé à 577 ± 213, correspondant à la gamme de valeurs typiquement observées chez les espèces avec un régime de reproduction facultativement xénogame. La production des inflorescences au bout des branches, la couleur jaunâtre et blanc verdâtre des fleurs et la quantité importante de nectar (1042 ± 117 µL) et de pollen produit représentent les structures florales qui prédisposent l'espèce à une pollinisation par les animaux. Les principaux pollinisateurs sont deux oiseaux (*Cyanomitra verticalis, Cinnyris coccinigastrus*) et trois abeilles (*Apis mellifera*, *Meliponula togoensis*, *Hypotrigona* sp.). En moyenne, 49% de fleurs par inflorescence parviennent à fructifier. Le ratio graine/ovule est estimé à 0.06 indiquant un taux d'avortement de 94% d'ovules par fleur. La productivité totale en fruits augmente en fonction de l'âge de l'arbre et varie en fonction de l'année, atteignant un pic de 346 ± 392 fruits pour les arbres ayant un diamètre de 60-80 (2008) et 240 ± 247 pour les arbres de 60-70 cm de diamètre (2009). Les graines ont une teneur en eau de 42.5 ± 2.9 %; elles sont récalcitrantes mais elles peuvent supporter la conservation à 20°C pendant 3 mois en prenant soins de les arroser régulièrement. La germination des graines est optimale à 30°C. Ces résultats indiquent qu'il n'est pas possible de conserver *ex situ* les ressources génétiques de cette espèce sous forme de banque de semences. Nous suggérons, en marge des efforts à déployer pour la reforestation et la conservation *in situ*, la création d'un verger de diverses provenances comme alternatives à la conservation en dehors de l'habitat naturel de *P. butyracea*.

Article soumis à *Annals of Botany*.

Reproductive biology of *Pentadesma butyracea* Sabine (Clusiaceae) in Benin

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Abstract

Background and aims: The main reproductive traits (phenological pattern, floral morphology, pollinator assemblage, seed production and germination) of a native threatened African food tree species, *Pentadesma butyracea*, were examined in Benin to gather basic data necessary to develop conservation strategies.

Methodology: Data were collected on phenological profile and its variation, floral traits associated to breeding system and P/O ratio, fruit and seed set, and on longevity of *P. butyracea* seeds in 2008 and 2009 on 77 adult individuals from three natural populations belonging to the Sudanian phytogeographical zone.

Principal results: *Pentadesma butyracea* flowered once a year from September to December during the dry season. Flowering entry displayed less variation among populations than among individuals within populations. However, due to a long flowering period (ca. 2 months per tree), the synchrony of different floral stages between trees was high, facilitating pollen exchange. Pollen-ovule ratio was 577 ± 213 indicating facultative xenogamy. The apical position of inflorescences, the yellowish to white greenish flowers and the high quantity of pollen and nectar per flower (1042 ± 117 µL) represent floral attractants that predispose the species to animal-pollination. The main pollinators were two sunbirds (*Cyanomitra verticalis*, *Cinnyris coccinigastrus*) and three Hymenoptera (*Apis mellifera, Meliponula togoensis, Hypotrigona* sp.). The mean fruit set reached 49%, and the absolute fruit production increased with tree size. Seeds were recalcitrant; however, hydrated storage was possible for more than 3 months. Germination of *P. butyracea* seeds was most successful and rapid at 30°C (50% after 9 days).

Conclusions: Our results indicate that *P. butyracea* is a facultative xenogamous plant species. The conservation of its genetic resources is not feasible through *ex situ* conservation of seed banks but *in situ* conservation strategies and/or *ex situ* conservation in orchards should be successful.

Keywords: breeding system, floral synchrony, fruit set, pollination, pollen-ovule ratio, recalcitrant seeds, conservation

Introduction

For the conservation of plant resources, a variety of approaches and techniques both *in situ* and *ex situ* have been proposed and implemented. For example, in Benin, situated in a savanna corridor interrupting the zonal African rainforest, the Department of Forest and Nature Protection relies heavily on both approaches to conserve the natural plant richness. Here, the *ex situ* approach finds more applications for economic and domesticated plants in some private or public botanical gardens or agronomic research centers, however, without an overall management plan. The *in situ* approach focuses on wild plants.

Important factors for successful *in situ* conservation of a plant species are continuous recruitment to maintain a mixed population age structure and sexual reproduction to preserve high genetic diversity as preadaptation to environmental change (Gugerli 1999). Failure of reproductive processes due to environmental changes is often the fundamental reason for species loss (Moza and Bhatnagar 2007). However, habitat fragmentation and overexploitation are the more apparent causal factors. Habitat fragmentation transforms large and continuous habitats into smaller patches, which can affect ecological and genetic processes (Young and Clarke 2000; Mustajarvi *et al.* 2001; Leimu *et al.* 2006; Broadhurst and Young 2007). This partition may alter plant reproductive processes by reducing the size of plant and pollinator populations and by altering the spatial arrangement of populations. Floral phenology and breeding system are key factors in plant reproductive biology. The role of the breeding system is crucial in the structuring and the transmission of genetic diversity from generation to generation (Ritland 1989) while at population level, floral phenology influences the mode of reproduction by shaping mating possibilities between synchronous individuals, affecting the degree of genetic structure. Flowering i.e. the transition from leaf to flower production, can be stimulated by internal (plant age or size) and external factors (day/night length, low temperature, fire and/or the presence of water; Erwin 2006). Knowledge of timing, duration, and intensity of plant phenology is crucial to understand the ecology and evolution of species and their inter- and intraspecific relationships. It has been shown that plant breeding systems are associated with particular floral traits. Patterns of floral phenology are considered to reflect evolutionary compromises in response to a set of selective forces, including the availability of gene dispersers (pollinators and seed dispersers). As a general trend, it is usual to predict the pollinator type of a given species based on its floral traits suggesting a tight evolutionary relationship between plants and their pollinators (Faegri and van der Pijl 1979; Fenster *et al.* 2004). Depending on the reproductive biology and population history of plant species, reduced pollinator service may have numerous negative impacts on

the plant population, including reproductive failure (Jennersten 1988; Aguilar *et al.* 2006). In addition, effective population sizes can decrease through reduced gene flow, leading to inbreeding and possibly an increase in selfing (Bawa 1990; Menges 1991; Aizen and Feinsinger 1994; Gitzendanner and Soltis 2000; Mustajarvi *et al.* 2001; Rymer *et al.* 2005; Leimu *et al.* 2006; Coates *et al.* 2007). These altered reproductive patterns may cause a loss of genetic diversity and/or reduced progeny fitness due to inbreeding depression (Karron 1989; Menges 1991; Barrett and Kohn 1991; Latta and Ritland 1994; Husband and Schemske 1996). Thus as a general rule, developing a conservation strategy requires an in-depth knowledge of the reproductive biology of the plant in focus (Bernardello *et al.* 1999).

The plant species *Pentadesma butyracea* Sabine (Clusiaceae) is a multipurpose tree used for the production and local trade of a yellow butter extracted from its seeds' kernel (Tchobo *et al.* 2007). However, this plant species is one of the ten most threatened food tree species in Benin and Togo (Dah-Dovonon 2002; Poidy 2002) due to habitat fragmentation and reduced recruitment due to overexploitation. For example, in some areas in Benin, seed harvesting can reduce available seeds for recruitment by 70% (Avocèvou *et al.* 2009). Currently, there is a lack of sylvicultural knowledge for its restoration and preservation in sub-Saharan Africa (Eyog-Matig *et al.* 2002). Socio-economic studies indicate that the next human generation will have only half of the resources (fruits, seeds, bark, branches and timber) of *P. butyracea* available today. An increasing pressure on *Pentadesma* resources results from the transfer of a subsistence product (subsistence farming by ancestors) to a commercial product (selling in all forms) (Sinsin and Sinandouwirou 2003) and its use in cosmetic trials by the French company L'OREAL based solely on naturally existing resources without cultivation efforts (Dencausse 1997). Thus this species becomes of particular interest for developing adequate sustainable management and conservation programs. Till now, *P. butyracea* has not been added to the list of protected species in Benin like *Parkia biglobosa*, *Vitellaria paradoxa* and *Dialium guineense* which are protected by this mean from felling, pruning, limbing, mutilation, uprooting and swidden bleeding.

The present study aims to elucidate the floral traits associated with breeding system and seed ecology of *P. butyracea* in order to contribute to the acquisition of fundamental data necessary to develop a successful sustainable managing strategy in the future. In the tribe Symphonieae, *Pentadesma* is the only genus for which the reproductive biology is still sparsely studied while other genera are well documented (Gill *et al.* 1999; Bittrich and Amaral 1996; Degen *et al.* 2001).

Material and methods

Study species, study area and sampling design

Pentadesma butyracea (yellow butter tree) belongs to the pantropical distributed family Clusiaceae including about 37 genera and 1610 species. Within Clusiaceae, *Pentadesma* belongs to the tribe Symphonieae, one of three tribes of the subfamily Clusioideae (Steven 2007) which has, however, been found to be paraphyletic based on *rbc*L sequences (Gustafsson *et al.* 2002). Clusiaceae is a particularly variable group displaying a wide spectrum of floral forms and associated pollination modes. Examples of such variable features in two well documented genera, *Clusia* and *Garcinia*, are floral phyllotaxis, petal number, petal fusion, sex distribution, type of androecium, stamen fusion, stamen and staminodes number, pollen surface, pistillodia of male flowers, carpel number and style presence and length, stigma position. Some of the floral diversity has been explained as (1) a result of adaptations to various types of pollinators and pollination mechanisms or (2) a floral developmental genetic mechanism that causes a relaxation of the floral bauplan, i.e. the underlying fundamental organization allowing for numerous deviations (Gustafsson 2000; Gustafsson *et al.* 2002). Another particularity in this family is the diversity of pollinator rewards which include nectar and pollen but also resin which is very rarely seen outside of the family Clusiaceae (Bittrich and Amaral 1997).

Pentadesma butyracea is a native African tree species occurring from 200 to 550 m of elevation, in Guineo-Congolian evergreen forests and gallery forests from Guinea-Bissau to the Democratic Republic of Congo (Bamps 1971). In Benin, where a savanna corridor interrupts the zonal West African rainforest, it occurs in the Sudanian phytogeographical zone as defined by White (1983) (Fig. 1, III), mainly in gallery forests, in savannah woodlands and at foot hills where humidity is elevated, and is found in highly aggregated stands (Sinadouwirou 2000). These forests are however fragmented due to their small sizes resulting from Quaternary climate crisis and especially depending on extensive agriculture and logging (Neuenschwander *et al.* 2011).

Its local names are Kpangnan in Nagot, Akoto in Anni, Yêkotchépouo in Otamari and Sesseido in Fulani. The tree is 10 to 25 m in height and can reach a diameter at breast height (dbh) of 110 cm. Flowers are whitish, fleshy, producing many stamens. The fruit of *P. butyracea* is a berry containing few seeds in a yellow pulp (Hawthorne and Jongkind 2006). *P. butyracea* often reproduces by asexual reproduction via root shoots.

In the area prospected, the climate is Sudanian with one rainy season (March-May to October) and nearly seven months of dry season. Annual rainfall in Benin ranges from ca. 900

mm to 1400 mm, with a peculiar geographic pattern: a less humid corridor from the South West to the North East in the Guineo-Congolian zone (900-1100 mm) and a wet patch in the Centre-West (1300 mm) in the Sudanian zone (Fig. 1, III). More precisely, the study area belongs to the agro-ecological zone from Centre-East and North-West of Sudanian zone (Dagbénonbakin et al. 2003). Daily relative humidity and temperature vary between 18 and 99 % and 18 and 42 °C, respectively. The yearly average potential evapotranspiration (ETP) is 1550 mm for the period from 1972 to 2001. Dry winds from the East, originating from the Sahara are frequent from November to January. Soils are tropical ferruginous with a breastplate of sandstone, or lateritic on a sandy subsoil. The specific climate conditions at the sampled habitats are described in Table 1.

To study the reproductive biology of *Pentadesma butyracea*, a total of 77 adult individuals (usually producing fruits) have been randomly sampled in 3 natural populations separated by 90 to 200 km (see Fig. 1 and Table 1). A population was defined by an accumulation of trees distant by less than 200 meters between adjacent trees and a minimum distance of 5 km to the next population. All three populations were in the proximity of a river. The population Penessoulou situated in the West is the single site included within a protected community forest where trees benefit protection from man made fires, contrary to the two other populations harbouring burned and unburned trees. Moreover, Penessoulou benefits higher annual rainfall (1381 mm) than Peperkou (1269 mm) and Igbo Aladja (1199 mm). The dbh of trees ranged from 8.1 to 106.1 cm (mean ± standard deviation = 37.0 ± 13.8 cm). The number of individual trees investigated varied between activities (see below).

Table 1. Characteristics of the *Pentadesma butyracea* populations studied. Habitat types: dry semi deciduous forest surrounded by savannahs (DDF), gallery forest at foot hills (GF). Lat = latitude; long = longitude.

Populations	Geographical coordinates (UTM, zone 31N)		Mean elevation (m)	Habitat	Annual rainfall (mm)	Adult population size	Number of individuals sampled
	Lat	Long					
Igbo Aladja	967239	415216	257	GF	1199	90	26
Penessoulou	1023542	336472	420	DDF	1381	27	20
Peperkou	1160673	332713	393	GF	1269	37	31
TOTAL						154	77

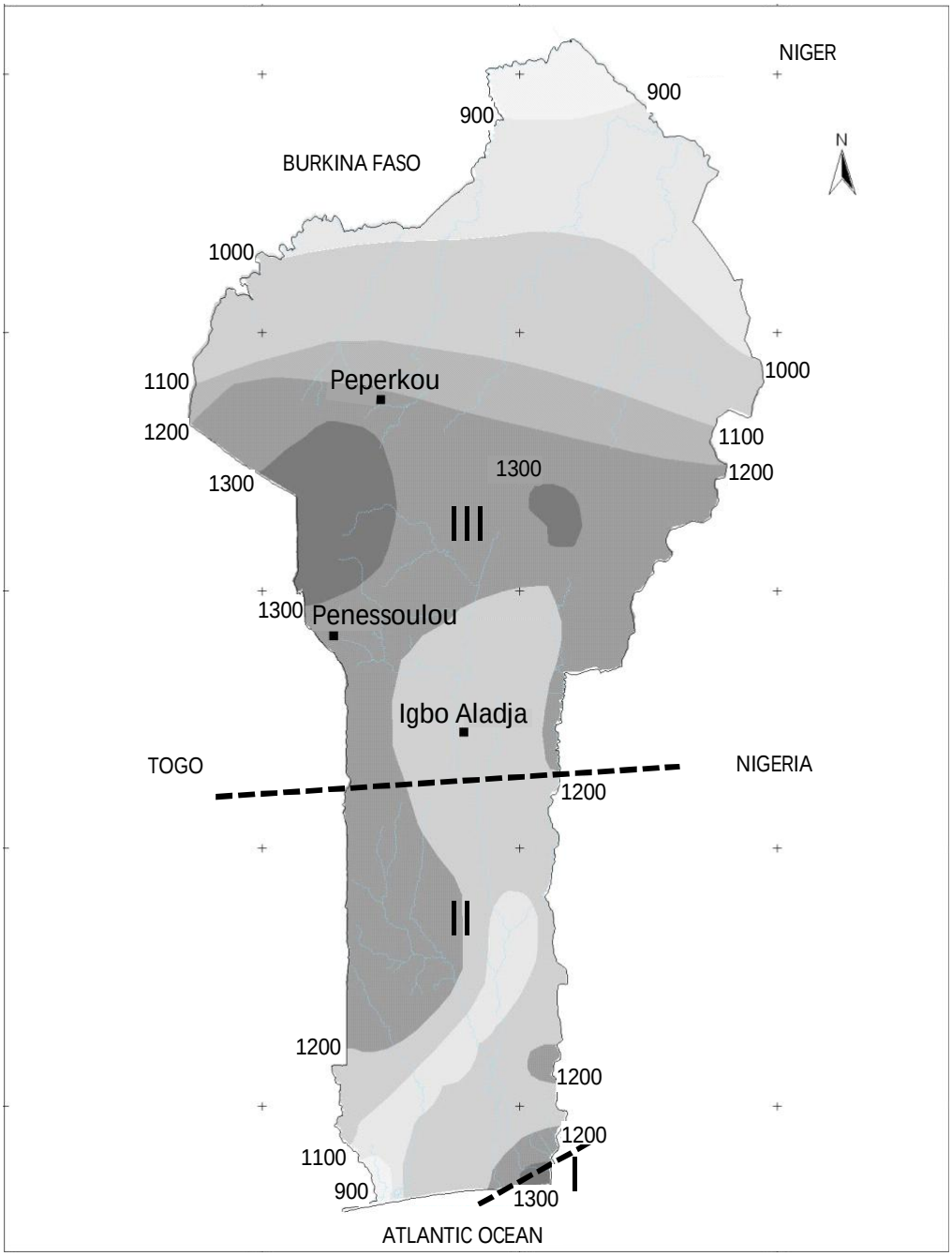


Fig. 1. Location of the three populations sampled to study the reproductive ecology of *Pentadesma butyracea* in Benin. Phytogeographical zones are adapted from White (1983): I = Guineo-Congolian, II = Guineo/Sudanian transition, III = Sudanian (hatched line separates phytogeographical zones). The different shadings show the distribution of rainfall from low (light shading) to high (dark shading; numbers refer to isohyets in mm, Akoegninou 2004).

Phenology and floral synchrony

Observations were carried out in two different phenophases (flowering and fruiting) during two successive years. The flowering was observed once a week from September to December in 2008 and in 2009 on 69 marked trees from three natural populations (26 trees in Igbo Aladja, 20 trees in Penessoulou and 23 in Peperkou). The fructification was observed once every two weeks on the same individuals from December to May in 2008-2009 and in 2009-2010.

Floral phenology

Four successive stages and eventual changes among stages were surveyed in each phenological phase as indicated below:

- Flowering: fl1 = floral initiation; fl2 = young bud; fl3 = bud well developed; fl4 = mature flower i.e. blooming flower (see Fig. 2 A - E).

- Fruition: fr1 = fruit set (= style dark-brown dehydrated, persisting ovary, stamen and calyx); fr2 = young green fruits in development (growing ovary with persisting calyx); fr3 = fruits brown, larger in size and well developed but not mature; fr4 = fruits mature (about 129 ± 32 mm long), brown colour, few days before falling down (see Fig. 3, A-D).

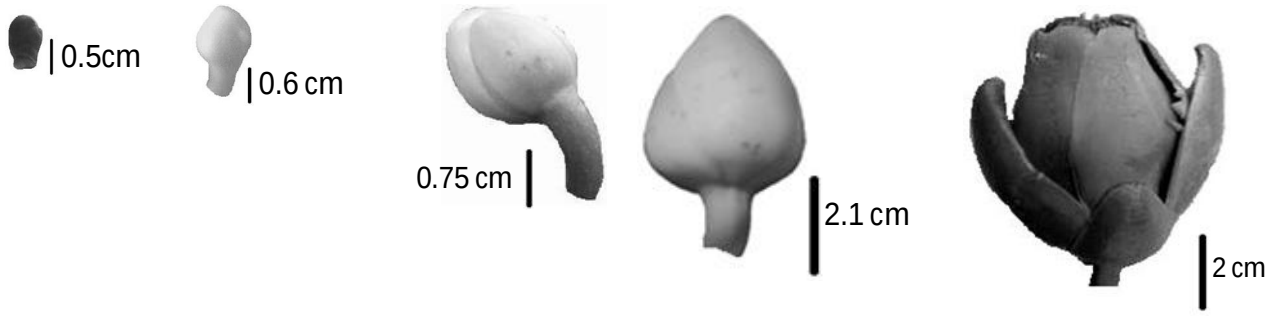


Fig. 2. Structure and phenological stages of *Pentadesma butyracea* flowers.
A = floral initiation (fl1); B-C = fl2, bud (13 to 17 days after fl1); D = fl3, developed bud (20 to 21 days after fl1); E = fl4, open flower (24 to 25 days after fl1)

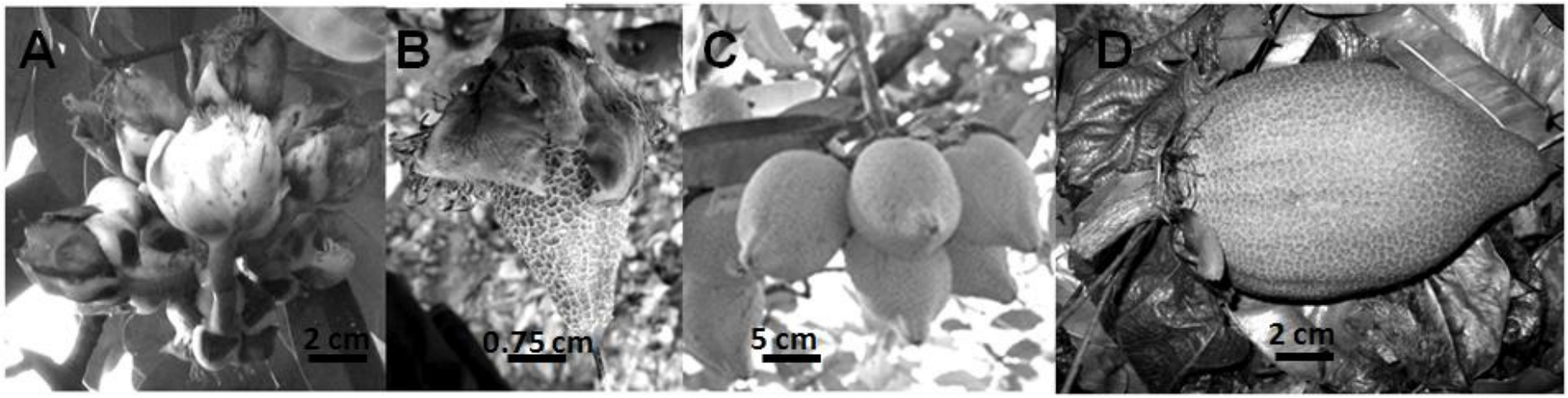


Fig. 3. Structure and phenological stages of *Pentadesma butyracea* fruition: A = fr1, fruit set (26 to 40 days after fl4); B = fr2, young fruits in development (27 to 38 days after fr1); C = fr3, fruits well developed but not mature (43 to 62 days after fr2); D = fr4, fruits mature (21 to 35 days after fr3).

Floral synchrony

The quantity of flowers and fruits in a given stage per tree was quantified by ordinal classes: 0 = absence of flowers or fruits in a given stage; 1 = 1 - 25 % of branches bearing organs in a given stage; 2 = 26 - 50 %; 3 = 51 - 75 %; 4 = 76 - 100 %. Data of the date of first flowering and stage duration were collected, as well as the synchrony of sexual maturity (homogamous, protogynous or protandrous flower), to construct the phenological diagram of each population. We also computed the floral synchrony index X_i of each individual per population and the index Z, which is the average X_i-index of synchrony of individual trees within a population, according to Augspurger (1983) as follows:

$$X_i = \left(\frac{1}{n-1}\right)\left(\frac{1}{f_i}\right)\sum_{j=1}^{n} e_{j\neq i}$$

$$Z = \left(\frac{1}{n}\right)\sum_{i=1}^{n} X_i$$

where n = number of individuals in the population, f_i = number of flowering days of the individual i, e_j = number of days on which individuals i and j flower in a synchronized way ($j\neq i$). X_i = 1 represents total floral synchrony where the flowering period of the individual considered coincides entirely with the flowering period of every individual $j\neq i$ of the population. X_i = 0 means that there is no phenological overlap between i and the other individuals. The stage fl3 was considered (instead of fl4) to determine these indexes to prevent potential errors of flower observations on extremely tall trees confounding open flowers (fl4) with wilted flowers. A student t test and ANOVA were conducted to assess whether differences occur among populations and among individuals at constantly inundated versus dry sites and, between trees affected or not by man made fires within population.

Flower morphological traits, pollen load and nectar reward

Seventy five inflorescences collected from 15 trees in the three populations were examined and the mean number of flowers per inflorescence was determined. Sixty flowers (randomly selected from different inflorescences) were dissected to determine whether all flowers were hermaphrodite, to describe the structure of the androecium and the gynoecium, and to count the number of stamens and ovules per flower.

The number of viable pollen grains was determined by counting red pollen grains colored by acetic carmine within an anther crushed between microscope object plate and cover slide under a binocular microscope. Samples of anthers were removed from 120 stamens (initially plunged in acetic carmine) collected from 40 flowers with 30 stamens at each of the following four different phenological stages: stage fl3 just before anthesis, stage fl4a at anthesis, stage fl4b 24 hours after anthesis and stage fl4c 48 hours after anthesis.

The quantity of nectar produced per flower (bagged before anthesis to avoid visit by pollinators) was measured by sequential removal of nectar present in a flower with a micropipette of 1200 µL. Removal was done three times, each time from a different flower (at first anthesis in the morning, in the evening at 6 p.m. and 24 hours after anthesis) on each of 30 flowers from 5 trees in Igbo Aladja and 5 trees in Peperkou.

Fruit set

We determined the average fruit set per inflorescence (n = 75 inflorescences from 25 trees) and the Seed/Ovule ratio (n = 75 flowers / fruits collected from 25 inflorescences/infrutescences in the year 2008). At phenological stage fr3 the total number of fruits produced per tree on a total of 77 trees (see Table 1 for number of trees sampled per population) was counted and the dbh of the trees was measured.

Pollinators

On one tree per population, four inflorescences were chosen for observation and marked to facilitate their identification. Pollinators and their behavior were observed for three days concordant with the life time of the observed flowers. The observations were conducted from anthesis at 6 a.m., 9 a.m., 12 a.m., 3 p.m. and 6 p.m. for 10 minutes each time. The choice of the days of observation was based on the flowering peak of the selected tree. The following data about the flower visitors were collected: species or taxonomic group, pollinator (come in contact with the sexual organs of the flower) or visitor (predate on floral rewards without being in contact with the sexual floral organs), time of visitation, frequency (number of visits per minute to an inflorescence) and duration of visit, and behavior. Hereafter, pollinators - mainly insects - were captured using a ladder and a handnet. Pollinators were preserved in 70° alcohol to perform their identification in the Biological Control Center for Africa of the International Institute of Tropical Africa (IITA). After identification, a total of 25 voucher specimens belonging to 3 species were kept in a private collection of the first author. Birds were determined by an ornithologist (Faculty of Sciences and Agronomy, Université d'Abomey-Calavi) based on pictures taken while visiting *P. butyracea*'s flowers.

Seed moisture content, germination capacity and storage characteristics

To estimate moisture content, germination capacity and storage characteristics of seeds, 127 mature fruits were collected randomly under trees from the three studied populations on May 24th, 2009. All healthy seeds were extracted (~ 953 seeds) from the fruits. The extraction of seeds took place 11 days after the collection of the fruits. Seeds were ridded from their coat. Weight and moisture content were determined gravimetrically at the Université d'Abomey-Calavi in Benin on 100 seeds. Moisture contents were determined by weighing before and after drying seeds in an oven at 103°C for 17 h. The moisture content is the difference of theses weight measurements given as the percentage of fresh weight (Willan 1992).

Two months after collection, seeds for germination test were sent to the Université Libre de Bruxelles (Belgium).. Since their collection, they were regularly wetted to avoid a loss of moisture (estimated to 1.51 % through drying experiments on n = 50 seeds) before trials. Nevertheless, seeds indicate a loss of water of about 1.5 % during transportation decreasing moisture content to 41 ± 2.2 % (n = 100 seeds) before trials. In Belgium, seeds were submitted to two treatments.

Treatment 1: Germination experiments - Germination capacity was determined at three different temperatures (20°, 25° and 30°C) by using five replicates of 10 seeds for each temperature. The chosen temperatures (20°C-30°C) were close to the mean minimal and maximal temperatures in the studied regions in Benin during the fruition period. Seeds were sown in peat and incubated for 103 days in individual incubator sets (Luminincubes) with 12 h light/12 h dark. These seeds were measured (length, width and thickness) and weighted before they were placed into peat filled pots. Kruskal-Wallis tests were applied to assess whether there is a significant difference in the number of germinated and non germinated seeds among different temperatures, and whether there is a difference in weight between germinated and non-germinated seeds.

Treatment 2: Storage trials - Storage experiments were performed using ~200 seeds that were stored at ambient temperature (ca. 20°C), and ~150 seeds stored at ca. 4°C in polyethylene bags to check the effect of conservation at low temperature on seed biology. Seeds were sub-imbibed only the first day of trial. After 35 days, the moisture content of 50 seeds stored at 25° and 50 seeds stored at 4° was measured and for each storage temperature five replicates of 10 seeds were germinated at 30°C which was the ideal germination temperature identified in treatment 1.

Results

Phenology and floral synchrony

Floral phenology

The branches that support flowers were often born at the basis of those of the previous year. From 13 to 17 days after initiation (stage fl1) buds reached stage fl2 (Fig. 2B). They were green and measured 1.4 - 5.7 cm x 0.8 - 3.0 cm. Stage fl3 occurred 20 to 21 days after fl1, color changed from green to green-yellowish and buds measured 8.3 cm x 4.5 cm. The stage fl4 occurred 24 to 25 days after fl1. Floral anthesis started in the evening at 5 pm. At this phase, a light pressure on the anthers produced an opening of the thecae and a display of pollen grains. In contrast, the 5 stigma papillae of the gynoecium remained closed. Only in the morning of the following day the latter separated. At this stage, anthers and stigma extended above the level of the petals at about the same height. Each lobe of the receptive stigma projected outwards. Anthesis was not synchronous between all flowers within an inflorescence: considering an inflorescence composed of about 5 cymes, it was always the terminal flower of each cyme and on overall the apical cyme that bloomed first. However, anthesis could happen at the same period for most of the overall apical flowers of the inflorescence i.e. flowering of cymes within an inflorescence was often overlapping. It took 3 to 5 days before all flowers of a cyme (and/or an inflorescence) were open. Flowers at stage fl4 lasted about 4 days followed by a progressive dropping of petals.

Fructification could be observed from December until April. At the stage fr1 (26 to 40 days after fl4 from December to January), flower fl4 was faded measuring 4.4 - 8.6 cm x 3.9 - 4.5 cm, its perianth had dehydrated, and corolla and the summit had become pink; sometimes numerous flowers fell down at this stage (see Fig. 3A). At the stage fr2 (27 to 38 days after fr1, January to February), the fruits´ color passed progressively from yellow to brown, perianth dehydration continued, some sepals and petals fell down from fruits but the androecium persisted; fruits measured 5.5 - 9.3 cm x 4.6 - 5.8 cm. At the stage fr3 (43 to 62 days after fr2 from February to April), fruit became brown, measuring 6.8 - 13.1 cm x 4.9 - 7.4 cm and kept this color until maturity (21 to 35 days after fr3 from April to May). The ripe fruits (measuring 8.3 - 17.9 cm x 6.8 - 11.3 cm, see Fig. 3D) started to drop down in April until July. Harvesting by humans only started in May.

Phenological diagram

The flowering (stages fl1 to fl4) occurred in a single yearly cycle, generally from September to December, sometimes until January for the late trees (Fig. 4). The onset of flowering varied

between populations: population Penessoulou always started and ended flowering first and population Peperkou flowered last (10 to 14 days delay between the mean dates of flowering initiation) (Fig. 4). Finally, there was a variation between years: in all three populations the flowering period was shorter in 2008 than in 2009 and flowering started three weeks later in 2008 (22 September) than in 2009 (01 September; cf. Fig. 4). Within a tree, the onset of flowering was not uniform in all branches and could reach a difference of up to 28 days between the first and last branches bearing flowers. Between individuals of the same population, the onset of flowering could be delayed (0 to 18 days shift): individuals standing in water and those on land showed the same temporal patterns but non-burnt individuals started flowering earlier than burnt trees ($p < 0.05$, see Table 2).

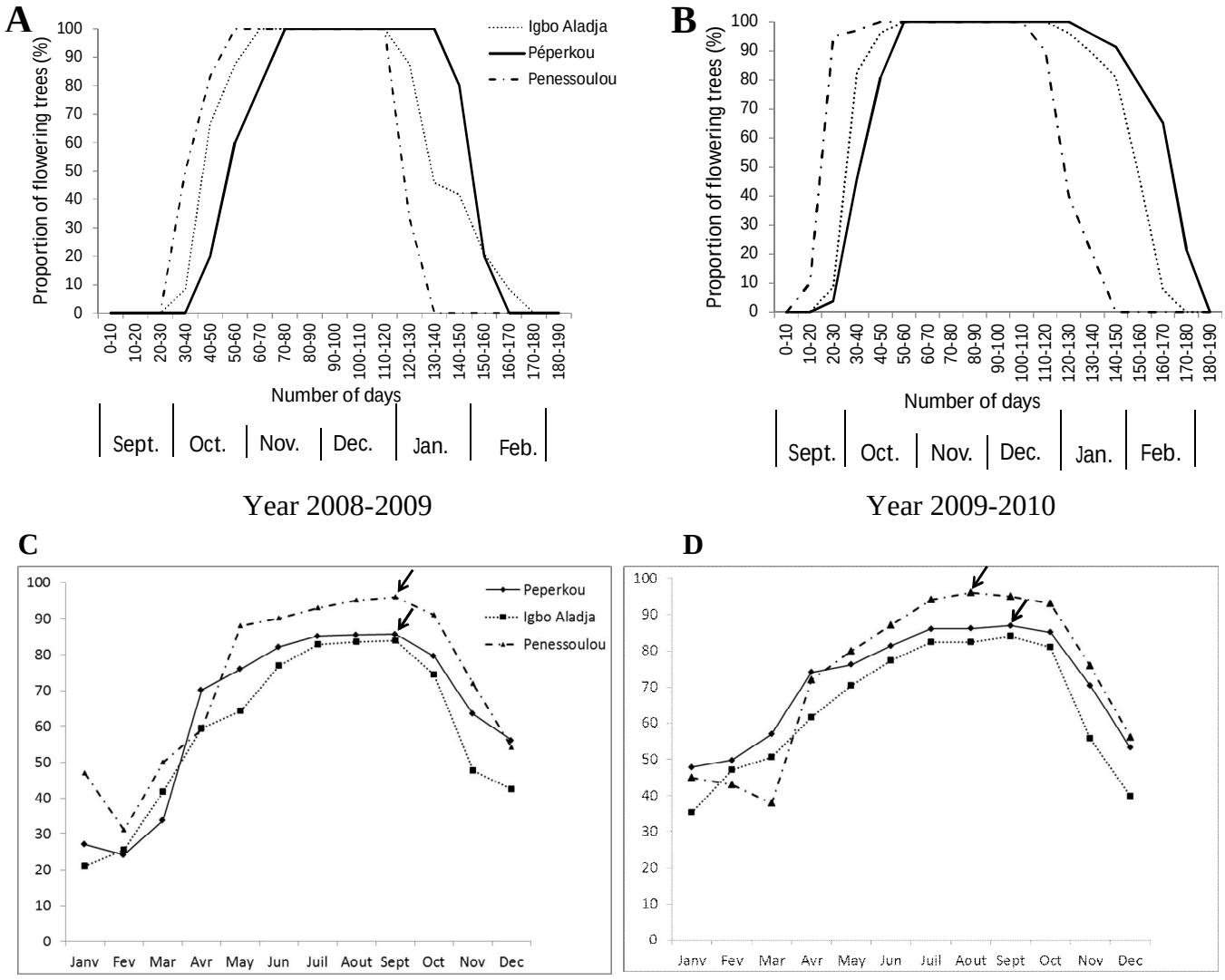


Fig. 4. Phenological diagram of the three populations of *Pentadesma butyracea* in Benin in 2008/09 (A) and 2009/10 (B). Graphs C and D showed monthly relative humidity for the

same populations in each of both years; arrows indicate months of the higher relative humidity.

Floral synchrony within populations

The synchrony index of an individual with respect to all others varied between 0.42 and 0.93. Their mean values (Z ± sd) were similar in Igbo Aladja and in Peperkou (0.71 ± 0.10 and 0.70 ± 0.13 respectively) but was higher (0.84 ± 0.08) in Penessoulou (Table 2). Trees that were burnt by man-made fires in the previous year, displayed a significantly lower synchrony index in Igbo Aladja ($F = 4.21$, $p = 0.041$, ANOVA) and Peperkou ($F = 2.60$, $p = 0.01$, ANOVA) than in Penessoulou (Table 2). In none of the three populations a difference in synchrony was detected between trees that grew in water and those situated on terra firme ($F = 0.41$, $p = 0.52$) (Table 2).

Table 2. Index of floral synchrony of *Pentadesma butyracea* individuals in comparison to all trees of the same population (year 2008).

	Trees out of water		***Trees in water***		***Trees burned***		***Trees unburned***	
	N	***Z ± sd***	***N***	***Z ± sd***	***N***	***Z ± sd***	***N***	***Z ± sd***
Igbo Aladja (N = 26)	10	0.71 ± 0.9 [a] (45 ± 7)[a]	16	0.73 ± 0.12[a] (39 ± 5)[a]	12	0.67 ± 0.12[a] (44 ± 7)[a]	14	0.75 ± 0.06[b] (34 ± 5)[b]
				Z ± sd = 0.71 ± 0.10 (36 ± 13)				
Peperkou (N = 23)	10	0.73 ± 0.13[a] (56 ± 9)[a]	12	0.66 ± 0.13[a] (51 ± 6)[a]	10	0.62 ± 0.16[a] (59 ± 7)[a]	12	0.74 ± 0.08[b] (50 ± 8)[b]
				Z ± sd = 0.70 ± 0.13 (53 ± 8)				
Penessoulou (N = 20)	5	0.84 ± 0.7[a] (32 ± 5)[a]	15	0.84 ± 0.8[a] (29 ± 3)[a]	3	0.80 ± 0.6[a] (35 ± 5)[a]	17	0.84 ± 0.8[a] (25 ± 4)[b]
				Z ± sd = 0.84 ± 0.08 (26 ± 5)				
All three populations together				Z ± sd = 0.74 ± 0.12				

The same letter denotes two groups of trees for which mean synchrony index (Z) does not differ significantly (α = 0.05) within population.

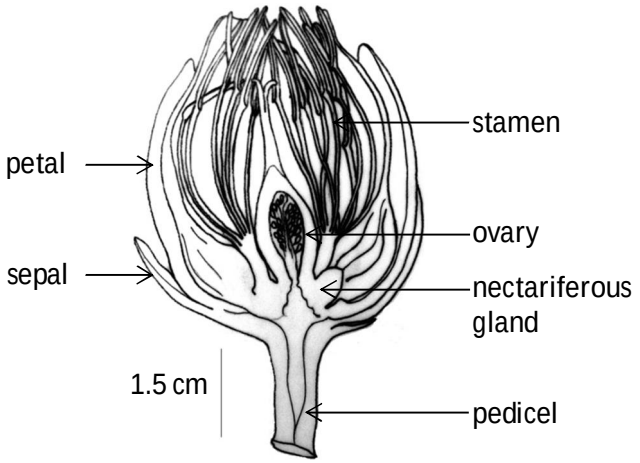
() Average number (± sd) of days of flowering across all individuals of a given category within a population

Inflorescence and floral traits

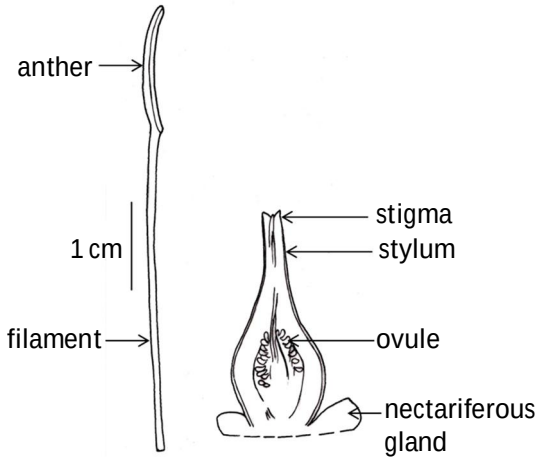
Inflorescences of *Pentadesma butyracea* were situated at the top of flowering branches and composed each by a cyme of 3 to 7 biparous cymes carrying 3 to 15 flowers (mean ± sd = 7 ± 3 flowers; n = 75 inflorescences). All flowers were very large (6 to 7 cm long and 5 to 6.5 cm in diameter), hermaphrodite, actinomorphic and yellowish or white greenish in color. The calyx was greenish, composed of 5 (pentamer) free, nested, unequal (two small sepals were external), oval sepals. The corolla was green-whitish, composed of 5 equal and free petals egg-shaped, oblong to oval, alternate with sepals, very concave, aestivation was twisted sinistrorse (Fig. 5). The androecium was constituted of 5 phalanges of epipetalous thread stamens fused at the base. Every phalange was composed of 49 ± 6 stamens and 0 to 5 staminodes. Thus every flower had on average 245 ± 30 stamens. Every stamen was composed of a filament (long of 4 to 6.5 cm) and an anther which dehisce introrsely (Fig. 4). Anthers were basifixed measuring 1.3 to 1.6 cm in length. The androecium showed a low spatial separation from the stigma. The inner anthers were at the same height as the stigma and even touching the latter while more distant stamens were shorter. On 20 % of phalange and 46 % of the flowers examined there were 1 to 5 highly variable distorted stamens found within a phalange (curved leaf, anther absent, etc., Fig. 5). The ovary was superior (see Fig. 4), usually composed of 5 carpels forming 5 locules. The stylum was short and ended in the stigma composed of five stigmatic papillae. Placentation was axile in two to three rows of ovules per locule. The number of ovules varied between 53 and 188 ovules per ovary (mean ± sd = 121 ± 22, n = 75 ovaries on 75 flowers). Ovules were anatropous.

Nectar reward

Every flower presented five cone-shaped nectariferous glands alternating with petal and stamen phalange. Each gland measured 0.8 x 0.5 cm with nectar seeping from its top. The mean total nectar volume measured was 1042 ± 117 µL per flower. Nectar was produced continuously during floral anthesis. The quantity decreased with flower aging from 1042 ± 117 µL at first anthesis in the morning at 06:30 a.m. to 694 ± 116 µL in the evening at 6:00 p.m. and to 447 ± 73 µL after 24 hours. It was observed that with the continuous opening of the flower, nectar flew out of the flower through openings appearing in between the petals.



A



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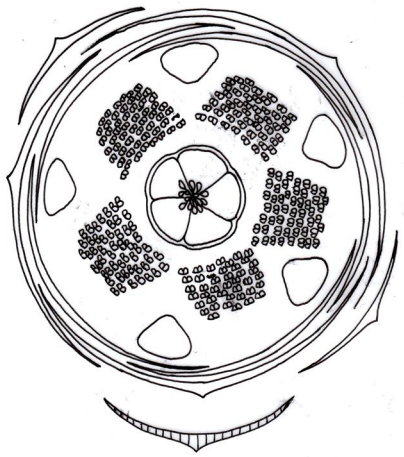
C



FF : (2+3) S + 5P + (41+50+51+55+53)E + 2St + 5C

D

Fig. 5. Some morphological characters of *P. butyracea* flower. A: Longitudinal section of flower; B: Detail of a stamen (left) and gynoecium (right); C: Five forms of staminodes surrounding a normal stamen; D: Floral diagram and formula of *Pentadesma butyracea.*

Pollen load and viability

Viable pollen grains in the anthers were often in packages of 2 to 17. They were circular and of variable sizes. Their mean number counted at stage fl3 was 287 ± 156 per anther (n = 30 examined anthers per floral stage and 120 anthers in total). For a number of 245 ± 30 stamens, a flower produced on average 70,000 ± 4,700 viable pollen grains. This number decreased significantly with the age of the flower from 287 ± 156 viable pollen grains per anther at anthesis to 21 ± 19 48 h after anthesis. Staminodes did not contain any pollen grains at all. Based on fresh anthers that had not yet lost any pollen grains (stage fl3), the P/O ratio was 577 ± 213.

Fruit and seed set

The mean number of flowers per inflorescence was 7 ± 3 (ranging from 3 to 15, n = 75 inflorescences) but fructifiscences had 3 ± 2 fruits (ranging from 0 to 9 fruits, n = 75 frutescences) resulting in a mean rate of free fertilization or fruit set of 49 ± 19 %.

The fruit of *P. butyracea* is brown at maturity, it has a pear shape, measuring 7.0 to 29.0 cm in length (mean ± sd = 12.9 ± 3.2 cm) and 2.8 to 15.0 cm in diameter (mean ± sd = 8.6 ± 2.3 cm). Its fresh weight varied between 263 and 1705 g (mean ± sd = 820 ± 333 g). Every fruit contained 1 to 34 seeds (mean ± sd = 8 ± 4 seeds, n = 70 fruits). The Seed/Ovule ratio was 0.06 indicating a mean rate of 94 ± 4 % ovule abortion per flower.

Seeds were dark brown, measuring 26 to 55 mm in length (mean ± sd = 38.2 ± 5.3 mm), 20 to 39 mm (mean ± sd = 29.0 ± 2.3 mm) in width and 16 to 33 mm (23 ± 3.2 mm) in diameter. Fruit and seed shapes were very polymorphic within and between individuals.

Trees produced 0 to 840 fruits (mean ± sd = 106 ± 135; n = 73 trees). Similar numbers of fruits per tree were produced in 2008 (mean ± sd = 92 ± 131) and in 2009 (mean ± sd = 89 ± 117). The mean number of fruits increased with tree diameter until a peak of 346 ± 392 (70-80 cm) and 240 ± 247 (60-70 cm) fruits in the years 2008 and 2009, respectively (see additional details on average fruit set of *P. butyracea* according to class diameter provided as Online supplementary material 1). Beyond the diameter of maximal fruit production, i.e. on old trees (dbh > 90 cm), fruit productivity decreased. Among populations, the mean number of fruits per tree tended to decrease with latitude.

Pollinators

The main flower visitors were birds and insects (Table 3). Birds were male and female sunbirds of the Nectariniidae: *Cyanomitra verticalis* and *Cinnyris coccinigastrus*. When anthesis started (fl4), sunbirds were the first animals to arrive and to gain access to floral

rewards. They landed on the inflorescence axis, held onto these axes and pecked the anthers of opened flowers, thereby harvesting pollen on their beak. They also actively sucked the nectar at the bottom of flowers usually by perforating the petal with their long and pointed beak. Here, they chose with high precision always the center of each petal that covered the nectariferous gland (cf. Online supplementary material 3). There was never more than one bird at a time on an inflorescence. The duration of their visit on an inflorescence varied between 3 and 7 seconds, harvesting a maximum of three flowers per inflorescence. When they left an inflorescence, they often returned, occasionally even within a minute.

Table 3. Complete list of pollinators identified on *Pentadesma butyracea* and kept as private collection.

Number of specimens or pictures	Species	Provenance	Identified by
10	*Meliponula togoensis* (Stadelman) (Hym.: Apidae)	Igbo Aladja (6 Nov. 2008), Penessoulou (4 Nov. 2008) and Igbo Aladja (21 Nov. 2008)	1
8	*Hypotrigona* sp. (Hym.: Apidae)	"	1
7	*Apis mellifera* L. (Hym.: Apidae)	"	1
6	*Cyanomitra verticalis*	Photographs from Igbo Aladja, Penessoulou, Péperkou	2
5	*Cinnyris coccinigastrus*	"	2

(1): G. Goergen, Biological Control Center for Africa, IITA-Benin
(2): O. T. Lougbégnon, Faculty of Agronomy Sciences, Université d'Abomey-Calavi, Benin

Insects included three species of Hymenoptera belonging to three different genera of the family Apidae: *Apis mellifera, Meliponula togoensis* and *Hypotrigona* sp. They collected either nectar or pollen or both in the flowers of *P. butyracea.* Similarly to birds, bees moved from one flower to another within the same inflorescence, however, they spent more time per flower (2 to 3 seconds), visited more flowers per inflorescence (3 to 5 flowers) and thus stayed in total longer within the same inflorescence (12 to 23 seconds). After the visitation to the anthers of a flower they showed pollen pincushions on their legs. To reach the nectar, these Hymenoptera took advantage of the openings at the base of the petals arranged by the birds (see Online Supplementary Material 3); 1 to 6 bees would enter the same flower at a

time. Here bees would stay 12 to 23 seconds to suck the nectar emitted by the nectar glands at the bottom of the flower.

Seeds: moisture content, optimal germination conditions, long term storage

Moisture content

The seeds sampled measured on average 41 ± 5.1 mm in length, 30 ± 2.7 mm in width and 24 ± 3.1 mm in diameter. Their fresh weight was 17.1 ± 3.0 g and their dry weight 9.8 ± 1.7 g. The moisture content was MC = 42.5 ± 2.9 % (see details about seed characteristics in Supplementary Material 2 provided online).

Initial germination

Figure 6 shows that germination occurred at different rates depending on the temperature and/or storage.

Germination occurred earlier at 30°C (first seeds germinated 9 days after incubation) than at 25°C and 20°C (first germination after 19 and 22 days, respectively) (Fig. 6). However, at the end of the study i.e. after 103 days of incubation, the same cumulated germination rate was found (50%) except at 25°C (40%). Non-germinated seeds (n = 85) were heavier and significantly bigger than those (n = 65) that germinated ($X^2 = 17.45$; $p < 0.001$) (see details on seeds characteristics in online Supplementary Material 2).

Storage trial

None of seeds stored at 4°C germinated. Already 41% of the seeds stored at 20°C started to germinate before the end of the five weeks storage trial. After five weeks, 32.5 % of the remaining seeds germinated (86 days of incubation at 30°C, see Fig. 6). The MC was 22.7 % for seeds stored at 4°C and 34.3 % for those stored at 20°C. In total, we found a maximal storage duration of 3.5 months (35 days of storage trials + 60 days of delay between collection in the field and start of storage trials).

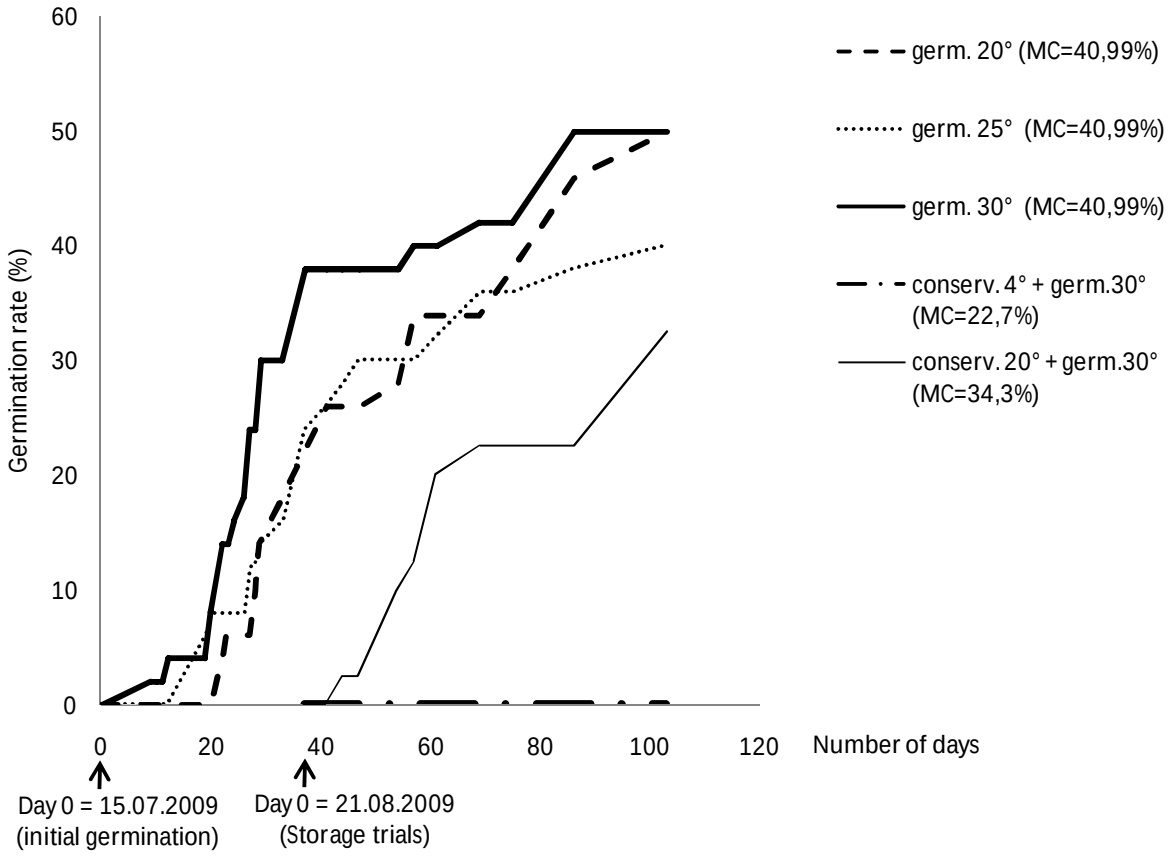


Fig. 6. Evolution of *P. butyracea* seed germination (germ.) rate according to time for different incubation temperatures and conservation treatments (initial and storage trials). MC, moisture content. Conserv., conservation.

Discussion

The phenological profile and its variation

P. butyracea starts to flower in September/October corresponding to the months where humidity, which is indirectly related to rainfall, reaches its maximum mean value (82 to 86 % air saturation according to year, data collected from http://www.tutiempo.net/en/Climate). From January to September a total of 770 mm of rainfall in 2008 and 1180 mm in 2009 (78 to 90 % of the annual precipitation) was recorded. This indicates that a maximum amount of water could be a required signal which induces flowering in *P. butyracea*. This is congruent with the general assumption that the main determinants of phenology of tropical tree species are temperature, light, rainfall and relative humidity (Frankie *et al.* 1974; Borchert 1994; Piechowski 2007).

The flowering period of *P. butyracea* was regular, once a year at the end of the rainy season and during the first two months of the dry season. This pattern was largely concordant

between the three study areas despite latitudinal differences probably due to their relative ecological similarity (concordant pattern of yearly rainfall and relative humidity, ferruginous soil). Flowering in the population Penessoulou occurred only slightly earlier than in the other two populations. Here, trees grow in the Sudania-Guinean transition zone where they benefit from both earlier rainy season and a more precocious air saturation than in Peperkou situated in the Sudanian zone.

This timing of flowering at the end of the rainy season and during the dry season is typical for the savannah zone (Sina 2006). In contrast moist forest tree species (e.g. *Carapa procera, Chrysophyllum sanguinolentum*) usually flower entirely during the rainy season (Auspurger 1983; Devineau 1999). Thus there is a phenological shift between these two habitats. However, this shift is absent in *P. butyracea.* Independently of forest type (e.g. semi-deciduous forest, gallery forest in savannah, evergreen forest …), flowering in *P. butyracea* occurs across its entire range in tropical Africa always during the dry season: in Guinea and Ivory Coast from February to April in wet forest and from July to September in drier forest (i.e. twice a year), and in Gabon from March to September (Aubreville 1959; Keay *et al.* 1964; White and Abernethy 1996).

The phenological difference in the three Benin populations between 2008 and 2009 (temporal shift of 22 days and shorter flowering period in 2009) might be explained by the unequal distribution of monthly rainfall especially the period of the large amount of rainfall (71% from July to September in 2008 vs. 82% from June to October in 2009) and lower amount of water available in 2008 (annual rainfall of 946 ± 74 mm) than in the year 2009 (1254 ± 232 mm). However, flowering ended at the same period in 2008 and 2009, probably as a response to the start of the winter period marked by a high evapotranspiration.

Floral synchrony

In *P. butyracea*, there was a minimum flowering period of two months for every tree as individual branches came into flowering consecutively. Consequently, synchrony of flowering between individual trees is often very high (Z = 0.70) within populations (Table 1), despite a difference in the onset of flowering that can reach two weeks. This value of flowering overlap is common in many other cross-pollinated plant species (Augspurger 1983; Guitian and Sanchez 1992; Sina 2006; Michalski and Durka 2007) and facilitates pollen exchange among trees within and among populations. The high level of floral synchrony increases the attraction of pollinators and could be the result of a selective pressure favoring cross-

pollination (Piechowski 2007). Thus in the case of an efficient pollination, a low level of genetic diversity could be expected within populations of this tree species.

Comparing burned and unburned trees in *P. butyracea* our results show that the burned trees bloomed later with often a short or discontinued period and thus had a lower Xi and Z in populations Peperkou and Igbo Aladja (Xi = 0.44 to 0.75, Z = 0.62 ± 0.16 and 0.67 ± 0.12 respectively). On the contrary, trees growing out of water included burned and unburned trees which tend to reduce the late effect of flowering entry in comparison with trees in water. This suggests that wildfires are creating significant biological stress which may delay or cancel the occurrence of flowering as found in grasses and trees (White *et al.* 1991; Erwin 2006).

Floral traits associated to breeding system and P/O ratio

In *P. butyracea* the attraction of pollinators is promoted by large flowers and the production of a large amount of nectar (1042 ± 117 µL). Additionally, a high amount of pollen is produced in each flower. The arrangement of numerous stamens in five phalanges is a monotypic floral trait of the six species belonging to Symphoniae except *Symphonia globulifera*. The high number of pollen grains per anther (287 ± 156) and the relatively high pollen:ovule ratio (P/O = 577) indicate a high allocation to male function, as generally found in predominantly out-crossing species (*Trichostema* spp, Spira 1980). The P/O ratios of most xenogamous, animal-pollinated plants are between 1200 and 8000 (Cruden 2000) suggesting that the yellow butter tree is facultative xenogamous. Indeed, *P. butyracea* seems to be self-compatible as shown by a tree planted out of its range in the south of Benin nine years ago and some other isolated trees (resulting from fragmentation of gallery forests) which commonly produce small fruits. Preliminary data using genetic markers also confirm that the species is mostly allogamous (Ewedje, unpublished results).

Pollinators

The pollination system of *P. butyracea* was of a general nature with two different bird species and three different insect species. The traits facilitating this include the open flower morphology and the offer of different rewards from an enormous quantity of nectar (1042 ± 117 µL) to a lot of pollen grains (69825 ± 4680) available per flower. Both birds and bees were attracted likewise by nectar and pollen.

Particular adaptations to bird pollination include the apical position and sturdiness of inflorescences on branches which allow an easy open access and a landing on the latter, the color (yellowish or white greenish) and size of flowers and the large amount of nectar and

pollen produced (Primack 1987). The concave corolla, however, seems to be more adapted to bees than to birds. Birds usually prefer a tubular corolla (Lange and Scott 1999; Freitas *et al.* 2006).

Comparing the distribution of *P. butyracea* and its pollinators, the bees are widely distributed beyond the range of *Pentadesma* whereas the birds' ranges correspond closely to the range of *P. butyracea* (Borrow and Demey 2001). There already exist further records of sunbird pollination of *P. butyracea* from Gabon (White and Abernethy 1996).

Concerning the pollination efficiency of the observed pollinators, the observed Hymenoptera are reported to have a significant effect on fruit set in many other entomophilous plants in some African tropical forests and savannas (Lobreau-Callen *et al.* 1986; Fohouo *et al.* 2001; Byarugaba 2004; Ewédjè 2005; Freitas *et al.* 2006; Sina 2006; Pauly *et al.* 2009). The same findings are reported in the case of the green-headed sunbird (*Cyanomitra verticalis);* indeed already a few visits by sunbirds were enough to increase seed set considerably over unvisited or bagged inflorescences of plant species (Lange and Scott 1999; Hargreaves *et al.* 2004; Ford and Johnson 2008). Pollen transport in *P. butyracea* via birds is probably accomplished on the bird´s beak (see also Frost and Frost 1981) or head (see also Wester and Claßen-Bockhoff 2007) where pollen adheres to when the bird pecks the anthers of opened flowers.

Regarding pollination behavior, the birds were faster during their visits than the insect pollinators and visited less flowers per inflorescence. Thus birds might facilitate less geitonogamy than insects.

Also pollen dispersal distances are potentially different between birds and insects. Sunbirds are thought to disperse outcross pollen over longer distances (Degen *et al.* 2001) than Hymenoptera. However, insects display large variations in their potential flight distances (Roubik 1989). In the case of an efficient pollination, the diverse pollinators spectrum (also primates and bats, see Gautier-Hion and Maisels 1994; White and Albernethy 1996; Petterson *et al.* 2004) combined with the high level of flowering synchrony within and between populations (Xi = 0.62 to 1 and Z = 0.62 to 0.89) could lead to substantial gene flow and a low genetic differentiation between populations.

Fruit and seed set

Our findings reveal that *P. butyracea* presents a high mean rate of fruit set (49 ± 19 %) from open-pollination of an inflorescence compared with other tropical tree species (Bawa and Webb 1984). The production of fruits increased with tree diameter. Generally, there is a tight

allometry between tree diameter, height and the crown volume (Alves and Santos 2002). So that, larger trees (except old trees of dbh = 90cm) may have a greater capacity to intercept sunlight for higher photosynthesis than smaller trees which are often situated under the forest canopy. Thus, larger trees would have more resources to produce a higher number of fruits than smaller ones.

Regarding the high number of pollen grains per ovule and flower, a high fertilization rate of ovules per fruit might be expected in this hermaphrodite plant (Sutherland 1986). However, we detected a high rate of unfertilized ovules per fruit (94 %). Similar results have been described in other plant species (Schemske and Horvitz 1988; Ley 2008). Our findings could be explained by:

(1) pollen limitation due to an insufficient pollinator visitation rate, a pollen loss during transport by pollinators, and/or a short pollen viability. To test this, further hand-pollination experiments should be conducted.

(2) a low acceptance of self-pollen, which could also be tested with hand-pollination experiments. This could result from a partial self-incompatibility system which favours allogamy. Moreover, asexual reproduction observed within *P. butyracea* might lead to a special accumulation of related trees increasing the transportation of self-pollen between these trees.

(3) a selective abortion, resulting from resource limitation, to favor high quality (i.e. out-crossed) fruits and seeds, (Kay 2006; Sun *et al.* 2007).

The longevity of* P. butyracea *seeds

Seeds of *P. butyracea* are killed when dried below a threshold moisture content of ~23% which falls into the range of recalcitrant seeds (Pritchard 2004). In recalcitrant seeds the embryo maintains a high tissue moisture content sustaining metabolic activity throughout ontogeny but bursts the seed tissues shortly after dispersal (Farnsworth 2000). Thus recalcitrant seeds are generally short-lived and die rapidly when they are dried or chilled. This is a trait commonly found in the family Clusiaceae (Carvalho *et al.* 1998; Daws *et al.* 2005; Orwa *et al.* 2009). In *P. butyracea* seeds are large which is typical for recalcitrant species (Hong and Ellis 1996), and might reduce the rate of seed desiccation. The moisture content in seeds of *P. butyracea* (MC = 42.5 ± 2.9 %) was similar to sheas (MC = 41 to 48 %, Gamene *et al.* 2004), much higher than in other recalcitrant seeds (e.g. *Khaya senegalensis* MC = 3.41 %; Gamene and Eriksen 2004; *Zanthoxylum zanthoxyloides* MC = 13.85%, *Parinari curatellifolia* MC = 17.49 %, *Lophira lanceolata* MC = 21.30 % and *Kigelia africana* MC =

26.98 % (Sanon *et al.* 2004)), and lower than the MC estimated in *Azadirachta indica* (70 %, Neya *et al.* 2004).

Germination of *P. butyracea* was more rapid at 30°C than at 20°C and was lowest at 25°C, but probably because the daily temperatures in this incubator was not constant. The temperature of 30°C or more are the likely soil temperatures that the dispersed seeds might experience in their natural environment (Daws *et al.* 2002). It might thus reflect a genetically fixed adaptation to the local environment. Still the germination rate obtained here at 30°C, is significantly lower than the one observed by Ouattara (1999) in Ivory Coast (germination rate of 95% of incubation at room temperature). This result was potentially due to the fluctuations of temperature and the loss of seeds' moisture content in the time between seed collection in the field and start of the trials in Belgium. For the further treatments at lower temperatures we would have expected a near linear relationship between temperature and germination. However, germination rate was lowest at 25°C and not at 20°C. That our results deviate from the expectation might be due to an inconstancy of temperature in the incubator at 25°C.

Concerning the potential long-term storage time of *P. butyracea* seeds we only tested five weeks here. But already after this short period only about 33 % of seeds germinated under optimal conditions. Similarly in other recalcitrant species only short storage times are reported (Shea trees, 11 months, Gaméné *et al.* 2004; *Aesculus hippocastanum:* 36 months (Pritchard *et al.* 1996). In all cases, already a feeble desiccation of 10% from initial moisture due to storage at 20°C (compare 20% in shea trees) results in a loss of seed viability. Thus, desiccation intolerance creates challenges for storing and preserving recalcitrant seeds and makes these seeds unsuited for long-term storage. The fact that all *P. butyracea* seeds stored fully hydrated at 20°C germinated in storage and the sensitivity of seeds to temperatures below 15°C (test results in Ouattara 1999) suggests that storage around 20°C seems optimal and that sub-imbibed storage may be beneficial for this species. Clearly further work on the effect of partial drying on recalcitrant seed viability and storability is required.

Implication for conservation

Currently, the highest threat to this species is the human being by overexploitation and competition for space. According to Avocèvou *et al.* (2009) and Djossa *et al.* (2007), seedlings (dbh < 5 cm) and saplings (dbh 5-10 cm) of this species and Shea tree (of which seeds are often mixed to those of butter trees to make butter when yearly harvesting decrease) occur within protected areas but are rare in harvested stands. Still, *P. butyracea* can partly compensate reduced seed-set by asexual reproduction via root shoots (more than 84 juveniles

firmly attached to the roots could be counted under a tree crown) and trees subjected to fraudulent logging activities, might regenerated with 6 to 11 sprouts from its stump (Ewedje, pers. obs.). However, an exact estimate of the contribution of asexual reproduction in *P. butyraceae* is still missing.

A further potentially important threat to the species survival might be population fragmentation. It is not known to which extend the observed pollinators are still able to maintain a regular gene flow between the left populations distant from each other. However, the maintenance of genetic diversity of the species as a preadaptation for environmental change is crucial for its survival and should be tested by further genetic work. Here especially the presence/absence of inbreeding effects should be investigated to estimate the potential for the fixation of detrimental mutations especially frequent in small populations (Robledo-Arnuncio *et al.* 2004).

The data has shown that only *in-situ* conservation and/or *ex-situ* conservation through orchard plantations are promising due to the recalcitrant nature of seeds unsuitable for long-term storage. The easy germination of seeds and the generalist pollination characteristics of the tree species (general pollination system, high pollen production, facultative outcrossing) will certainly facilitate any conservation effort. In the case of Benin, the creation of an orchard of *Pentadesma butyracea* is considered within the coastal forest at Ahozon within the touristic site.

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Contributions by the authors

All authors have made a substantial contribution to the actual scientific work.

Conflicts of interest

No conflicts of interest.

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Supplementary Material 1

Average fruit set of *P. butyracea* according to diameter class

Diameter Classes (dbh) [cm]	Number of trees	Fruit set per tree in year 2008 (mean ± sd)	Fruit set per tree in year 2009 (mean ± sd)
0-10	3	0 ± 0	8 ± 10
0-20	5	7 ± 13	11 ± 15
20-30	6	32 ± 41	13 ± 22
30-40	18	46 ± 41	52 ± 53
40-50	22	72 ± 64	88 ± 61
50-60	10	138 ± 127	130 ± 94
60-70	9	252 ± 213	240 ± 247
70-80	2	346 ± 392	126 ± 31
80-110	2	68 ± 02	65 ± 1
Total	**77**	**92 ± 131**	**89 ± 117**

Supplementary Material 2

Seed characteristics of randomly sampled *P. butyracea* seeds and of seeds used for germination trials separated by germinated and non-germinated seeds.

Seed samples	Range (Mean value ± SD)						Kruskal-Wallis test (variable = fresh weight)
	Length (mm)	Width (mm)	Diameter (mm)	Fresh weight (g)	Dried weight (g)	MC (%)	
Random seed sample (n = 100)	29.0 – 57.2 (41.06 ± 5.07)	23.6 – 38.5 (30.35 ± 2.74)	6.20 – 9.57 (7.96 ± 0.63)	11.2 – 28.0 (17.1 ± 3.02)	6.2 – 15.4 (9.8 ± 1.7)	36.14 – 51.36 (42.50 ± 2.88)	-
Germinated seeds (n = 65)	36.45 ± 6.40	26.62 ± 5.51	24.72 ± 3.23	14.93 ± 5.61g	-	-	df = 1; $X^2 = 17.45$; p-value = $2.94\ 10^{-5}$
Non-germinated seeds (n = 85)	40.52 ± 6.32	28.93 ± 4.17	24.61 ± 2.76	18.29 ± 5.05	-	-	

Supplementary Material 3

Petals of *P. butyracea* flowers perforated by sunbirds.

CHAPITRE VII.-

SYSTÈME DE REPRODUCTION, DISPERSION DE GÈNES ET STRUCTURE GÉNÉTIQUE À FINE ÉCHELLE D'UN ARBRE ALIMENTAIRE MENACÉ, *PENTADESMA BUTYRACEA* SABINE (CLUSIACAE) AU BÉNIN

Manuscript not submitted

Système de reproduction, dispersion de gènes et structure génétique à fine échelle d'un arbre alimentaire menacé, *Pentadesma butyracea* Sabine (Clusiacae) au Bénin

La définition des stratégies de conservation des ressources phytogénétiques des espèces traditionnelles sous-utilisées, telles que *Pentadesma butyracea,* repose sur la connaissance de leur système de reproduction. En effet, celui-ci influence la structuration de la diversité génétique au sein et entre les populations et le maintien de cette diversité au cours du temps. Nous avons fait l'étude de la structure génétique à fine échelle des adultes et des familles de graines de *Pentadesma butyracea*, du taux d'allogamie et des niveaux de corrélation de paternité. Cette étude a été réalisée au Bénin, dans une population de petite taille et dans trois autres de grande taille, à l'aide de marqueurs microsatellites.

P. butyracea est une espèce auto compatible, majoritairement allogame (*tm* = 0,88-0,95). L'hétérozygotie observée au sein des graines est similaire à celle des adultes, montrant l'absence de dépression de consanguinité. La structure génétique spatiale des populations parentales de grande taille estimée par la statistique *Sp* (*Sp* = 0,003-0,038) indique que la dispersion des gènes est géographiquement limitée. De même, la baisse de la corrélation de paternité entre familles de graines en fonction de la distance suggère une dispersion limitée du pollen. Le taux d'immigration du pollen est estimé à près de 0,21 dans une population et la distance moyenne de dispersion du pollen est inférieure à 100m dans deux populations. La structure génétique à fine échelle au sein de chaque population indique aussi une dispersion limitée des graines. Notre étude a détecté une corrélation de paternité plus élevée respectivement aux niveaux intra-fruit vs. inter-fruits (0,42 ± 0,11 vs. 0,32 ± 0,13), arbres proches vs. arbres éloignés, semences fécondées la même année vs. semences de différentes années (0,24 ± 0,25 en 2009 vs. *rp* = 0,13 ± 0,06 en 2010), puis au sein d'une population de petite taille vs. population de grande taille (*rp* = 0,37 vs. *rp* = 0,23).

En conclusion, nos résultats suggèrent que les populations de petite taille peuvent subir une plus forte limitation en pollen (faible diversité des sources). Néanmoins, les populations semblent relativement interconnectées. Aucun signal de dépression de consanguinité n'est détecté, probablement grâce à une purge d'allèles délétères sous l'effet d'évènements de fondation passés.

Dans le cadre de la conservation *ex-situ* des semences ou d'enrichissement des populations existantes, nos résultats suggèrent que la collecte de fruits/graines doit être réalisée dans plusieurs directions pour représenter au maximum la diversité d'un arbre-mère. De plus, pour éviter d'échantillonner des arbres apparentés, la distance à observer entre arbre-mères devra être d'au moins 10 mètres.

Breeding system, gene dispersal and small-scale spatial genetic structure of a threatened food tree species, *Pentadesma butyracea* Sabine (Clusiacae) in Benin

Ewédjè E.B.K, Ahanchédé A. and Hardy O.J.

Abstract

Defining appropriate strategies for the conservation of genetic resources of under-studied food tree species, such as the butter tree *Pentadesma butyracea*, requires a good knowledge of mating patterns at the population level. Therefore, the fine-scale genetic structure of adults and maternal sibships, outcrossing rate and levels of correlated paternity were estimated for a small and three large populations of *P. butyracea* in Benin using microsatellite markers. Similar outcrossing rates (88-95%) were found in all populations, showing that *P. butyracea* is mainly an outbreeder species despite its selfing capacity. We found no evidence of a decay of the observed heterozygosity between seeds and adults, suggesting the absence of inbreeding depression. The spatial genetic structure detected among adults within the large populations (Sp = 0.003 to 0.038) shows that gene dispersal is spatially limited at this scale. Similarly, the decay of the degree of correlated paternity between sibships with spatial distance highlights limited pollen dispersal distances. According to parentage analyses following the neighborhood model, the mean pollen dispersal distance within the large populations was estimated to range between 50m and 450m, but up to 21% pollen might immigrate from external sources. The smallest population displayed slightly higher correlated paternity than the large populations (rp = 0.37 vs. rp = 0.17-0.30), suggesting pollen limitation in the former. In one population sampled two consecutive years, the rate of correlated paternity within maternal sibship varied with year ($rp \pm sd = 0.24 \pm 0.25$ in 2009 vs. $rp = 0.13 \pm 0.06$ in 2010) and was small between years ($rp \pm sd = 0.04 \pm 0.08$), suggesting a variable effective number of fathers due to asynchronous phenology among adults trees. In conclusion, our results suggest that small populations might suffer pollen limitation but that large populations are connected through gene flow and that inbreeding depression does not appear as a major threat, possibly because the populations of Benin have already undergone a purge of deleterious alleles through an historical bottleneck.

Keywords: correlated paternity, kinship coefficient, mating system, *Pentadesma butyracea*, pollen dispersal

Introduction

Assessing the spatial genetic structure, the mating system and the gene flow patterns in natural populations are central topics of evolutionary and conservation biology for the following reasons. (i) Mating patterns influence the structure of genetic diversity within and among populations and the maintenance of that diversity over time. The mating system is typically characterized in plants by the outcrossing rate and by the level of correlated paternity within maternal sibships. Together with gene flow, these parameters condition the departure from random mating and the significance of genetic drift under the isolation-by-distance model (Ritland 1989). The mating system depends on the particular attributes of the species' reproductive biology and also on the population spatial structure (Boshier 2000). Thus, changes in population size and degree of isolation are expected to affect the mating system. Therefore, the mating system of many tropical tree species is likely to be altered by deforestation and change of land use (Myers 1986). In small or isolated populations, high rates of correlated paternity and relatively low rates of outcrossing in self-compatible plants are expected as pollen is derived primarily from a few sources (Mitton 1992). (ii) Trees generally carry a heavy genetic load of deleterious recessive alleles because of their large number of cell divisions between generations and their greater difficulty of purging deleterious alleles (Williams and Savolainen 1996), so that inbreeding, as caused by selfing, may lead to reduced fertility (hence poorer regeneration), slower growth rates in progeny, limited environmental tolerance and increased susceptibility to pests or diseases (Boshier 2011). Reducing the possibility or impact of inbreeding and maintaining genetic diversity in trees is important, and may be critical to seed collections that are used in tree breeding and *ex situ* conservation.

The spatial distribution of genetic diversity depends on gene flow which favors the genetic connectivity between populations, genetic drift and natural selection. The magnitude of gene flow depends on seed and pollen dispersal and the mode of reproduction (asexual vs. sexual, selfing vs. outcrossing). Pollen dispersal is often a major contributor to gene flow while seed dispersal is often more limited and largely determines the spatial genetic structure at the finest scale (Miyazaki & Isagi 2000, Hardy *et al.* 2004). An outcome of restricted gene flow is isolation by distance IBD (Wright 1969), i.e. an increase in genetic differentiation with spatial distance. Information about the spatial genetic structure of tree populations helps to elucidate the forces driving genetic dynamics. It has important implications for the management and

conservation of forest genetic resources (Epperson 1992), as well as for assessing the expected impacts of forest exploitation and fragmentation (Young & Merriam 1994).

We characterize in this paper the mating system, the inbreeding, the gene dispersal pattern and the small-scale spatial genetic structure of the food tree species *Pentadesma butyracea* Sabine, which is threatened in Benin by the ongoing fragmentation of its natural habitat and a reduced recruitment due to seeds overexploitation. The exploitation of its resources (seeds, timber) becomes so important that it is now vulnerable and falls among the ten priority food tree species for conservation in Togo and Benin (Eyog-Matig *et al.* 2002). However, in spite of fragmentation, *P. butyracea* can reproduce asexually through root shoots. Generally, fragmented populations are predicted to experience stochastic loss of rare alleles because only a small portion of the original gene pool remains after fragmentation (Young *et al.* 1996). This may increase local inbreeding levels in progeny cohorts and interpopulation genetic divergence (Ohara *et al.* 2006). *Pentadesma butyracea* is considered to be a facultative xenogamous species according to the number of pollen grains produced per ovule (P:O = 577) and its floral structure delivering both nectar and pollen as rewards (Ewédjè, unpublished data), but its outcrossing rate has never been quantified precisely. Flowers are pollinated by birds and insects (Ewédjè, unpublished data) and seeds are assumed to be dispersed by vertebrate species. So, gene flow should be extensive. As we lack knowledge about seeds dispersers, their impact on the genetic structure of populations must be investigated.

To improve our knowledge on the mating system, the spatial genetic structure and the consequence of inbreeding of *P. butyracea* in Benin, we used microsatellite markers to conduct detailed investigations in one small and three large populations to address the following questions:

- What are the levels of inbreeding in adults and progeny and do they reveal evidence of inbreeding depression?
- What is the pattern of pollen dispersal (outcrossing rate, correlated paternity through space and time, within population pollen dispersal distances, pollen immigration rates in populations)?
- What is the spatial genetic structure within populations of *P. butyracea*?

Materials and Methods

Study populations

We sampled one small (11 adults) and three large (90-144 adults) *P. butyracea* populations of Benin in 2009 and 2010 (characteristics summarized in Table 1). The four studied sites belong

to a single agro-ecological zone defined as Centre-East and North-West of the Sudanian zone (Dagbénonbakin *et al.* 2003). The small population (Nioro) represents a remnant stand consisting of 11 isolated adult trees scattered over an area of c. 0.05 ha. The three large populations (Igbo Aladja, Kouba and Setou) are situated in linear gallery forests over an area of 0.55 ha, 0.64 ha and 0.80 ha, respectively (Fig. 1). Each of the four sampled populations is surrounded by two to three other small populations (unsampled) distant by 3 to 23 km. All adult trees were sampled in each population by collecting leaf dried in silica-gel. In May-June 2009 and/or 2010, two to seven fruits were directly collected per tree on 17 to 19 mother trees in each of the large populations and on eight trees in the small population (Table 1). Seeds were extracted from each fruit and germinated to sample young leaf for DNA extraction (after drying them in silica-gel).

DNA isolation and PCR protocols

Total DNA was extracted from silica gel – dried or fresh leaf using (1) NucleoSpin Plant kits (Macherey-Nagel, Düren, Germany) for seedlings and (2) CTAB procedure (Doyle & Doyle 1987) for adult trees. The latter procedure was more successful in adult leaf probably because it is more efficient to cope with problematic chemical compounds such as polysaccharides. Nevertheless, some of the adult trees could not be genotyped.

We performed polymerase chain reaction (PCR) using 11 microsatellite loci that were amplified in two multiplexed reactions using the QIAGEN Multiplex kit in a final 15 µL reaction volume following Ewédjè *et al.* (in press). As one locus amplified weakly in multiplexed reactions and two loci failed to amplify in many individuals, they were discarded from the analyses. Allele sizes were read and analyzed using Peak Scanner Software Version 1.0 (Applied Biosystems).

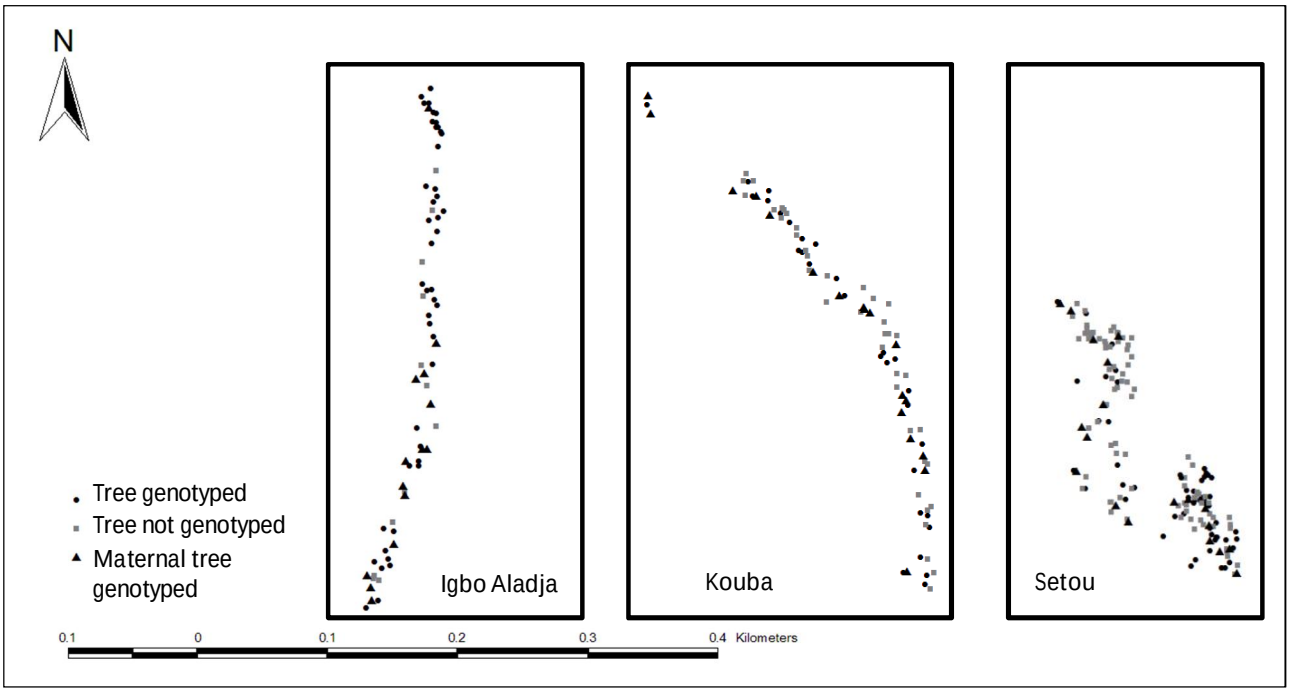

Fig.1. Spatial distribution of trees within each of three large populations Igbo Aladja, Kouba, Setou.

Table 1: Characteristics of the four populations sampled

Populations	Geographical coordinates (UTM, zone 31N)		Mean elevation (m)	Habitat[1]	Annual rainfall (mm)	Adult population size	Number of genotyped individuals	Number of maternal sibships (total number of seeds genotyped)	
	Lat.	Long.						Year 2009	Year 2010
Igbo Aladja	967239	415216	257	GF	1199	90	85	12* (114)	10* (58)
Kouba	1023542	336472	420	DDF	1381	91	64	-	17 (107)
Setou	1160673	332713	393	GF	1269	144	103	-	19 (93)
Nioro	1027648	334795	429	GF	1269	11	11	5 (17)	3 (15)

[1]GF: gallery forest, DDF: dry semi deciduous forest surrounded by savannahs

*4 mother trees were sampled both year 2009 and 2010

Data analyses

Parental and progeny genetic diversity and inbreeding

The diversity at each locus was assessed by the number of alleles per locus and gene diversity (Nei 1978) in all populations using SPAGeDi 1.3 (Hardy and Vekemans 2002). Allelic richness was estimated using Fstat version 2.9.3.2 (Goudet 2002).

For each population we computed the observed heterozygosity per locus and the average inbreeding coefficient and we tested for deviation from the Hardy – Weinberg equilibrium (HWE) separately for adult trees and progeny. Results were adjusted for multiple comparisons. Due to the presence of null alleles, we checked whether departure from HWE at a given locus might be explained by the presence of null alleles using the software INest under the population inbreeding model (Chybicki and Burczyk 2009). INest provided estimates of the frequency of null alleles as well as corrected estimates of the inbreeding coefficient and the gene diversity (expected heterozygosity) accounting of null alleles. Finally, we tested whether the observed heterozygosity differed between adults and progeny using an ANOVA where population and locus were treated as random effects.

Spatial genetic structure

Within each of the large populations, we characterized the fine-scale spatial genetic structure among adults by computing pairwise kinship coefficients with J. Nason's estimator (Loiselle et al. 1995) and averaged them over a set of distance intervals (the upper distances of the intervals were: 10, 20, 40, 60, 100, 200 and 520 m). Test of significance were obtained by randomizing the spatial positions among trees using SPAGeDi (Hardy & Vekemans 2002). We then quantify the spatial genetic structure by the *Sp* statistic according to Vekemans and Hardy (2004) as follows, $Sp = -b / (1- F_1)$, where b is the regression slope of pairwise kinship coefficients on the logarithm of spatial distance and F_1 the kinship coefficient between neighbours (here we used the average of the first distance interval).

Mating system parameters estimation and pollen dispersal

To compare mating system variations of the four populations, the genotypes of sibship families and adults were analyzed by both software MLTR version 3.4 (Ritland 2009) and NM+ version 1.0 (Chybicki and Burczyk 2009), assuming that null alleles can occur. MLTR is based on the multilocus mixed-mating model of Ritland and Jain (1981) and on the correlated mating model of Ritland (1989). It allowed estimating the selfing rate, the biparental inbreeding, and the correlation among paternal gametes within maternal sibships

(correlated paternity). MLTR provided variances of estimators by bootstrapping over families 500 times. The program NM+ provided maximum-likelihood estimators of gene dispersal and mating system parameters by modeling spatially dispersal processes while accounting for null alleles when computing parentage probabilities. It estimated the selfing rate, the rate of pollen immigration (i.e. the proportion of seeds sired by unsampled adults) as well as the mean pollen dispersal distance within population assuming that the dispersal kernel followed a two-dimensional exponential function.

Within and among-sibship correlated paternity: effect of isolation by spatial distance, phenology and year.

The module Kindist of the software Poldisp (Robledo-Arnuncio *et al.* 2007) was used to compute the average correlated paternity within and among maternal sibships following the procedure described in Robledo-Arnuncio *et al.* (2006). Estimates are based on the computation of multilocus pairwise kinship coefficients between the paternal gametic genotypes of offspring pairs. The pollen-pool spatial genetic structure was characterized by the decrease of among- maternal families correlated paternity with spatial distance, which is a signature of limited pollen dispersal.

For the population Igbo Aladja where sibships were collected in 2009 and 2010, the average correlated paternity within and among maternal sibships was computed within and between years and regressed on spatial distance between mothers and sampling year difference (0 or 1) in order to evaluate the impact of phenological asynchronism between adults.

Finally, to obtain insights on the diversity of the pollen loads received by individual flowers, we also compared the average correlated paternity within vs. among fruits for maternal sibships for which several fruits and several seeds per fruit were available.

As Polpdist does not take into account the possible presence of null alleles, we compared estimates obtained with all loci with those obtained after removing loci containing a null allele at a frequency above 10%.

Results

Genetic diversity and inbreeding coefficient variations within large parental populations

Detailed information on allele sizes ranges, observed and expected heterozygosities and null allele frequencies per locus and population are given in Online supplementary materials 1, 2 and 3. Null alleles were detected in each population for some of the loci, contributing to the apparent heterozygote deficiency at three to four loci according to population. Indeed, the

software INest revealed that the apparent departures from HWE can be explained by a relatively high frequency of null alleles (0.1 to 0.3). Among the eight microsatellites loci, five showed low levels of diversity (H_E ranging from 0 to 0.5) whereas the three other ones were more polymorphic (H_E ranging from 0.53 to 0.67). The H_E estimations based on the raw genotype scoring were always lower than those computed with the software INEst which accounts for null alleles.

There was significant heterogeneity in diversity indices among the sampled populations: the allele number, allelic richness and expected heterozygosity were higher in the population Kouba, medium in Setou, lower in Igbo Aladja and very low in Nioro (Table 2). Adults displayed an uncorrected multilocus inbreeding coefficient significantly different from zero in Kouba (F_{IS} = 0.34) and Igbo Aladja (F_{IS} = 0.31, Table 2), and a similar pattern was found with the progeny (Kouba: F_{IS} = 0.26; Igbo Aladja: F_{IS} = 0.14, Table 2). However, corrected estimates (F_{IS}') accounting for null alleles were all very close to zero (Table 2).

Apart from the small population Nioro where the observed heterozygosity (H_O) of adults was much higher than the progeny's H_O (but this may be due to the very small sample size of adults n= 8), there was no clear trends in the three large populations. Multi-way ANOVA test indicates significant difference of observed heterozygosity among loci ($p < 0.001$) but not between adults and progeny ($p = 0.12$, $F = 2.48$), suggesting no signal of inbreeding depression.

Table 2: Genetic diversity and inbreeding coefficient parameters. *N*a, number of alleles; *A*, allelic richness; H_E and H_O (expected and observed heterozygosity estimated after INest). F_{IS}' and F_{IS}, inbreeding coefficient estimated respectively with and without accounting for null alleles following the method implemented in INest.

	Diversity estimators Mean value (SD)							Inbreeding coefficient Mean value (SD)			
	Adult stage				Sibship family			Adult stage		Sibship family	
	*N*a	*A*	H_E	H_O	*A*	H_E	H_O	F_{IS}	F_{IS}'	F_{IS}	F_{IS}'
Igbo	3.2 (0.53)	2.38	0.41 (0.24)	0.28 (0.24)	2.85	0.40 (0.20)	0.34 (0.19)	0.31* (0.11)	0.01	0.14* (0.06)	0.00
Kouba	4.7 (0.89)	3.16	0.48 (0.19)	0.31 (0.22)	2.75	0.46 (0.19)	0.34 (0.18)	0.34* (0.08)	0.15	0.26* (0.07)	0.07
Setou	3.9 (1.52)	2.31	0.44 (0.22)	0.31 (0.34)	2.36	0.37 (0.25)	0.30 (0.25)	0.16 (0.10)	0.01	0.19 (0.05)	0.01
Nioro	1.87 (0.64)	1.87	0.33 (0.25)	0.49 (0.41)	2.36	0.32 (0.25)	0.24 (0.23)	-0.48 (0.23)	0.00	0.25 (0.11)	0.00

*p < 0.05 and **p <0.001 indicate significant deviation from HWE.

Spatial genetic structure within parental populations

Within each of the large populations, mean pairwise kinship coefficients between individuals were significantly positive at short distance (< 10 m: *F*ij = 0.26 in Igbo Aladja, *F*ij = 0.16 in Kouba and *F*ij = 0.08 in Setou, see Fig. 2) and appear to decay more linearly with the logarithm of distance than with the linear distance (Fig. 2). Their regression slopes on the logarithm of the distance were significantly negative, giving the following *Sp* statistic: 0.003 in Setou, 0.008 in Kouba and 0.038 in Igbo Aladja. These results showed that *P. butyracea* displayed SGS patterns apparently consistent with isolation-by-distance expectations with the two linear populations more structured than Setou which extends more in two dimensions (Fig. 1).

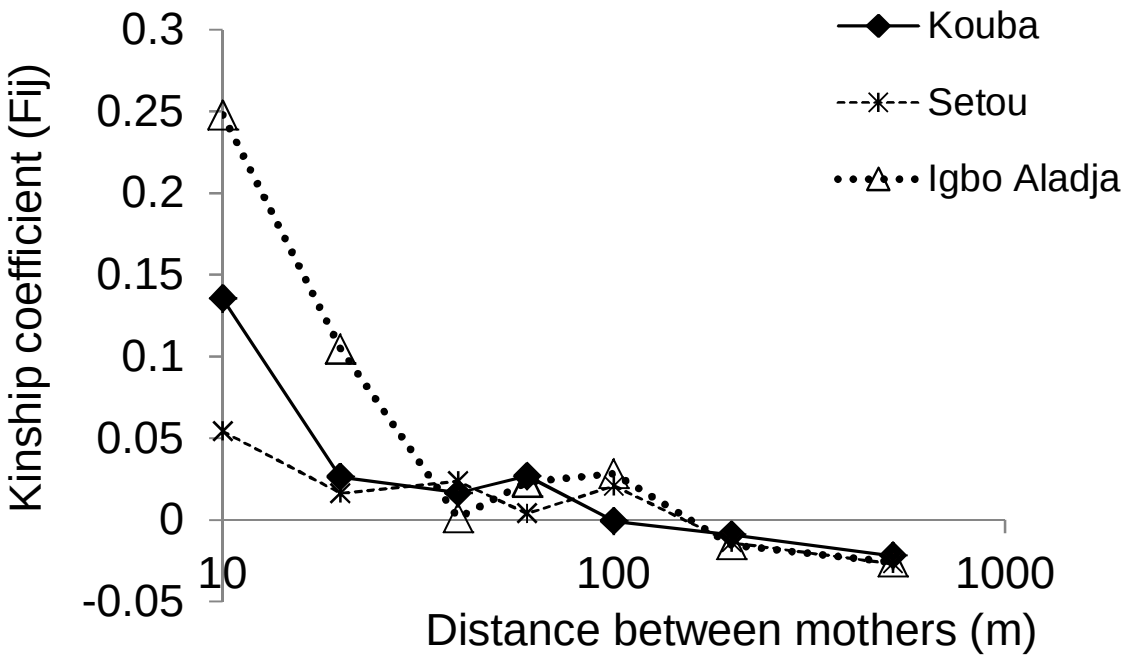

Fig. 2. Comparison of the spatial genetic structure of *P. butyracea* in three populations

Mating system parameters and pollen dispersal

The multilocus outcrossing rate estimated by MLTR was *tm* = 0.89 in the small population and it ranged from 0.78 to 0.93 in the large populations (Table 3). Slightly higher estimates were obtained using NM+ which accounts for null alleles but they remain inferior to 1, indicating that selfing does occur (Table 3). The highest selfing rate was observed in population Kouba (multilocus estimates *s* = 0.22 and *s* = 0.12 according to MLTR and NM+, respectively). Single locus estimates of the outcrossing rate (*ts*) remained important (0.78 to 0.98) and the difference between multilocus and single locus estimates (*tm* – *ts*) was negative (-0.15 to -0.02), suggesting the absence of biparental inbreeding. Indeed, the difference (*tm* - *ts*) reflects the amount of apparent selfing rate at a single locus resulting from mating between relatives rather from true self-fertilization. These results indicated that *P. butyracea* is a predominatly outcrossing and self-compatible plant species.

The correlated paternity within maternal sibships (*rp*) estimated by MLTR showed moderate values ($0.24 < rp < 0.37$) for the large populations (i.e. effective number of fathers per family, $1/rp$, = 2.7 to 4.1) which were substantially and significantly smaller than the *rp* estimated in Nioro ($rp = 0.79$, $p < 0.05$, suggesting $1/rp = 1.2$ effective number of fathers per family, see Table 3). Multiple comparison tests showed no significant differences of *rp* among the large populations ($p > 0.05$). The same estimates of *rp* from Poldisp gave 0.17, 0.23, 0.30 and 0.37 in Igbo Aladja, Sétou, Kouba and Nioro respectively (see Table 3) and it was more important within sibship families than among sibship pairs where *rp* was near zero except in Kouba (0.2). In comparison of both programs used to compute this biological parameter, values recorded after Poldisp were lower (especially in Nioro and Igbo Aladja) than those compute by MLTR (see Table 3); the highest value of *rp* was recorded in population Nioro as previously estimated with MLTR. Even if locus displaying more than 10% of null allele were discarded from analyses, the estimates from Poldisp remain similar except the population Igbo Aladja where it decreases from 0.17 to 0.08 probably due to data collected in two years.

Assuming that pollen dispersal follows an exponential kernel, the mean effective pollen dispersal distance within population estimated by NM+ reached *dp* = 50 m, 84 m and 450 m in Setou, Kouba and Igbo Aladja, respectively (see Table 3). Pollen immigration rate estimates ranged from *mp* = 0.19 to 0.31, displaying values substantially larger than the proportion of non-genotyped adults in two populations (see Table 3), suggesting genetic connections with surrounding populations.

Table 3: Estimates of mating system parameters according to the methods implemented in the software MLTR[1], NM+[2] and Polpdist[3]. *N*s = average number of seeds analysed per family; *tm*, multilocus outcrossing rate; *ts*, single locus outcrossing rate; *mp*, pollen immigration rate; *dp*, mean pollen dispersal distance within population.

Population	**Familly number**	***N*s**	**Outcrossing rate**				**Correlated paternity, *rp***				***mp*[2]**	**Proportion of non genotyped adults**	***dp*[2] (m)**
			***tm*[1] mean (SD)**	***tm*[2] mean (SD)**	***ts*[1] mean (SD)**	***tm*-*ts*[1]**	**Tree level[1]**	**Tree level[3]**	**Within fruit[3]**	**Among fruits[3]**			
Igbo Aladja	18	7	0.93[a] (0.08)	0.95[a] (0.03)	0.92[a] (0.11)	0.002 (0.05)	0.35[a] (0.11)	0.17	0.32 (0.45)	0.17 (0.28)	0.21 (0.06)	0.06	450 (193)
Kouba	17	6	0.78[a] (0.05)	0.88 [a] (0.05)	0.78[a] (0.08)	-0.003 (0.05)	0.24[a] (0.10)	0.30	0.44 (0.45)	0.27 (0.34)	0.27 (0.07)	0.30	84 (19)
Setou	19	5	0.85[a] (0.09)	0.94 [a] (0.07)	0.98[a] (0.11)	-0.13 (0.05)	0.37[a] (0.13)	0.23	0.35 (0.41)	0.34 (0.22)	0.19 (0.07)	0.28	50 (10)
Nioro	8	4	0.89[a] (0.23)	0.91 [a] (0.15)	0.96[a] (0.28)	-0.07 (0.06)	0.79[b] (0.46)	0.37	0.57 (0.03)	0.50 (0.46)	0.31 (0.10)	0.00	412 (1448, 69)

[a] Test of means comparison ANOVA; means followed by the same letter within a column are not significantly different at $p < 0.05$

Analysis of correlated paternity within- and among maternal sibships: spatial effects

Following estimates of Poldisp, the mean value of correlated paternity within sibships was higher within fruit than among fruits especially in the two populations Igbo Aladja (*rp* = 0.32 vs. *rp* = 0.17) and Kouba *(rp* = 0.44 vs. *rp* = 0.27) revealing that seeds were sired by more donors among fruits of the same family than within fruits (5.8 vs. 3.1 donors in Igbo Aladja and 3.7 vs. 2.2 donors in Kouba).

From a spatial point of view, our results showed a weak but significant decay of shared correlated paternity between sibships with spatial distance: Pearson coefficient R^2 = 0.09 in Kouba (p = 0.03), R^2 = 0.03 in Igbo Aladja (p = 0.01) and R^2 = 0 in Setou (p = 0.93).

Factors promoting correlated paternity in population Igbo Aladja

Within maternal sibships, correlated paternity was significantly higher in the year 2009 (mean *rp* = 0.24) than in 2010 (mean *rp* = 0.13) suggesting more contribution of pollen donors in 2010. Between these years, correlated paternity was much reduced (mean *rp* = 0.04), suggesting few shared paternal trees.

Between maternal sibships, the correlated paternity tended to decrease with the spatial distance between mothers as well as with the difference in flowering years (see Fig. 3). Linear regression analysis revealed a significant multivariate determination coefficient (R^2 = 0.35), the two factors displaying similar importance (univariate R^2 = 0.13 and R^2 = 0.12 for log(distance) and year difference, respectively, p = 0.0016 for multivariate R^2).

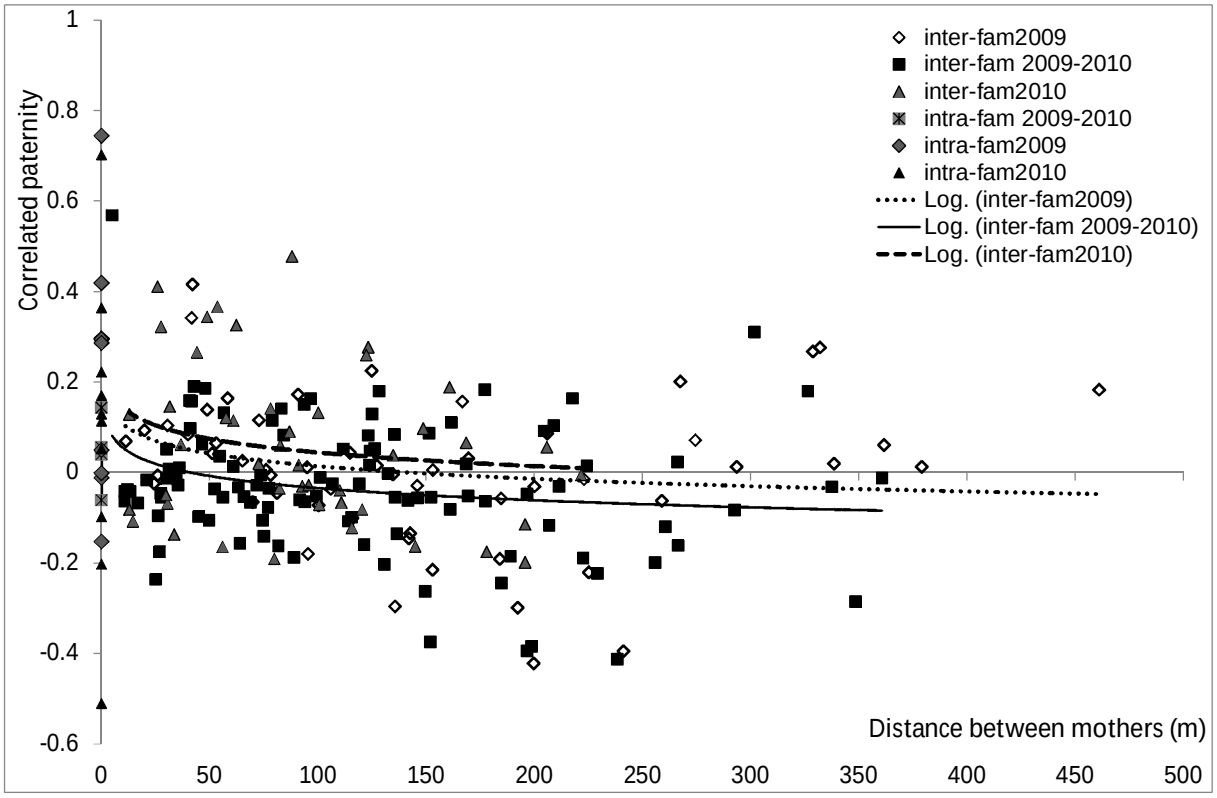

Fig.3. Correlated paternity between maternal sibships according to the distance separating the mother trees in the population Igbo Aladja. Comparisons within and between two flowering years (2009 and 2010) are distinguished and regression lines according to the logarithm of the distance are shown. Values on the vertical axis (distance = 0) indicate correlated paternity within maternal sibships.

Discussions

In spite of selfing occurrence, our results confirm that *P. butyracea* is essentially an outbreeder. Pollen dispersal is limited; at most 31% of pollen came from outside the population (in the case of the small population Nioro) and estimates of mean pollen dispersal distances in two large populations were inferior to 100m. The small-scale spatial genetic structure detected in each population also indicates that seed dispersal must be limited. We found higher correlated paternity within fruit versus between fruits, between nearby trees versus distant trees, between seeds fertilized the same year versus different years, and within a small population versus large populations. As expected from the predominantly outcrossed mating system and inbreeding coefficient across adults and progenies, when corrected for the presence of null alleles, was low and of same order of magnitude (averaging 0.005 to 0.07), indicating the absence of inbreeding depression.

Inbreeding coefficient variations

Comparison of observed diversity between adults and progenies suggests very low inbreeding depression except in Nioro. Inbreeding depression often resulted from the exposure of deleterious alleles when plants are homozygous (Dudash and Fenster 2001, Cheptou and Donohue 2011). It indicates the existence of selection against more genetically related neighbors so that the observed heterozygosity in adult stage becomes higher than that one in progenies which contrasts our findings. In this case study, *P. butyracea* would prevent inbreeding depression by long pollen dispersal associated to maintenance of preferential outcrossing.

Mating patterns

It should be noted that MLTR and NM+ gave slightly different estimates of outcrossing rates (Table 3). This may result from an algorithm bug in MLTR because this program gave the same estimates when the option asking to account for null alleles was selected or not. Hence, we put more confidence in the estimates provided by NM+ and Poldisp.

According to our findings, *P. butyracea* is largely outcrossed with at least 0.88 to 0.95 outcrossing rate, values close to estimates (*tm* = 0.92, Degen *et al.* 2004) of *Symphonia globulifera*, another tropical tree species of the same tribes of Symphoniae. This suggests that ovules received a large amount of pollen from other trees and/or that allopollen preferentially fecundate them, even in small populations such as Nioro, matching with common feature of high outcrossing rate in tropical tree species (Ward *et al.* 2005). Similar result was found in

Entandrophragma cylindricum despite low densities (Lourmas et al. 2007) while high selfing rate was observed in reduced population sizes of other species such as *Pinus sylvestris* (Robledo-Arnuncio *et al.* 2004) and *Carapa procera* (Doligez and Joly 1997). In effect, relatively low rates of outcrossing are expected in small or isolated forest stands as pollen is derived primarily from a few sources, whereas outcrossing is expected to increase in dense and large stands (Mitton 1992). These results were congruent with the facultative xenogamous mating system deduced from the P:O ratio of this species (Ewedje *et al.* unpublished).

Levels of correlated paternity

In the three large populations, about 24 to 37% (vs. lower values of 17 to 30% estimated by Poldisp) of sib-pairs were full-sibs. In other words, the reciprocal of correlated paternity (1/rp) indicate an effective number of 2.7 to 4.1 (3.3 to 5.8) sires per mother tree, that lies in the range of other tree species: *Acacia melanoxylon, Quercus lobata, Albizia julibrissin, Dinizia excelsa, Symphonia globulifera* (Muona *et al*. 1991, Sork *et al*. 2002, Irwin *et al*. 2003, Dick *et al*. 2003, da Silva Carneiro *et al.* 2007) but it was lower than in *Sorbus torminalis* (Oddou-Muratorio *et al.* 2004). Interestingly, analyses detected 1 to 3 donors which sired seeds within fruit and 2 to 6 donors siring those between fruits. These results were in the same order of magnitude as in the tropical tree species *Pachira pinata* (Quesada *et al*. 2001), and suggesting that various pollen pools contributed to genetic diversity within a tree. In other words, outcrossed pollen sampled by maternal trees tended to be diverse (Hardy *et al.* 2004, Robledo-Arnuncio *et al.* 2004). The correlated paternity was however very high (but moderate from estimates of Poldisp) in Nioro, probably due to its small size (n = 11 adult individuals), decreasing the effective number of pollen donors. These findings pointed out the evidence of pollen limitation in small vs. large populations and indicating that population size reduction might have been one key factor affecting the mating system of this outcrossed tree species. Indeed the number of local compatible mates and total pollen availability decreased under reduction of population size, which will increase the likelihood of correlated mating and self-fertilization (Surles *et al*. 1990).

Factors affecting correlated paternity

We found that the percentage of offspring pairs sharing the same father decreased with the spatial distance among mothers. In other words, the spatial location of maternal trees determines the sources of the pollen they sample, confirming dispersal limitation by distance due to a proximity advantage for pollen from the nearby and/or related individuals.

Independently to spatial distance, our results found the time as a second factor affecting the level of correlated paternity. First, the pollen pools siring maternal sibships were significantly different showing more diversity of pollen donors in 2010 (*rp* = 0.13 ± 0.06) than in 2009 (*rp* = 0.24 ± 0.03). Second, the level of correlated paternity within maternal sibship was low between years (*rp* = 0.04). These patterns probably reflect a variance in the intensity of flowering of adult trees and/or in their phenological overlap between years.

Both parameters (the time and the spatial distance) are reported as crucial factors affecting mating events (Burczyk and Prat 1997, Gömöry *et al.* 2000). The population Igbo Aladja was composed by ~60 % of burnt and ~40% of non-burnt trees and it was previously shown that fires create stress that delay flowering entry (Ewédjè, unpublished data). The heterogeneity of flowering phenology among both groups of trees is important because even when there are many potential mating partners over the whole flowering period, mate availability was limited. Other studies revealed male fertility variation and asynchronous phenology and (probably) the presence of self-incompatibility system as factors affecting correlated paternity in plant species (Burczyk and Prat 1997, Hardy *et al.* 2004).

Pollen dispersal

Information about gene dispersal is needed to understand the distribution and evolutionary dynamics of species. Our approach to assess gene flow through pollen dispersal gave estimates of the mean pollen dispersal distance within population ranging from 50 to 450 m. Apart from the population Nioro of small size where estimates might be biased, our results are congruent with the assumption that pollen dispersal often seem to be negatively correlated with the tree density (Degen *et al.* 2004, Stacey *et al.* 1996): the high tree density at short distance in Setou leading to short pollen dispersal (*dp* = 50 m) in comparison with Igbo Aladja (*dp* = 450 m) for example. This occurs if pollinator behaviour fitted optimal foraging strategy according to a cost-benefit function (Waddington and Holden 1979).

However, our results indicate important pollen dispersal. For example in the locus Pent16, three opened-pollinated seeds from the population Igbo Aladja (*mp* = 0.21) presented the allele 223 commonly observed in the population Kouba and apparently absent from the adults of this population which was nearly exhaustively genotyped. The gene flow estimated here falls in the same range of other tropical trees of which diaspores are heavy and dispersed by animals and gravity such as *Chrysophylum* sp. (*dp* = 413 m), *Symphonia globulifera* (*dp* = 141m), *Carapa procera* (*dp* = 182 m), *Entandrophragma cylindricum* (*dp* = 385 m).

According to Obayashi *et al.* (2002), *Apis* and *Trigona* bees identified as this plant species pollinators, dispersed pollen on long distance. Long distance pollen flow could mitigate the effects of physical isolation, as revealed in other plant species such as *Pithecellobium elegans* (Chase *et al*. 1996) and *Gliricidia sepium* (Dawson *et al.* 1997).

Finally, our results describing variation of outcrossing rate among populations within the same species were congruent with many other studies: For example, Aldrich and Hamrick (1998) estimated an outcrossing rate of 0.920 in a continuous forest, 0.886 in fragmented forests, and 0.731 for pasture trees, for *Symphonia globulifera*. In the same way, Robledo-Arnuncio *et al.* (2004) found heterogeneity of correlated paternity ranging from 0 to 19.6 while outcrossing rate decreases from 0.98 to 0.74 according to five population sizes of *Pinus sylvestris*. A possible reason of departure from complete outcrossing in *P. butyracea* would be constraints in the expression of mating system which depends on vectors for pollen removal and pollen receipt. For example, a particular case of high selfing was observed in a tree planted out of its range in the south of Benin nine years ago; it commonly produces small fruits of which all seeds germinate. A similar mating system variation (attributed to the absence of pollinators and alteration in population density by fragmentation) is observed in *Symphonia globulifera* (da Silva Carneiro *et al.* 2007). Hence, facultative outcrossing could be considered as an aspect of reproductive plasticity in *Pentadesma butyracea*.

Spatial genetic structure within parental populations

Isolation by distance models predict that linear populations, as those of *P. butyracea* (in particular Igbo Aladja and Kouba, Fig. 1), should display at drift-dispersal equilibrium a linear decay of the mean pairwise kinship coefficient between individuals with spatial distance (Rousset 2000). However, we observed a decay more linear with the logarithm of distance (Fig. 2). This pattern might result from a much more limited seed than pollen dispersal distances. Indeed, such asymmetry causes the kinship-distance curve to be more concave (Heuertz *et al.* 2003). Usually, seeds are heavy and dispersed by elephants (White and Albernethy 1996) but these dispersers were infrequent in populations studied in Benin due to hunting and forest degradations. The high and frequent anthropic pressures did not facilitate dispersal by rodents which consumed seeds.

Our study showed a spatial aggregation of related individuals on short distance and similar SGS patterns resulting from IBD are common in some tree species (Born *et al.* 2008; Hanson *et al.* 2008) often resulting from feeble dispersal ability. In agreement with theoretical

expectations (Rousset 2000, Vekemans & Hardy 2004), the linear habitat observed in Igbo Aladja and Kouba could explain their stronger SGS compared to Setou which lies in a more bi-dimensional habitat (Fig. 1 and 2).

In conclusion, this study supports the expectation that *Pentadesma butyracea* is a predominantly outcrossing species. These results indicate that it is important to maintain large populations to prevent increases in inbreeding and to maintain pollinator communities. Attention must be paid due to the constraints of fires and ongoing fragmentation (such as in Nioro) that have been detected to influence the correlated paternity, suggesting the definition of a legislative framework to protect such natural habitats existing out of common protected forests (parks etc.). Depending on opened-pollinated seeds from fruits sampled in all directions of trees, correlated paternity estimated here within and among fruits suggest a relative moderate number of pollen donors and that seeds resulted from as important genetic mixing. Due to the restricted expansion of this plant species, this study at fine scale presents the advantage to be more precise revealing that in the context of *ex situ* conservation and/or reforestation, fruits harvesting must be done from maternal trees distant by at least 10 m (to reduce inbreeding effect) while the conventional method of FAO suggests a minimum of 100 m to get the maximum of genetic variation (Palmberg 1985).

Online supplementary material 1: Genetic diversity and inbreeding coefficient parameters in Igbo Aladja. *N*a, number of alleles; *A*, allelic richness; H_E and H_O (non-corrected expected and observed heterozygosity estimated after INest). F_{IS}, inbreeding coefficient estimate after INest.

	Adult stage						Sibship family			Adult stage	Sibship family
	*N*a	Size	Freq. nul	*A*	H_E	H_O	*A*	H_E	H_O	F_{IS}	F_{IS}
Pent14	4	171-184	0.00	2.40	0.49	0.56	2.96	0.51	0.45	-0.41	0.12
Pent13	3	164-170	0.01	2.27	0.55	0.47	2.33	0.52	0.53	0.15	-0.02
Pent1	3	119-125	0.00	1.27	0.02	0.02	1.33	0.02	0.00	-0.003	1**
Pent17	3	211-217	0.08	2.09	0.20	0.12	2.32	0.20	0.17	0.40	0.12
Pent22	4	283-300	0.00	3.59	0.67	0.69	3.18	0.58	0.55	-0.04	0.06
Pent12	3	151-165	0.02	2.96	0.55	0.54	3.81	0.55	0.50	0.02	0.09
Pent16	4	215-221	0.31	2.44	0.58	0.22	3.05	0.50	0.24	0.61**	0.50**
Pent18	4	228-257	0.19	2.05	0.19	0.08	3.83	0.30	0.27	0.56*	0.08
Average (SD)	3.2 (0.53)	-	-	2.38	0.41 (0.24)	0.28 (0.24)	2.85	0.39	0.34	0.31* (0.11)	0.14* (0.06)

*p < 0.05 and **p <0.001 indicate significant deviation from HWE.

Online supplementary material 2: Genetic diversity and inbreeding coefficient parameters in Kouba. *N*a, number of alleles; *A*, allelic richness; H_E and H_O (non-corrected expected and observed heterozygosity estimated after INest). F_{IS}, inbreeding coefficient estimate after INest, underlined F_{IS} is estimated from INest with account of null allele.

	Adult stage						Sibship family			Adult stage	Sibship family
	*N*a	Size	Freq. nul	*A*	H_E	H_O	*A*	H_E	H_O	F_{IS}	F_{IS}
Pent14	7	165-184	0.02	4.2	0.66	0.66	3.0	0.64	0.57	0.001	0.11
Pent13	4	164-170	**0.24**	2.3	0.60	0.34	2.9	0.48	0.20	0.42**	0.58**
Pent1	3	116-127	**0.16**	1.7	0.09	0.03	1	0.09	0.02	0.65**	0.79**
Pent17	4	202-220	0.08	2.5	0.32	0.25	2.5	0.51	0.35	0.22	0.30
Pent22	6	283-303	**0.20**	4.2	0.49	0.22	3.0	0.60	0.36	0.55**	0.39**
Pent12	5	145-165	0.05	3.9	0.62	0.43	3.7	0.51	0.50	0.33	0.01
Pent16	5	215-223	0.04	3.6	0.66	0.61	3.0	0.58	0.48	0.07	0.18*
Pent18	3	225-231	0.00	2.7	0.28	0.23	2.8	0.26	0.23	0.16	0.09
Average (SD)	4.7 (0.89)	-	-	3.16	0.48 (0.19)	0.31 (0.22)	2.75	0.46 (0.19)	0.34 (0.18)	0.34* (0.08)	0.26* (07)

*p < 0.05 and **p <0.001 indicate significant deviation from HWE.

Online supplementary material 3: Genetic diversity and inbreeding coefficient parameters in Setou. *N*a, number of alleles; *A*, allelic richness; H_E and H_O (non-corrected expected and observed heterozygosity estimated after INest). F_{IS}, inbreeding coefficient estimate after INest, underlined F_{IS} is estimated from INest with account of null allele.

	Adult stage						Sibship family			Adult stage	Sibship family
	*N*a	Size	Freq. nul	*A*	H_E	H_O	*A*	H_E	H_O	F_{IS}	F_{IS}
Pent14	4	171-186	0.01	3.9	0.59	0.67	4.0	0.71	0.64	-0.12	0.09
Pent13	5	162-172	0.03	2.9	0.61	0.55	2.0	0.49	0.44	0.09	0.10
Pent1	3	116-123	**0.11**	1.3	0.05	0.01	1.0	0.08	0.00	0.79**	1.00
Pent17	1	214	0.28	1	0.25	0	1.0	0.12	0.00	1.00	1.00
Pent22	5	287-303	0.04	2.6	0.52	0.29	2.7	0.42	0.37	0.44	0.13
Pent12	3	151-165	0.00	3.0	0.68	0.92	3.9	0.67	0.55	-0.35	0.19
Pent16	2	217-219	0.04	1.9	0.35	0.26	2.0	0.39	0.37	0.26	0.06
Pent18	5	225-257	**0.23**	1.8	0.24	0.05	2.2	0.08	0.04	0.80**	0.48
Average (SD)	3.9 (1.52)	-	-	2.3	0.44 (0.22)	0.31 (0.34)	2.3	0.37 (0.25)	0.30 (0.25)	0.16 (0.10)	0.19 (0.05)

*p < 0.05 and **p <0.001 indicate significant deviation from HWE.

Online supplementary material 4: Genetic diversity and inbreeding coefficient parameters in Nioro. *N*a, number of alleles; *A*, allelic richness; H_E and H_O (non-corrected expected and observed heterozygosity estimated after INest). F_{IS}, inbreeding coefficient estimate after INest

	Adults (n= 11)							Progeny (n=30 from 5 families)			
	*N*a	Size	Freq. nul	*A*	H_E	H_O	F_{IS}	*A*	H_E	H_O	F_{IS}
Pent14	3	171-184	0.11	3	0.62	0.54	0.12	3.9	0.65	0.56	0.14
Pent13	2	168-170	0.05	2	0.52	1.00	-0.91	3	0.56	0.56	-0.005
Pent1	2	123-125	0.09	2	0.41	0.54	-0.31	2	0.32	0.25	0.21
Pent17	1	214	0.19	1	0.00	0.00		1	0.06	0.00	1.00
Pent22	2	287-303	0.17	2	0.09	0.09	0.00	1	0.00	0.00	1.00
Pent12	2	151-165	0.07	2	0.48	0.72	-0.50	3.9	0.49	0.22	0.56**
Pent16	2	217-219	0.05	2	0.52	1.00	-0.91	3	0.45	0.34	0.23
Pent18	1	228	0.18	1	0.00	0.00		1	0.06	0.00	1.00
Average (SD)	1.87	-	-	1.87	0.33 (0.25)	0.49 (0.41)	-0.48 (0.23)	2.36	0.32 (0.25)	0.24 (0.23)	0.25* (0.11)

*p < 0.05 indicate significant deviation from HWE.

CHAPITRE VIII.-

DISCUSSION GÉNÉRALE

Chez *Pentadesma butyracea*, l'étude de la caractérisation et de la répartition spatiale des lignées génétiques chloroplastiques et nucléaires indiquent une séparation ancienne des populations d'Afrique centrale et d'Afrique de l'ouest, et que les populations du Dahomey Gap résulteraient des forêts denses humides de l'Afrique de l'Ouest (Haute Guinée). Trois pools génétiques ont été détectés et leurs limites géographiques correspondent aux trois types d'habitats (Haute Guinée, Dahomey Gap et Basse Guinée). La diversité génétique est faible dans le Dahomey Gap, modérée dans le Haut-Guinéen mais élevée dans le Bas-Guinéen. Dans le couloir sec, les populations ont subi des évènements de fondations et/ou de dérive génétique. Les deux pools génétiques identifiées contrastent avec deux groupes éco-morphologiques au Bénin, suggérant que la variabilité morphologique observée pourrait résulter soit d'une plasticité phénotypique, soit d'une pression de sélection contre la baisse de la pluviométrie.

P. butyracea est une espèce auto-compatible majoritairement allogame. La corrélation de paternité est plus élevée respectivement aux niveaux intra-fruit vs. inter-fruits, au sein d'une population de petite taille vs. population de grande taille.

8.1.- *Pentadesma butyracea* – une ou plusieurs espèces ?

Nos résultats ont permis de détecter trois pools génétiques grâce à des marqueurs nucléaires (microsatellites) au sein de *P. butyracea* dont la répartition géographique correspond à trois régions adjacentes (forêts d'Afrique de l'Ouest, savanes d'Afrique de l'Ouest, et forêts d'Afrique centrale). En outre, les séquences chloroplastiques et nucléaires montrent une profonde séparation, impliquant une barrière aux flux de gènes, entre les deux blocs forestiers d'Afrique de l'Ouest et d'Afrique centrale, et l'origine de cette divergence serait antérieure aux changements climatiques de l'Holocène. Une datation fournirait plus de précision sur cette période. De plus, ces séquences montrent que des individus de *P. butyracea* originaires d'Afrique centrale sont plus proches de l'espèce *P. grandifolia* (présente en Afrique centrale également) que des individus de *P. butyracea* de l'Afrique de l'Ouest (Figure 1). Finalement, on observe des différences marquées du point de vue morphologique entre les individus de *P. butyracea* d'Afrique de l'Ouest et d'Afrique centrale (Aubreville 1959, Keay *et al.* 1964, White and Abernethy 1996, Ewédjè *et al.* 2012). Ces résultats suggèrent qu'il serait utile de revoir la taxonomie du genre *Pentadesma* car il paraît fort possible que *P. butyracea* mériterait d'être subdivisé en deux espèces. En effet, la spéciation allopatrique est souvent due à l'accumulation de mutations de lignées incompatibles (Reiseberg & Willis 2007, Schluter 2009). En général ces divergences sont accompagnées d'un isolement postzygotique

intrinsèque (c'est-à-dire qu'il peut en découler des hybrides stériles non viables à cause d'un ensemble de gènes complémentaires qui interagissent) selon le modèle de Bateson-Dobzansky-Muller (Orr 1996, Sobel *et al.* 2010). Ceci pourrait être très probable dans le cas de *P. butyracea* au regard de ses 56 chromosomes et de la présence de plus de deux allèles à certains loci. En absence de dispersion de gènes sous l'effet de la barrière installée, différents allèles deviennent fixés dans diverses populations par la sélection et/ou la dérive génétique.

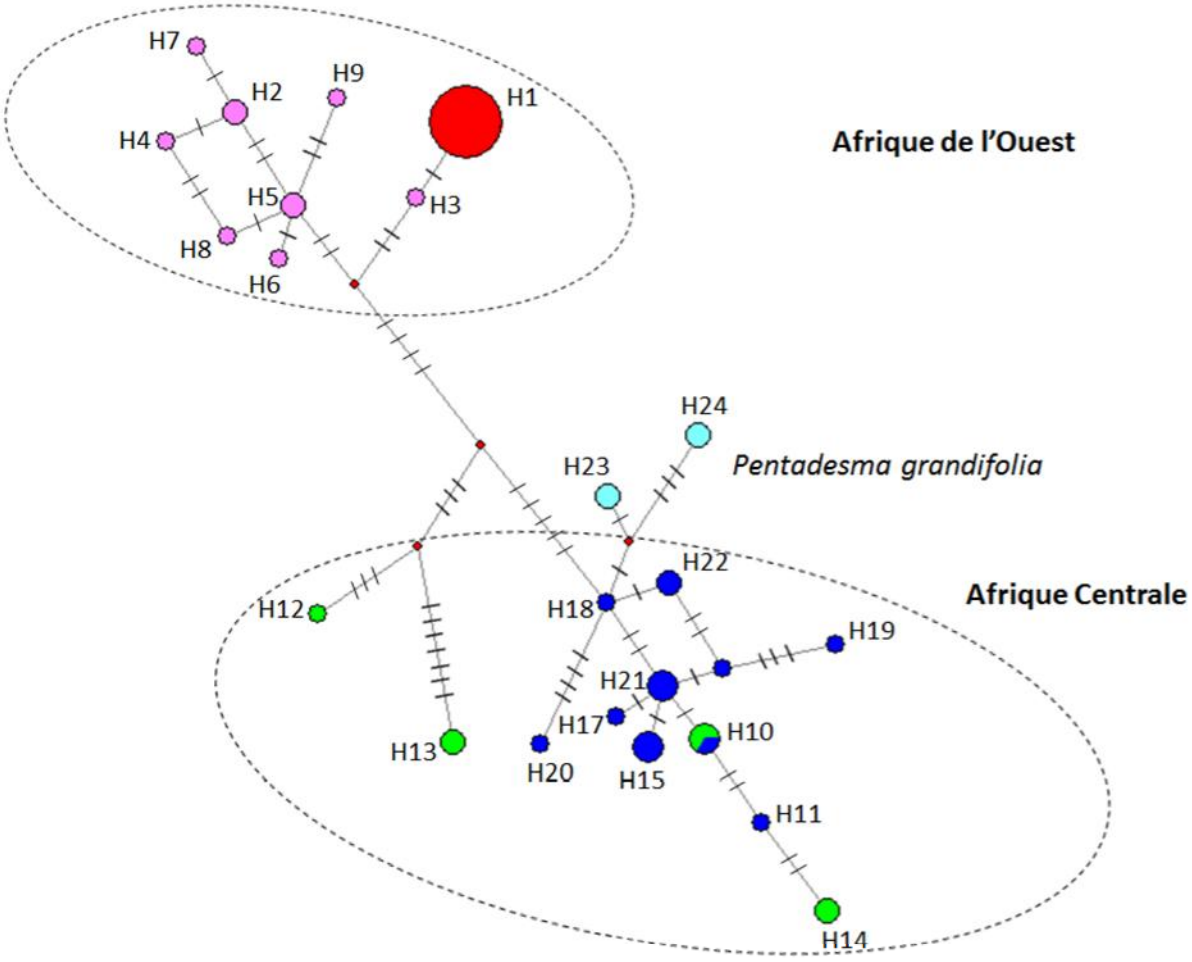

Figure 1 : Réseau d'haplotypes d'ADN chloroplastique de *Pentadesma butyracea* (H1 : Dahomey Gap ; H2-H9 : Haute Guinée ; H10, H12-H14 : Cameroun ; H10, H11, H15-H22 : Gabon) montrant la position des deux haplotypes de *P. grandifolia* (H23-H24).

L'isolement géographique accompagné d'un isolement génétique depuis un long terme entre populations au sein d'une espèce est en effet, un précurseur majeur de la spéciation allopatrique (Robbrecht 1996, Sobel *et al.* 2010). L'existence de lignées chloroplastiques distinctes appartenant à des populations isolées ont suggéré et/ou détecté des sous-espèces au sein des taxons (Allal *et al.* 2011, Besnard *et al.* 2011, Koffi *et al.* 2011). Dans le cas de *P. butyracea,* bien que la floraison demeure synchrone dans les deux blocs forestiers, l'isolement géographique intervenu depuis longtemps pourrait suffire à favoriser des incompatibilités génétiques au sein d'hybrides. De plus, des différentiations morphologiques entre les deux groupes (par exemple au niveau des feuilles) concordent avec la divergence génétique décrite. Les feuilles provenant de l'Afrique Centrale (Gabon et Cameroun) sont coriaces et montrent des nervures nettement plus saillantes que celles de l'Afrique de l'Ouest (Ewédjè E.B.K, pers. obs.). Malgré que nous n'ayons pas étendu l'étude détaillée de la variabilité morphologique de *P. butyracea* à l'ensemble de son aire de distribution comme ce fut le cas au Bénin, la littérature existante révèle une distinction morphologique entre les deux régions. En Afrique de l'Ouest, les caractères morphologiques de l'arbre, des feuilles, fruits et graines présentent des dimensions plus grandes que celles de l'Afrique Centrale précisément au Gabon : feuilles (12-24cm x 4-7cm en Afrique occidentale vs. 11cm x 4cm en Afrique Centrale), arbres (35 m vs. 30 m de hauteur), fruits (6,0-29,0cm x 2,8-17,0cm vs. 15,0cm x 10,0cm), nombre de graines (1-31 vs. 1-10) (Aubreville 1959, Keay *et al.* 1964, White and Albernethy 1996, Ewédjè *et al.* 2012).

En marge de la concordance entre les marqueurs moléculaires et les caractères morphologiques, l'analyse du réseau d'haplotypes de l'ADN chloroplastique (Fig. 1) montre que l'espèce apparentée *Pentadesma grandifolia* est plus proche de *P. butyracea* de l'Afrique Centrale que ne l'est *P. butyracea* de l'Afrique de l'Ouest. Ces résultats nous amènent à suggérer la nécessité d'une nouvelle délimitation taxonomique au sein de cette espèce, après un complément d'étude de la variabilité morphologique étendue à toute l'aire de distribution de l'espèce.

8.2.- L'origine des populations de *P. butyracea* du Dahomey Gap remonte-t-elle à la période de l'Holocène humide ?

Si les populations d'Afrique de l'Ouest et d'Afrique centrale sont très divergentes au point de suggérer l'existence d'espèces distinctes, les populations des régions de savanes d'Afrique de l'Ouest sont clairement apparentées à celles des forêts d'Afrique de l'Ouest. En effet, les

forêts denses humides tropicales ont connu plusieurs phases d'extension et de contraction au cours de leur histoire en réponse aux oscillations climatiques. Selon les données palynologiques, les dernières conditions climatiques chaudes et humides de l'Holocène (entre 9000 et 6000 ans, Assi-Kaudjhis *et al.* 2010, Demenocal *et al.* 2000) étaitent très favorables à une importante montée des niveaux lacustres et mangroves et une large expansion des forêts tropicales. Les carottages effectués jusqu'à une profondeur de 990 m (dont la datation remonte à 7529 ± 66 années, Tossou 2002) à la latitude de 6°35'N au Bénin, indiquent la présence de nombreux taxons de forêts denses humides telles que *Celtis, Triplochiton, Mansonia* … confirmant l'expansion maximale de ces forêts. La présence de certaines espèces forestières au-delà de cette latitude suggère une plus large extension vers le nord à des latitudes assez élevées. En effet, selon les travaux de Anhuff *et al.* (2000) sur l'histoire de la végétation africaine, les forêts ombrophiles humides de l'Afrique de l'Ouest étaient étendues jusqu'à la latitude de 11°N et celles de l'Afrique Centrale ont occupé l'extrême sud au Bénin au cours de l'Holocène humide (8000 ans BP). Mais plus tard, il y a 3000 ans, ces formations ont regressé en réponse aux crises climatiques devenues sèches de l'Holocène, laissant place aux savanes qui ont atteint la côte. Au cours du retrait, des reliquats (de forêts de l'Afrique de l'Ouest) ont survécu auxdites crises climatiques dans ce couloir sec. Ceci représente l'hypothèse qui explique le mieux la distribution actuelle de *P. butyracea* dans le Dahomey Gap.

8.3.- Quelles stratégies de conservation pour les ressources génétiques de *Pentadesma butyracea* au Bénin ?

Au Bénin, des signaux de dérive génétique sont associés à la faible taille des populations. On y observe également une faible fréquence des jeunes tiges d'avenir et un vieillissement des arbres. Cependant, *P. butyracea* est soumis à de fortes demandes des communautés locales surtout dans le contexte actuel de pression démographique galopante. Pour répondre à ces défis, nous préconisons une approche multidimensionnelle de conservation (soutenue par un cadre législatif de protection des habitats naturels de *P. butyracea*) qui intègre les communautés locales et l'Agence des Eaux et Forêts en charge de la gestion et des recherches forestières. Notre étude contribue à déterminer les paramètres génétiques et de reproduction pour la conservation des ressources génétiques de l'espèce. Elle permet de désigner quelles populations il faut conserver en priorité. Dans le cadre d'une conservation *ex situ* et d'un enrichissement des populations existantes, elle montre quelles stratégies il faut développer en

vue de capturer un maximum de variation génétique depuis l'échantillonnage des semences jusqu'à l'acquisition des plantules.

La conservation des ressources phytogénétiques vise à assurer le maintien de la variation génétique intra-spécifique et permettre ainsi à l'espèce de s'adapter aux changements futurs de l'environnement. Elle repose sur une variété d'approches et de techniques *in situ* et *ex situ*.

Les stratégies de conservation *in situ* sont orientées sur la protection des écosystèmes naturels afin de conserver toute la diversité des gènes, des espèces et des processus écologiques. Elle représente à ce titre l'unique méthode qui garantit une adaptation continue aux conditions locales, même si celles-ci viennent à se modifier, dans des limites compatibles avec la survie de l'espèce (évolution des facteurs physicochimiques de l'environnement, réchauffement et pollution de l'atmosphère, modification rapide du paysage phytosanitaire). Cependant, elle exige beaucoup de moyens (finances, personnel, etc. et de sacrifice) qui sont loin d'être acquis, surtout dans le contexte actuel de la crise économique. De plus, *P. butyracea* se situe généralement hors des aires classées et protégées par l'administration forestière. En conséquence, l'espèce subit une utilisation excessive des ressources, la dégradation des peuplements et la perte d'habitat.

Les stratégies de conservation *ex situ* reposent sur les banques de gènes (graines et/ou pollens, Linington & Pritchard 2001), des vergers, des jardins botaniques et de case etc. en vue de la sauvegarde de l'espèce en dehors de son habitat naturel. A l'opposé de la conservation *in situ,* il n'est pas permis à l'espèce d'évoluer sous la pression de l'environnement et elle pourrait devenir incapable de résister par exemple à des parasites. Par ailleurs la cryo-préservation de graines exige un minimum de contraintes et de suivi à long terme, qui sont loin d'être réunies dans le contexte africain et en particulier béninois comme les coupures de courant ou le manque de crédits etc. Cependant, les jardins botaniques et de case et les vergers qui constituent une autre forme de conservation *ex situ*, permettent de surmonter certaines contraintes. La situation idéale serait de mettre en place parallèlement ces deux types de conservation pour garantir le maintien de la diversité et la survie des espèces.

8.3.1.- Conservation in situ

Le développement et la mise en œuvre d'une stratégie efficiente de gestion durable des ressources génétiques de *P. butyracea* requièrent l'intégration de nos résultats sur la variabilité génétique intra-spécifique de l'espèce et ceux obtenus par l'étude des paramètres de biologie de reproduction.

L'analyse du système de reproduction et l'évaluation du niveau de diversité et de la structure génétique ont révélé que

(1) *P. butyracea* est une espèce auto-compatible et majoritairement allogame (*tm* varie entre 0,88 entre 0,95) avec un niveau de corrélation de paternité relativement faible (*rp* entre 0.17 et 0.37). L'allogamie est assurée par des disperseurs de pollen efficaces (oiseaux souimangas et abeilles) et est aussi conditionnée par des facteurs environnementaux (synchronisme floral élevé des arbres reproducteurs),

(2) *P. butyracea* possède une diversité génétique intra-population modérée (A = 2,04 ± 0,21; H_E = 0,34 ± 0,07 ; Tableau 1) accompagnée d'une différenciation génétique entre populations faible qui traduit un important flux de gènes (F_{ST} = 0,13) dans une situation d'équilibre entre la dérive génétique et la migration.

Dans le contexte de la conservation des espèces, la richesse allélique est reconnue comme un facteur important d'identification des populations à conserver en priorité (Marshall & Brown 1975, Petit *et al.* 1998, Neel & Ellstrand 2003, Sina 2006). En effet selon ces auteurs, la composition allélique initiale détermine les limites de réponse à la sélection à travers les générations alors que la réponse de l'espèce à une sélection immédiate est liée à l'hétérozygotie attendue. La richesse allélique est un caractère qui régit la capacité des organismes à réagir à des changements de milieu. En outre elle est très fortement dépendante de la taille effective des populations, ce qui est moins le cas de l'hétérozygotie (Petit et al. 1998). En général pour les populations de *P. butyracea*, pour un même locus, la plupart des populations partagent presque les mêmes allèles mais à des fréquences très dissemblables.

Tenant compte de la richesse allélique moyenne (A = 2,04) et de la taille de population moyenne associée, la protection de *Pentadesma butyracea* au Bénin doit (1) porter prioritairement sur les populations ayant un taux d'hétérozygotie plus élevé (sauf Bensékou situé à l'extrême nord-est, H_E = 0.28) tout en veillant à ce que ces populations représentent les deux groupes génétiques pour garder la diversité exprimée entre populations et (2) tenir compte des deux groupes morphologiques car ceux-ci pourraient être différenciés pour des gènes adaptatifs (au cas où la différentiation morphologique est héritable). Sur cette base, neuf populations (représentant 56% du total) méritent d'être retenues pour la conservation *in situ* (Tableau 1). Cet ensemble inclut deux populations du pool génétique Est, dont une population de grande taille (Igbo Aladja avec 90 individus) au sud de l'aire de distribution de l'espèce, et une autre de petite taille, Bensékou, qui présente la plus forte richesse allélique (A = 2,35). En effet, contrairement à toutes les autres populations échantillonnées au Bénin et qui se développent dans des rivières, Bensékou héberge le seul site de *Pentadesma butyracea* trouvé

sur un fleuve dans une forêt galerie très fermée (fleuve Sota, cf. Fig. 2 Introduction). Cependant, cette richesse allélique demeure inférieure à celle estimée dans les populations de la Haute Guinée (A = 3,02) ou de la Basse Guinée (A = 3,09). Cinq des neuf populations retenues, appartiennent au groupe éco-climatique du Nord, caractérisés par des traits morphologiques de petite taille ; parmi celles-ci figurent la population Tchiapéta (A = 2,20 ; H_E = 0,43) très isolée car encerclée par une chaîne de collines et présentant des fruits ellipsoïdes tordus endémiques. Si aucune disposition urgente de conservation n'est prise, cette population qui ne renferme que 12 individus disparaitra dans moins de deux ans au regard de sa destruction pour les activités de maraîchage par les populations locales.

Nos résultats indiquent l'absence de dépression de consanguinité liée à une purge des allèles récessifs délétères résultant de la taille relativement réduite des populations. Ceci nous amène à proposer, en cas de besoin d'enrichissement d'un site, l'utilisation de semences de ses propres individus. La stratégie de collecte de fruits/graines par arbre-mère et la distance minimale d'échantillonnage à observer entre arbre-mères que nous conseillons sont indiquées ci-dessous.

Tableau 1 : Indices de diversité des populations étudiées au Bénin, indiquant les populations retenues pour la conservation (*). *N :* taille de la population ; *Gp :* pool génétique (1= Est, 2 = Ouest) ; *Gm :* groupes morphologiques (a = Sud, b = Nord) ; *He*: hétérozygotie attendue (Nei 1978); *Na*: nombre moyen d'allèles par locus; *A*: richesse allélique moyenne par locus

n°	Population	*N*	*Gp*	*Gm*	*Na*	*A*	H_E
1	Igbo Aladja*	90	1	**a**	3,20	2,07	0,41
2	Kouba*	91	2	b	4,80	2,57	0,48
3	Setou*	144	2	**a**	3,50	2,02	0,41
4	Nioro	11	2	a	1,87	1,79	0,33
5	Bassila*	86	2	**a**	2,25	2,07	0,34
6	Kikele	18	2	a	2,00	1,86	0,30
7	Boribansifa	9	2	b	2,00	1,93	0,38
8	Bongou	19	2	b	2,12	1,96	0,30
9	Koda	18	1	a	2,25	1,99	0,34
10	Penessoulou*	27	2	**a**	2,25	2,08	0,35
11	Tandafa*	21	2	b	2,50	2,00	0,36
12	Tassigourou	19	2	b	2,00	1,95	0,36
13	Tchiapeta*	12	2	b	2,25	2,20	0,43
14	Tchoundegou*	15	2	b	2,62	2,19	0,39
15	Yagua	43	1	b	1,25	1,70	0,13
16	Bensekou*	23	1	b	1,87	2,35	0,28
	Moyenne				***2,42***	***2,04***	***0,34***

8.3.2.- Conservation ex situ

Type de semences

Les semences de *P. butyracea* ont une teneur en eau de 42,5 ± 2,9 % et sont récalcitrantes : leur conservation au froid n'est pas bonne, contrairement aux semences orthodoxes qui

supportent le stockage à long terme. Ceci constitue une grande contrainte à la création d'une banque de semences de l'espèce. Nos essais de stockage ont montré que la température seuil pour la conservation à moyen terme (environ 3 mois) est de 20°C, ce qui est plus élevé que les 15°C recommandé habituellement pour conserver les semences récalcitrantes (Cromarty *et al.* 1990). Toutefois, l'espère montre une forte reproduction végétative à travers les rejets de racines et des bourgeons de tiges, et des essais de culture *in vitro* et de cryoconservation pourraient constituer des alternatives à la préservation *ex-situ* à long terme de l'espèce. Cependant, tenant compte des moyens et infrastructures disponibles au Bénin, nous conseillons de centrer la conservation *ex-situ* de l'espèce sur la création de vergers.

Récoltes de graines destinées aux besoins de reboisement et à la conservation ex situ à long terme

- Mode de récolte des fruits/graines sur l'arbre

En plus du caractère majoritairement allogame de l'espèce, la valeur moyenne de la corrélation de paternité est modérée au sein d'un fruit (rp = 0,37 soit 2,7 donneurs de pollens) et faible entre fruits (rp = 0,17 soit 5,8 donneurs de pollens). Ces estimations ont été acquises à partir d'une collecte de fruits effectuée dans toutes les directions de chaque arbre échantillonné. Ces résultats signalent la nécessité de collecter plus d'un fruit par arbre-mère dans plusieurs directions pour piéger le maximum de diversité génétique. On pourra par la suite mélanger les semences de tous les fruits collectés de chaque arbre de manière à obtenir un lot de graines issues d'un bon brassage génétique représentatif de l'arbre-mère. Malgré le bon niveau de synchronisme floral individuel (Xi = 0,62 – 0,84 dépendant de l'état sanitaire et de l'entrée en floraison des arbres), la récolte des semences doit intervenir à un moment où la majorité des arbres portent des fruits mûrs en abondance.

Quant aux semences collectées pour le reboisement, elles doivent comprendre un mélange de semences des différents arbres échantillonnés de chaque population. Par contre, les semences récoltées en vue de la conservation à moyen terme pourraient être séparées par arbre-mère, pour une évaluation des descendants en cas de nécessité.

- Distances à observer entre les arbre-mères pour la récolte

Au regard de la petite taille des populations de *Pentadesma butyracea* et de leurs dimensions spatiales, la méthode conventionnelle de la FAO ne peut être appliquée ici (distance de 100 m entre arbre-mères, Palmberg 1985). Pour éviter d'échantillonner les arbres apparentés et réduire les risques liés à la dépression de consanguinité, les résultats découlant de la structure

génétique à fine échelle nous permettent de recommander une distance minimale de 10 m entre pieds-mères.

L'analyse de l'estimateur de la différentiation F_{ST} révèle une valeur relativement faible (0.1) mais néanmoins significative, qui suggère la nécessité d'un échantillonnage étendu à toute l'aire de distribution de l'espèce au Bénin. L'échantillonnage dans les neuf populations identifiées précédemment, par exemple, présente l'avantage de prendre en compte aussi la majorité de la variation éco-morphologique.

- Température optimale de germination

Nos essais de germination en conditions contrôlées ont montré une augmentation du taux de germination avec la température de 20 à 30°C. Une température de 30°C correspondant à 50% de germination après quatre mois de collecte des fruits est optimale. Cependant, ces résultats restent faibles par rapport aux taux de germination en conditions naturelles puisque les semences présentent un taux de plus 95% de germination aussitôt après la récolte, en température ambiante (Ouattara 1999).

- Effets de provenance et homogénéité des pools génétiques

La création d'un verger à partir des semences de plusieurs provenances du Bénin (pool génétique Est, pool génétique Ouest) permettrait de réaliser un suivi de provenance et de détecter la part de la variabilité morphologique résultant de la diversité génétique par rapport à la plasticité phénotypique. Cet essai pourrait être étendu aux semences provenant des deux blocs forestiers pour évaluer leur évolution adaptative en milieux plus secs et identifier les caractères soumis à la sélection.

CHAPITRE IX.-

CONCLUSION GÉNÉRALE ET PERSPECTIVES

9.1.- Conclusion générale

Pentadesma butyracea est une espèce typique des forêts denses humides de l'Afrique tropicale, répandue dans les deux blocs forestiers guinéo-congolais depuis la Sierra Leone jusqu'au Gabon. On le rencontre aussi dans des forêts galeries en savane au-delà de la latitude d'extension des forêts humides où les ressources de l'espèce sont utilisées à diverses fins par l'Homme, contribuant ainsi au développement socio-économique des communautés locales en Afrique de l'Ouest. Dans cette région, l'étude de l'histoire de ses populations revêt une importance écologique capitale du fait de sa position atypique dans le couloir sec du Dahomey qui est assimilé à une barrière écogéographique à l'échange d'espèces et de gènes entre les deux blocs de la forêt dense humide africaine. Ce travail nous a permis d'atteindre les objectifs fixés pour établir les bases génétiques nécessaires à la gestion et à l'utilisation durable des ressources génétiques de l'espèce.

En effet au cours de nos travaux, nous avons pu isoler des marqueurs microsatellites nucléaires de cette espèce (chapitre 2) qui se sont révélés transférables sur d'autres espèces du même genre. Leur exploitation combinée à celle de marqueurs universels chloroplastiques et nucléaires a été d'une grande contribution pour comprendre l'histoire des populations du Dahomey Gap. L'étude de la phylogéographie (chapitre 3, objectif 1) indique une séparation ancienne des populations d'Afrique centrale et d'Afrique de l'ouest, qui pourraient en fait constituer des espèces distinctes. Nos résultats indiquent par contre que les populations du Bénin, et du Dahomey Gap en général, seraient des populations résiduelles des forêts denses humides de l'Afrique de l'Ouest, peut-être lorsque celles-ci ont connu leur extension maximale durant l'Holocène humide avant se rétracter en formant le Dahomey Gap. Une forte dérive génétique en a résulté dans ce couloir sec, ce qui s'est traduit par une baisse de diversité génétique par rapport aux populations des forêts humides.

Cette faible diversité au Bénin met en exergue la nécessité de définir à court terme des stratégies de conservation pour maintenir le patrimoine génétique restant (chapitre 3) au regard de la permanence des menaces climatiques et anthropiques actuelles (objectif 2). Au Bénin, alors que les analyses morphométriques *in situ* mettent en évidence deux groupes corrélés au gradient climatique nord-sud (chapitre 4), les analyses par les microsatellites indiquent l'existence de deux pools génétiques partiellement introgressés suivant un axe est-ouest, et une corrélation entre taille des populations et leur diversité génétique (chapitre 5). Nous avons ainsi identifié neuf populations de plus grande diversité (et représentant 56% des

populations étudiées) qui méritent d'être gérées de façon durable. Ces populations ont l'avantage d'appartenir aux deux pools génétiques de faible différentiation et de contenir le maximum de la variabilité morphologique observée au sein de l'espèce au Bénin (arbres de grande taille produisant de gros fruits et de grosses graines de diverses formes et ceux de petite tailles tout en incluant par exemple la population très isolée Tchiapéta dont les arbres produisent des fruits spécifiques, chapitre 4, objectif 2).

L'étude de la biologie de reproduction et des paramètres du système de reproduction (objectif 3, chapitres 6 et 7) a montré que *P. butyracea* est une espèce auto-compatible majoritairement allogame et apparemment insensible à la dépression de consanguinité. Les populations présentent un synchronisme floral élevé pouvant favoriser entre elles l'échange de pollen. La dispersion du pollen et des graines est toutefois limitée. La conservation *ex situ* des semences à long terme (à 20°C) et en chambre froide n'est pas possible. Nos résultats ont montré que dans le cadre d'une conservation *ex situ* sous forme de création de vergers ou d'enrichissement des populations existantes, la récolte de fruits doit être opérée tout autour de l'arbre en prenant soins d'échantillonner sur les arbres distants d'au moins 10 mètres.

Mesures actuelles de conservation

Au regard de la biologie des semences et de la fragmentation continue des populations en dépit des efforts de sensibilisation, un verger de *Pentadesma butyracea* est mis en place dans un espace de 2 hectares dans le domaine réservé à l'écotourisme au sein de la forêt d'Ahozon au sud du Bénin. Les plantules utilisées proviennent des semences collectées dans les deux groupes morphologiques (Tchiapeta, Tandafa, Kouba, Igbo Aladja, Nioro, Bensékou) et intègrent les deux pools géniques identifiés au Bénin.

9.2.- Perspectives

Les forêts tropicales denses humides africaines ont laissé deux principales signatures sur l'histoire des populations de *P. butyracea* : (1) une profonde divergence impliquant une ancienne barrière aux flux de gènes entre les deux blocs forestiers pourrait être accompagnée d'accumulation de mutations de deux lignées incompatibles. Cependant, n'ayant pas une aire sympatrique, des essais de croisement artificiel entre individus des deux lignées et/ou avec des individus de *P. grandifolia* apporteraient des enseignements précieux. Ceci pourrait se faire à moyen terme dans le verger de diverses provenances en cours au Bénin. La manifestation d'une vigueur hybride (hétérosis) chez les descendants pourrait contribuer à mieux comprendre la biologie de ces espèces et peut-être offrir des opportunités

d'amélioration végétale dans un programme de domestication de l'espèce. (2) Les populations du Dahomey Gap peuvent être perçues comme des vestiges que les forêts denses humides de l'Afrique de l'Ouest ont laissés lors des crises climatiques de l'Holocène moyen. Il serait toutefois intéressant de prospecter le reste des formations végétales sèches de l'Afrique de l'Ouest en particulier en Côte d'Ivoire pour vérifier si le pattern génétique observé en milieu sec demeure le même. En outre, la révision du taxon *Pentadesma butyracea* et l'étude de la phylogénie du genre *Pentadesma* pourraient être envisagées.

A l'instar d'autres espèces ligneuses alimentaires telles que le baobab (*Adansonia digitata*), le Tamarinier (*Tamarindus indica*), le néré (*Parkia* biglobosa), ou le karité (*Vitellaria paradoxa*), la conservation et la gestion durable des ressources génétiques de *P. butyrcaea* requièrent des connaissances supplémentaires tant en biologie qu'en sciences humaines et sociales. En particulier, l'extension des connaissances scientifiques de la biodiversité requiert un effort majeur mobilisant des scientifiques de toutes les disciplines. Ceci implique la nécessité de constituer une équipe de recherches pluridisciplinaires au niveau national pour définir les actions à mettre en œuvre dans l'espoir de contribuer à la sécurité alimentaire et à la génération de revenus en milieu rural et urbain. Il s'agira par exemple de : (i) Définir et mettre en œuvre des stratégies appropriées de conservation (échantillonnage, collecte, entreposage de semences, mise en place de plantations conservatoires, etc.) ; (ii) Caractériser les propriétés nutritionnelles du beurre et du mésocarpe charnu et sucré du fruit mur; (iii) Etudier la production et explorer la filière de l'arbre à beurre, y compris les analyses socio-économique (opportunités du marché (inter-) national pour les graines et le beurre, documenter les caractéristiques du marché actuel, développer des stratégies de marketing (inter-) nationales; (iv) Caractériser, évaluer et améliorer génétiquement l'espèce; (v) Développer les techniques culturales améliorées (taille, irrigation, engrais, etc.) ; (vi) Réaliser une caractérisation morphologique et génétique du matériel végétal dans différentes zones agro-écologiques dans les autres pays (Togo, Ghana, Côte d'Ivoire, Cameroun, Gabon) pour déboucher à l'identification d'accessions adaptées à différentes zones agro-écologiques et leur résistance/tolérance au stress biotique, décrire les maladies et ravageurs, identifier le potentiel de production, documenter la pollinisation et la fructification, etc. Cette approche tout en s'inscrivant dans la logique de politique de la diversification et de promotion des ressources génétiques à grande valeur économique est en phase avec les enjeux nationaux et internationaux de la conservation et de l'utilisation durable des ressources génétiques forestières. Le principal objectif est de répondre aux besoins actuels des populations sans compromettre ceux des générations futures.

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***Ewédjè E-EBK**, Ahanchédé A and Hardy OJ (*en préparation*) Genetic diversity of *Pentadesma butyracea* Sabine (Clusiaceae) in Benin.

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