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Chloroplast and microsatellite markers in Astronium urundeuva (Allemão) Engl. and close species of Anacardiaceae: toward the definition of a species complex?

Sofia Caetano, Louis Nusbaumer & Yamama Naciri

Abstract

CAETANO, S., L. NUSBAUMER & Y. NACIRI (2008). Chloroplast and microsatellite markers in Astronium urundeuva (Allemão) Engl. and close species of Anacardiaceae: toward the definition of a species complex? *Candollea* 63: 115-130. In English, English and French abstracts.

Tree species belonging to genus *Astronium* Jacq. *(Anacardiaceae* R. Br.) can be sometimes difficult to identify on the sole basis of the morphological traits of the leaves. Individuals collected from Paraguay and Argentina were *a priori* identified in the field as *Astronium urundeuva* (Allemão) Engl., *Astronium balansae* Engl. and *Astronium fraxinifolium* Spreng., although no flowers nor fruits were found at the time of sampling. These were analysed with six microsatellites and two chloroplast markers. Using a bayesian approach on the microsatellite data, each individual was assigned to one of three distinct clusters that subsequently appeared to correspond to the previously described species. *Astronium fraxinifolium* was identified as the most differentiated species with both microsatellites and chloroplast data, although its leaf morphology is close to the one of *Astronium urundeuva.* The lower differentiation levels reported among *Astronium urundeuva* and *Astronium balansae,* despite their different leaf morphologies, was attributed to a more recent divergence of

Résumé

CAETANO, S., L. NUSBAUMER & Y. NACIRI (2008). Chloroplaste et marqueurs microsatellites chez Astronium urundeuva (Allemão) Engl. et autres espèces apparentées d'Anacardiaceae: vers une définition du complexe d'espèces? *Candollea* 63: 115-130. En anglais, résumés anglais et français.

Les espèces d'arbres appartenant au genre *Astronium* Jacq. *(Anacardiaceae* R. Br.) sont parfois difficiles à identifier sur la seule base des caractères morphologiques des feuilles. Des individus, *a priori* identifiés lors de la récolte comme étant *Astronium urundeuva* (Allemão) Engl., *Astronium balansae* Engl. et *Astronium fraxinifolium* Spreng., bien que ne présentant ni fleurs ni fruits au moment de l'échantillonnage, ont été collectés au Paraguay et en Argentine. Ils ont été analysés à l'aide de six marqueurs nucléaires microsatellites et de deux intergènes chloroplastiques. Sur la base des résultats obtenus sur les microsatellites, et en utilisant une approche bayésienne qui ne fait aucune utilisation de l'appartenance spécifique supposée, chaque individu a été assigné à l'un des trois groupes désignés par l'analyse. Ces trois groupes se sont avérés être exactement superposables aux trois espèces supposées. *Astronium fraxinifolium* est l'espèce la plus différenciée des deux autres, à la fois pour les microsatellites et les marqueurs chloroplastiques, bien que la morphologie de ses feuilles soit très

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the species or to a case of introgressive hybridization. This study underlines the advantages of using a Bayesian assignement procedure whenever molecular studies based on one particular species is to be undertaken. It furthermore confirms the species status of the three analysed taxa, and represents a first step toward a more comprehensive analysis of the three species.

Key-words

ANACARDIACEAE – *Astronium* – Species complex – Chloroplast markers – *trn*H-*psb*A – *trn*S-*trn*G – Nuclear microsatellites – Bayesian statistics

proche de celle d'*Astronium urundeuva.* La plus faible différentiation génétique trouvée entre *Astronium urundeuva* et *Astronium balansae,* bien que leurs feuilles soient assez différentes morphologiquement, est expliquée par une divergence plus récente des deux espèces ou à des phénomènes d'introgression et d'hybridation entre espèces. Cette étude souligne l'intérêt des procédures d'assignement Bayésiennes lorsque des études moléculaires sur une espèce doivent être entreprises. Elle confirme par ailleurs le statut d'espèce des trois taxons analysés, et constitue un premier pas en direction d'une analyse plus poussée de ces espèces.

Introduction

The conceptual recognition of species constitutes the starting point for many detailed investigations (MALLET, 1995), such as biodiversity assessments, red list publications, establishment of conservation strategies or genetic studies focusing on the evolutionary processes within well-defined species. Because different approaches can be used in species definition, alternative concepts co-exist, among which the biological concept of Mayr, the Hennigian concept, the phylogenetic concept or the evolutionary concept (WHEELER & MEIER, 2000). The basic procedure of accurate and efficient delimitation of species relies on the identification and comparison of diagnostic morphological or chemical characters, which are inferred to be invariant or to vary within a specified range among samples of different geographical origins (WIENS & SERVEDIO, 2000). In practice both uncertainty and controversy about how to recognize species nevertheless exist, and are mostly related to four different issues: the concept of species presently used, the intrinsical properties of the species, the processes liable for their existence, and the methods used for inferring species boundaries (QUEIROZ, 2005).

Population researchers are frequently interested in describing the genetic structure of a group of populations more or less connected by gene flow, and in such a case, it is of major interest to ensure that no other reproductive entity is present in the data set to be analysed. DNA barcoding has been invoked to provide a rapid and reproducible identification of the species (CHASE & al., 2005; CHASE & al., 2007; KRESS & ERICKSON, 2007), but much debate about its performance still exists and this method is not yet widely accepted. Accordingly, DNA barcoding, as currently applied, is unsuitable in particular situations where closely related species coexist and/or hybridization and introgression is occurring. Natural hybridization and introgression are indeed common phenomena of evolutionary importance that retain much of the researchers' attention across many fields within biology (ARNOLD, 1992; ANDERSON & THOMPSON, 2002).

Due to the continuous improvement of the techniques for developing species-specific markers (SELKOE & TOONEN, 2006) and to the recent development of numerous new statistical analyses, such as bayesian assignment techniques (PRITCHARD & al., 2000; ANDERSON & THOMPSON, 2002), other methods allowing the identification of related species have been introduced (DUMINIL & al., 2006). Hence, whenever nuclear microsatellite markers are available in a group of congeneric species, the correspondence between genotypic clusters and the previously described taxonomic species can be easily checked. Additionally, correspondence between nuclear microsatellite genotypes and other sources of genetic information, such as chloroplast haplotypes, can be very useful in cases of introgression. In this study, we aim at validating the strategy proposed by DUMINIL & al. (2006) within the genus *Astronium* Jacq. *(Anacardiaceae)*. Based on molecular tools, we seek to differentiate three congeneric species, *A. urundeuva* (Allemão) Engl., *A. balansae* Engl. and *A. fraxinifolium* Spreng., and to detect possible introgression among them. No clear agreement exists concerning the species name of *A. urundeuva* and *A. balansae,* with two recent revisions that either classified the species into *Astronium* genus (e.g., MUÑOZ, 1990) or moved it into the genus *Myracrodruon* Allemão (SANTIN & LEITÃO-FILHO, 1991). Several aspects concerning the phylogenetic relationships among the two genus are still unresolved, so it is difficult to know which classification is the most suitable, biologically speaking. We will accordingly use the *Astronium* genus name in the following study.

A total of 13 *Astronium* species have been described in Central and South America, distributed from Mexico and the Antilles to Argentina (MUÑOZ, 1990). *Astronium urundeuva* is widespread in Eastern and central Brazil, Bolivia, Paraguay and Northwest Argentina. *Astronium balansae* has been described in Paraguay and Northwest Argentina, whereas *A. fraxinifolium* has been found from Central Brazil to the Eastern Bolivia and Western Paraguay. Close ecological requirements have been found for *A. urundeuva* and *A. fraxinifolium,* and because they also share similar distribution patterns (Fig. 1), co-occurrence has been reported in different patches of dry forests (e.g. Monte Occidental and Parque Chaqueño in Paraguay (MUÑOZ, 1990) or southwest Brazil (Oliveira-Filho, *pers. comm.)*. On the other hand, *A. balansae* is rather reported in more dense and less dry forests, although some overlapping distribution exists in Paraguay with the two former species in the most humid parts of Parque Chaqueño or with *A. urundeuva* in Parque del Rio Paraguay. All three species are dioecious, wind or insect pollinated and produce small seeds that are wind dispersed. In Paraguay and North Argentina, the flowering period overlaps for the three species during September and October.

For a taxonomist, species differentiation is ideally based on floral characteristics (MUÑOZ, 1990), but in the absence of flowers and/or fruits, which often happens when sampling for population genetic purposes, identification of the species is still possible using a combination of several other characters. In the particular case of these three *Astronium* species, vegetative criteria often overlap (e.g. *A. urundeuva* is 5-30 m high, *A. balansae* 6-25 m and *A. fraxinifolium* 3-16 m), and the morphological traits of the leaves are the most useful character for recognizing the different species when the trees are in a vegetative state. After MUÑOZ (1990) and after a comparative survey of herbarium samples in the Geneva collection, the leaves of *A. urundeuva* measure 10-30 cm long and the leaflets are ovate to oblong-ovate, entire to slightly crenulated-dentate, shortly petiolulate (0.2-0.4 cm long excepted terminal one reaching 2 cm long), subacute to

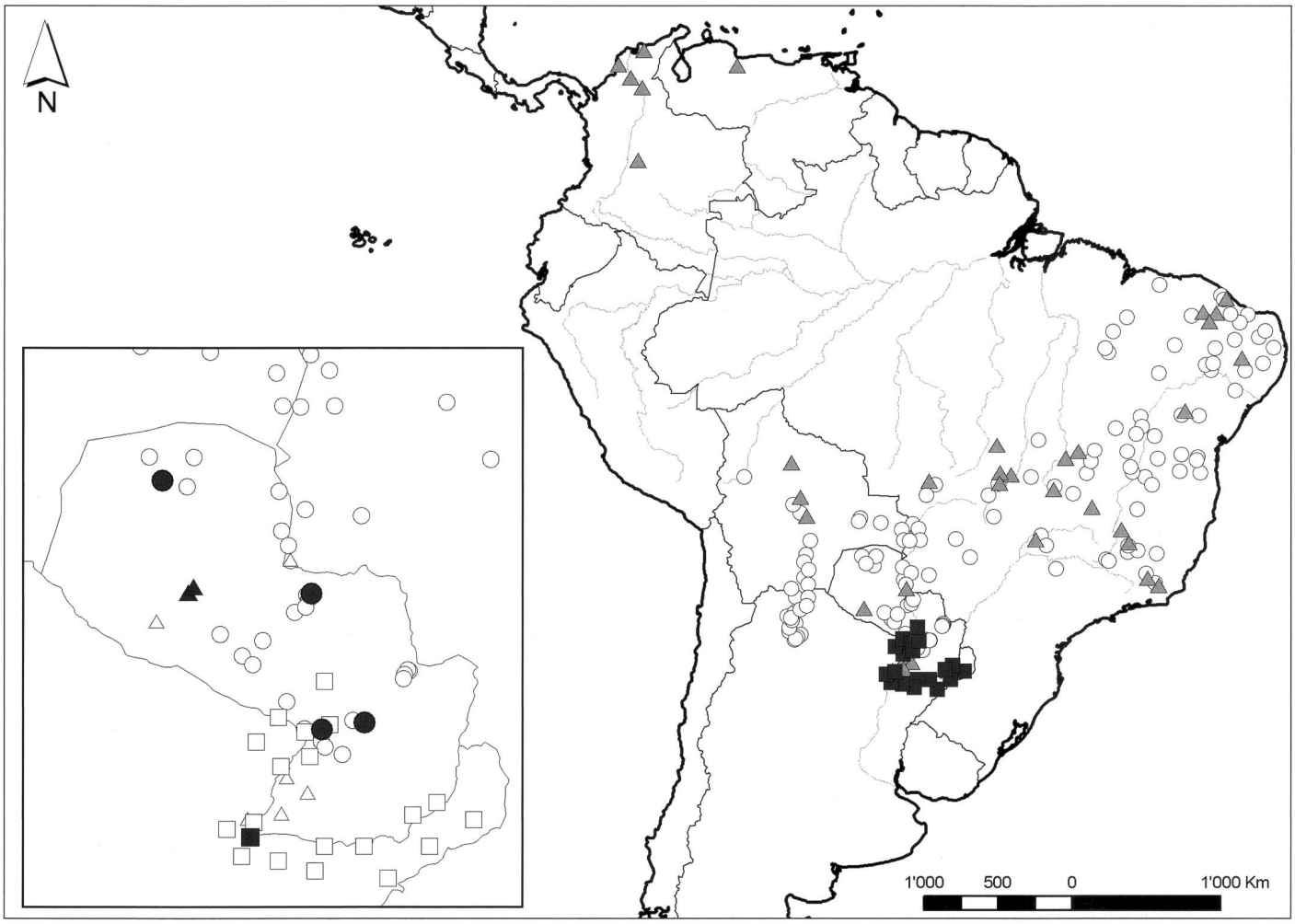

Fig. 1. – Geographical range of the three *Astronium* species (○: *A. urundeuva* (Allemão) Engl.; △: *A. fraxinifolium* Spreng.; ■: *A. balansae* Engl.). In the detailed map, the same symbols described before for the three species have been kept: their distribution is represented by white symbols and the seven populations used in this study appear in black.

acuminate and mucronate at the apex, concolour to slightly discolour and generally pubescent. The leaves of *A. fraxinifolium* measure 12-30 cm long and the leaflets are ovate to triangular or oblong to ovate, entire to slightly undulate-dentate, shortly petiolulate (0.2-0.4 cm), slightly acuminate, concolour to discolour and glabrous to hairy especially at the base of the main nerve. The leaves of *A. balansae* are 10-17 cm long and the leaflets are oblique lanceolate, distinctly dentate, long petiolulate (0.3-1.2 cm), acuminate at the apex, distinctly discolour and glabrous.

Despite the clear identification key for adult *Astronium* individuals, confusion can still persist due to the plasticity inherent to each species, depending on the geographic location, age of the tree and age of the selected branches, which can lead to possible sampling errors, especially when one single species is the object of the study. In this perspective, the risk of confusion among *A. urundeuva* and *A. fraxinifolium* is indeed considerable, because their leaves are very alike. This risk is furthermore increased by their similar geographical range and ecological requirements. On the other hand, recognition of *A. balansae* is generally easier (morphologically distinct leaves and occurrence in a different habitat), and confusion is more uncommon.

Because of their chemical composition, *Astronium* species have been investigated for pharmacological ends and several properties have been identified: *A. urundeuva* has been screened for immunomodulatory activity (Deharo & al., 2004), tested as an antioxidant agent acting in anti-inflammatory processes (Desmarchelier & al., 1999), and proved to be successful in treating gastrointestinal transit disturbances (Menezes & Rao, 1988). *Astromium balansae* revealed the presence of bactericidal components (Salvat & al., 2004). Moreover, this genus is traditionally known for its very good timber quality, which

has resulted in the exploitation of several species for commercial purposes (BARANY & al., 2003). In this perspective, it seems obvious that a clear identification of the species is fundamental, either for pharmacological researches, or for the implementation of conservation strategies, even in the absence of flowers.

In this study we used Bayesian assignment analysis of multilocus nuclear genotypes in different populations of *Astronium* sp. in Paraguay and Argentina, to identify genotypic clusters. Recognition of *A. urundeuva, A. balansae* and *A. fraxinifolium* individuals was systematically done *a priori,* based on the morphological traits of the leaves, but this information was only used *a posteriori* to check for the consistency of the assignment results. Moreover, we examined the correspondence between nuclear and chloroplast molecular markers to search for possible cases of introgression.

Material and Methods

Astronium individuals were *a priori* identified on the basis of morphological characteristics of the trees, in particular their leaves. Identification of the three species was done in the field by local botanists: Dr. D. Prado, from the Rosario University (Argentina) for *A. balansae,* and K. Elizeche, forest engineer at the Asuncion University (Paraguay) for both *A. fraxinifolium* and *A. urundeuva.*

A total of 165 non flowering adult individuals were sampled within seven populations:

1. 33 *A. fraxinifolium* from two populations in Paraguay;
2. 19 *A. balansae* from one Argentinean population;
3. 113 *A. urundeuva* from four populations in Paraguay (Table 1 and Fig. 1).

Genomic DNA was extracted with the DNeasy Plant Kit (Qiagen) and used as template for both chloroplast and microsatellite markers amplification.

Microsatellite markers

Each individual was genotyped with six nuclear microsatellites (Table 2) developed from *Astronium urundeuva: Auru.*A392, *Auru.*B209, *Auru.*D094, *Auru.*D167, *Auru.*D282 (CAETANO & al., 2005) and *Auru.*H207 (CAETANO & al., 2008). Analyses were performed on an ABI377 automated sequencer using Genescan software (Applied Biosystems), and Genescan-400 Rox (Applied Biosystems) as size standard for each individual PCR product.

Table 1. – Description of the seven *Astronium* populations used in this study (Nb Ind.: number of individuals).

Population	Code	Longitude	Latitude	Nb Ind.	ID Species
San Luis	Pa_StL	-57.439	-22.624	46	*A. urundeuva*
Cerro Léon	Pa_CLe	-60.317	-20.432	33	*A. urundeuva*
Altos	Pa_Alt	-57.238	-25.257	14	*A. urundeuva*
Cordillera	Pa_Cor	-56.420	-25.117	20	*A. urundeuva*
Paso de Patria	Ag_PsP	-59.815	-22.604	19	*A. balansae*
Loma Plata	Pa_LmP	-59.717	-22.490	13	*A. fraxinifolium*
Laguna Capitan	Pa_LgC	-58.610	-27.330	20	*A. fraxinifolium*

Table 2. – Characterization of the six microsatellite loci over the seven populations and the three *Astronium* species (N_A: number of alleles; Allele Size: size of alleles [in base pairs]; H_T: total expected heterozygosity; F_{IS}: average allelic correlation within individuals relative to each population averaged over populations; F_{ST}: measure of genetic divergence among the seven populations; **: $p < 0.01$, ***: $p < 0.001$).

Locus	GeneBank	N_A	Allele Size	H_T	F_{IS}	F_{ST}
*Auru.*A392	AY640260	14	178-214	0.823	0.003	0.308***
*Auru.*B209	AY509817	19	186-236	0.873	-0.004	0.280***
*Auru.*D094	AY640267	8	105-127	0.697	-0.105	0.448***
*Auru.*D167	AY640268	3	134-138	0.551	-0.166	0.277***
*Auru.*D282	AY640270	33	177-245	0.945	0.066	0.139***
*Auru.*H207	AY509818	19	127-173	0.848	0.173**	0.271***

Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested with 10000 iterations using ARLEQUIN 3.11 software (EXCOFFIER & al., 2005). Genetically homogeneous groups of individuals were detected using a Bayesian-based approach, implemented in the Structure program (PRITCHARD & al., 2000). With Structure, the number of clusters K was tested in the range of one to seven (i.e. the total number of populations studied), assuming the admixture model with correlated allele frequencies. Ten independent runs for each value of K were performed, using a burn-in and MCMC lengths of 30000 and 100000 iterations, respectively. Convergence was achieved in all runs. The number of clusters was determined by calculating the ΔK statistic (EVANNO & al., 2005) and samples were then placed into the cluster for which they showed the highest assignment probabilities, as far as this probability was higher than 50%. Differentiation among the identified clusters, as well as between the seven populations was estimated using pairwise F_{ST} and computed in ARLEQUIN 3.11 software.

A hierarchical analysis of molecular variance was also used to estimate the fraction of variance due to differences among clusters, among populations within clusters and among populations (EXCOFFIER & al., 1992). In order to estimate null allele frequencies within each identified cluster, we then estimated the maximum-likelihood allele frequencies from the observed data, using 10000 iterations and an Expectation-Maximization (EM) algorithm implemented in ARLEQUIN. Measures of diversity were calculated within population and within cluster: number of polymorphic loci (N_{PL}), observed number of alleles (N_A) and private alleles (N_{PA}), observed heterozygosity (H_O) and expected heterozygosity (H_E) corrected for small samples. Allelic richness (R_S) was computed using the rarefaction method implemented in FSTAT software (GOUDET, 2007).

Chloroplast Markers

The chloroplast DNA (cpDNA) polymorphisms were analysed by sequencing two different spacers in all *Astronium* spp. individuals: the HA locus using *trn*H and *psb*A primers, the SG locus using *trn*S and *trn*G (HAMILTON, 1999). PCR products were sequenced from both ends using BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 377 automated sequencer (Applied Biosystems). Each haplotype sequence was deposited in GenBank (Table 3). Nucleotide sequences were aligned using Clustal W (THOMPSON & al., 1994) implemented in the BIOEDIT program (HALL, 1999) and revised manually.

Genotypic linkage among loci was tested with the ARLEQUIN package (EXCOFFIER & al., 2005), and the results allowed the combination of the two haplotypes for each individual. In order to visualize the relationships between the combined haplotypes, a median-joining network was drawn using NETWORK software (BANDELT & al., 1999). Insertion-deletion events (indels), as well as microsatellites, were included in the analysis, as their usefullness in intrageneric studies has already been shown (HAMILTON & al., 2003; INGVARSSON & al., 2003).

Table 3. – Characterization of chloroplast haplotypes found for *trn*H-*psb*A and *trn*S-*trn*G spacers (S: substitution; ID: indel; mutation, including indels of one base pair, and indel positions, are numbered from the end of the *trn*H and *trn*S primers, respectively).

		trnH-psbA (607bp)																
Haplotype	**Gene Bank**	**ID 36**	**S 60**	**S 147**	**ID 192**	**S 218**	**S 273**	**S 295**	**S 342**	**S 352**	**S 399**	**ID 415**	**S 467**	**S 481**	**S 500**	**S 510**	**ID 535**	**ID 540**
A	EF513743	–	T	T	–	A	C	A	T	A	T	–	C	G	A	A	G	–
B	EF513744	C	T	T	–	A	C	A	T	A	T	–	C	G	A	A	G	–
C	EF513745	C	T	T	–	A	C	A	G	A	T	–	C	G	A	A	G	–
D	EU053211	–	C	G	T	T	T	G	T	C	G	A	G	C	C	G	–	A

		trnS-trnG (650bp)					
Haplotype	**Gene Bank**	**S 108**	**S 230**	**ID 253**	**S 308**	**S 468**	**S 601**
A	EF513746	C	C	A	T	G	G
D	EU053212	A	A	–	C	T	T

Results

Bayesian assignment tests indicated that the most likely number of clusters was $K = 3$, as suggested by the distribution of ΔK values (Appendix 1, supplementary data). Assignment probabilities were very high, with 98% of the values being higher than 0.9. Four individuals showed lower performances (assignment probabilities to cluster A ranging between 0.616 to 0.878) but they still displayed a sharp difference with the second best cluster (between 0.081 and 0.381) and could, therefore, still be easily assigned (Appendix 2).

Our results indicated complete correspondence between taxonomic species and genotypic clusters: cluster A included all 113 individuals *a priori* identified as *A. urundeuva,* and clusters B and C corresponded respectively to *a priori* identified as *A. balansae* and *A. fraxinifolium.* Differentiation between the three Bayesian drawn clusters was very high ($F_{CT} = 0.344$; $p < 0.01$) accounting for 90% of the total differentiation overall samples ($F_{ST} = 0.383$; $p < 0.0001$). This is a further indication that the three clusters correspond to distinct biological entities, for which the average differentiation between conspecific populations was comparatively small ($F_{SC} = 0.059$, $p < 0.0001$). *Astronium fraxinifolium* was the most differentiated cluster/species among the three, measured in terms of pairwise F_{ST}, which is also reflected by the differential sharing of alleles among clusters (Table 4). The highest frequency of null alleles (i.e. alleles that do not amplify because of mutations in the primer regions) was observed in *A. fraxinifolium* (0.169 ± 0.074 averaged over loci, *A. urundeuva:* (0.027 ± 0.043), *A. balansae* (0.026 ± 0.042). Accordingly, clusters corresponding to *A. urundeuva* and *A. balansae* were found at Hardy-Weinberg equilibrium, as evidenced by their non significant F_{IS}, but the opposite was true for *A. fraxinifolium* (Table 5).

Analysing the clusters/species separately, differentiation among populations within *A. fraxinifolium* cluster ($F_{ST} = 0.088$; $p < 0.0001$) was about twofold the level reported among *A. urundeuva* populations ($F_{ST} = 0.048$; $p < 0.0001$, the pairwise values varying between 0.021 and 0.075). F_{ST} could not be computed for *A. balansae,* as it was represented by a single population. In terms of diversity, *A. urundeuva* was the most diverse species (Table 5), as reflected by the highest diversity indices, namely R_S and H_E.

Four chloroplast haplotypes were detected with *trn*H-*psb*A spacer (HA) and only two with *trn*S-*trn*G (SG, Table 3), which resulted in four combined haplotypes (Fig. 2). Based on consensus sequences, the HA fragment was 607bp long, and the haplotypes were characterized by four indels of 4, 5, 7 and 13bp, one mononucleotide repeat displaying two variants and twelve nucleotide substitutions. For SG, which corresponded to a 650bp fragment, the two haplotypes were characterized by five nucleotide substitutions, and two variants of a mononu-

Table 4. – Pairwise F_{ST} (below the diagonal) and number of alleles shared among each cluster of *Astronium* Jacq. (above the diagonal), estimated with six microsatellite loci. All F_{ST} values were highly significant ($p < 0.001$).

	A. urundeuva	***A. balansae***	***A. fraxinifolium***
A. urundeuva	–	15	9
A. balansae	0.295	–	2
A. fraxinifolium	0.346	0.631	–

Table 5. – Variability indices in the three *Astronium* species based on microsatellite and chloroplast data (N_I, N_P: number of individuals and number of populations; N_{PL}, N_A, N_{PA}, N_{NA}: total number of polymorphic loci, of alleles, of private alleles, and of null alleles respectively ± standard deviation; R_S: mean allelic richness averaged over populations ± standard deviation; H_O, H_E: observed and expected heterozygosities averaged over populations ± standard deviation; F_{IS}: the average allelic correlation within individuals relative to each species, all populations confounded and corresponds to a measure of inbreeding; F_{ST}: measure of genetic divergence among populations; for the chloroplast data the number of individuals displaying the combined haplotypes AA, BA, CA and DD is indicated (the first letter refers to *trn*H-*psb*A and the second one to *trn*S-*trn*G); *: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$).

	Microsatellite data											**Chloroplast data**			
	N_I	N_P	N_{PL}	N_A	N_{PA}	N_{NA}	R_S	H_O	H_E	F_{IS}	F_{ST}	AA	BA	CA	DD
A. urundeuva	133	4	6	71	49	0.027 ± 0.043	8.2 ± 4.2	0.701 ± 0.119	0.723 ± 0.140	-0.003	0.048***	52	59	2	–
A. balansae	19	1	3	17	4	0.026 ± 0.042	3.3 ± 3.4	0.270 ± 0.301	0.278 ± 0.299	0.029	–	–	19	–	–
A. fraxinifolium	33	2	4	28	20	0.169 ± 0.074	4.3 ± 4.2	0.375 ± 0.283	0.439 ± 0.338	0.108*	0.088**	–	–	–	33

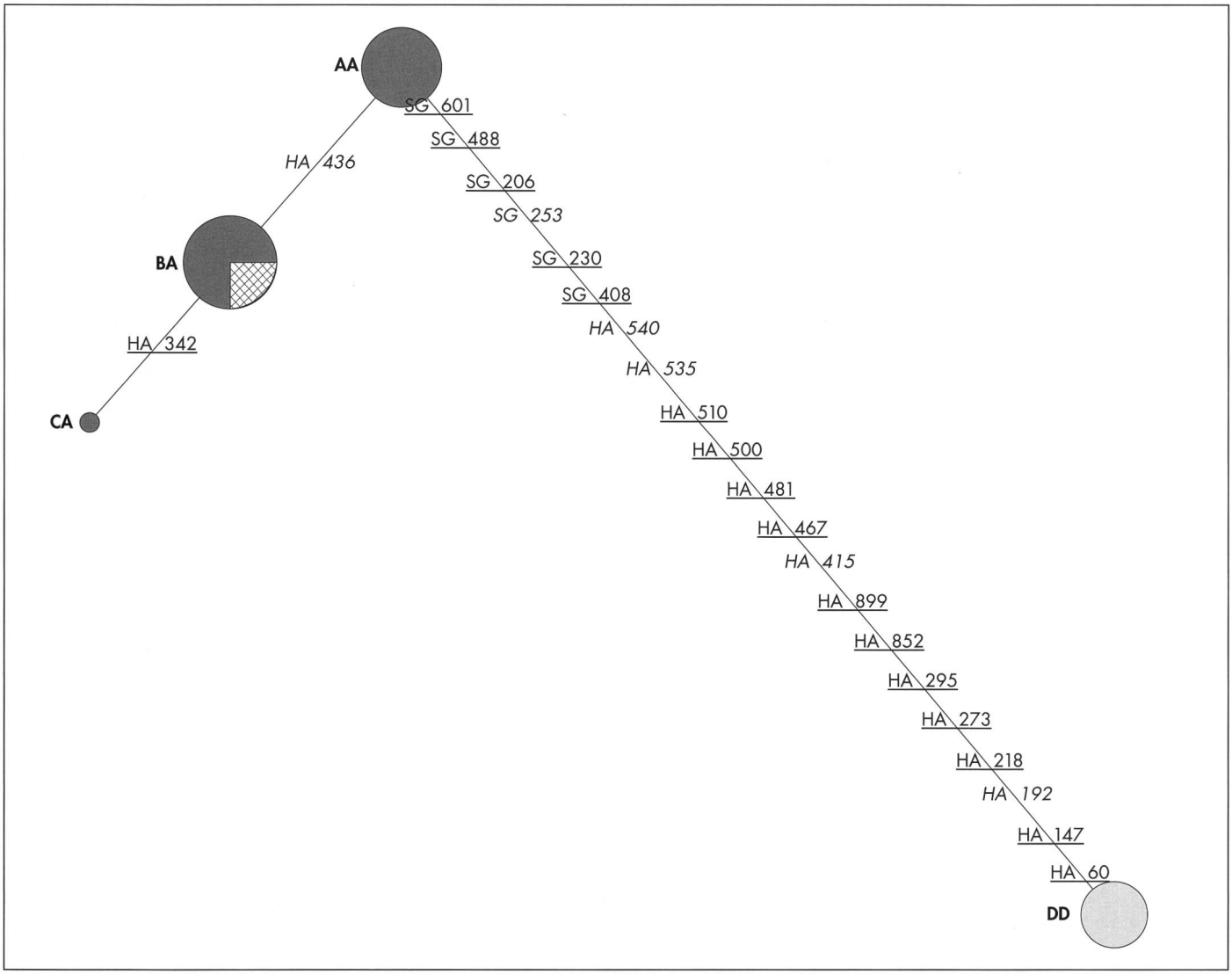

Fig. 2. – Median Joining Network of *Astronium* haplotypes considering all mutations. The radius of each circle is proportional to the number of individuals displaying the haplotype and colours assigned correspond to each species (dark grey: *A. urundeuva* (Allemão) Engl.; diagonal cross: *A. balansae* Engl.; light grey: *A. fraxinifolium* Spreng.). Mutation names starting with HA and SG referred to mutations observed in *trn*H-*psb*A and *trn*S-*trn*G, respectively. Indels are in italics and substitutions are underlined.

cleotide repeat. We found absolute correspondence between combined haplotype DD and the cluster C, previously identified as being entirely constituted by *A. fraxinifolium* individuals. Combined haplotypes AA and CA corresponded to some *A. urundeuva* individuals, assigned to cluster A, and for the haplotype BA, correspondence was found with the remaining *A. urundeuva* individuals constituting cluster A and all *A. balansae* individuals assigned to cluster B (see Appendix 2 for details).

Discussion

A good delimitation of the three *Astronium* species was obtained using the nuclear markers and the Bayesian approach. However a major inconsistency concerning the identification of *A. urundeuva* and *A. balansae* was observed when considering the chloroplast results. Three haplotypes A, B and C, were found in the first species with the HA spacer, whereas all *A. balansae* individuals displayed haplotype B. Moreover, the locus SG could not discriminate these two species (haplotype A for both). On the other hand, whether with HA or SG, *A. fraxinifolium* displayed a new haplotype, D. Consequently, on the basis of both the higher microsatellite pairwise F_{ST} and the chloroplast results, *A. fraxinifolium* was the most

differentiated cluster/species, whilst a much weaker level of differentiation was reported between *A. urundeuva* and *A. balansae,* with possible confusion between the species when using the chloroplast markers alone. These results raise two additional topics in the discussion; the first concerns the sharp contradiction with the morphology, according to which *A. urundeuva* and *A. fraxinifolium* are closer to each other, and the second the status of species complex for *A. urundeuva* and *A. balansae.*

Divergence of A. fraxinifolium

Both types of markers agree well in identifying *A. fraxinifolium* as the most differentiated species. Given that discernment between *A. fraxinifolium* and *A. urundeuva* based on morphological traits of the leaves is tricky, and that these species share the same ecological conditions and often occur in sympatry, these results are rather reassuring. On the one hand, such results indicate that the close morphological traits of the leaves are most probable related to some selection pressure due to the same ecological features being shared among species. This is a clear example where the morphological similarity does not reflect the genetic relationships between species, as evaluated by *a priori* neutral markers. On the other, these results reveal that genetic structure surveys based on microsatellites (Caetano & al., 2008) can be performed in all confidence, as delimitation of the species has been safely accomplished in places where a confusion might have had occurred (e.g. Central Brazil to Eastern Bolivia and Western Paraguay).

Relationship between A. urundeuva *and* A. balansae

The second issue concerns the relationship between *A. urundeuva* and *A. balansae,* and four major hypotheses can be put forward to explain the discrepancy between chloroplast and nuclear genes:

- it could result from homoplasy of this chloroplast region (i.e. identity by state but not identity by descent);
- it could reflect the existence of a still shared ancestral haplotypes;
- it could reflect the differential evolutionary rates of the chloroplast and nuclear polymorphisms analysed here;
- it could reflect chloroplast capture, *i.e.* the introgression of the chloroplast from one species to another through interspecific hybridization. Hybridization is indeed known to be one of the major factors leading to genetic incongruence between nuclear and cytoplasmic markers in plants (Soltis & Kuzoff, 1995), as illustrated by numerous examples reporting cases of chloroplast capture in several taxa (Soltis & Kuzoff, 1995; Comes & al., 1997; Manen & al., 2002; Okuyama & al., 2005).

While it is highly unlikely that homoplasy is in the origin of such results, especially given the nature of the mutation differentiating haplotypes HA_A and HA_B (indel of 7bp long), we are unable at this point to undoubtedly decide which of the second, third or fourth explanation is the more plausible. Since *A. urundeuva* and *A. balansae* appear to be closer to each other than to *A. fraxinifolium,* it can be hypothesized that they have diverged more recently than did *A. fraxinifolium.* In this context, the BA haplotype shared between *A. urundeuva* and *A. balansae* could indeed be viewed as an ancestral one still occurring in the two species despite their split into differentiated biological entities. Closely related to this second explanation is the differential mutation rates reported in the literature for chloroplast spacers and nuclear microsatellites that are known to evolve much faster. The rate of indel mutations in the intergenic regions of chloroplast genomes has for instance been recently estimated to be $\approx 0.8 \pm 0.04 \times 10^{-9}$ per site per generation (Yamane & al., 2006), whilst mutation rates for nuclear microsatellites are in general substantially higher, of the order of 10^{-4}-10^{-2} per generation (Jarne & Lagoda, 1996; Hancock, 1999).

The fourth explanation, i.e. introgressive hybridization between *A. urundeuva* and *A. balansae* could be hypothesized, but the direction in which this event would have occurred cannot be assessed, because of the poor representation of *A. balansae* in the dataset. It is interesting to notice that the four individuals more poorly assigned to *A. unrundeuva* cluster were all secondarily assigned to *A. balansae* cluster, with p ranging from 0.081 to 0.381. Generally speaking, the introgression signal displayed by individual loci is still poorly understood (Okuyama & al., 2005) and depends on each locus evolutionary history, although it is known that chloroplast capture is generally promoted by cyto-nuclear incompatibilities and facilitated by partial selfing events under certain conditions (Tsitrone & al., 2003). In our case, such hybridization between *A. urundeuva* and *A. balansae* could be (or have been) promoted by their overlapping flowering period, the ability of pollen to be wind-dispersed and the co-occurrence of the two species in Parque del Rio Paraguay and in Parque Chaqueño, although they have different ecological requirements in terms of soil humidity.

It should be noticed finally, that the last three explanations are not exclusive since the more recent the two species are, the more chance they have to hybridize, because the barrier to gene flow is not strong enough, the more chance they have to share ancestral alleles or haplotypes, and the more chance they have that no mutation occurred since they diverged, specially with the chloroplast. In this perspective, it is reasonable to question a species complex status for *A. urundeuva* and *A. balansae,* and we therefore strongly encourage a more detailed study in light to what has been done in Europe for *Quercus petraea* (Matt.) Liebl. and *Q. robur* L. (Muir and Schlötterer, 2006).

This result is in agreement with the finding of SANTIN & LEITÃO-FILHO (1991) that *A. urundeuva* and *A. balansae* share some ovary features, a reason why they suggested moving the two species together to *Myracrodruon* genus.

Diversity within species

A higher level of diversity was found within *A. urundeuva* when compared to the two other species, as reflected by both gene diversity and allelic richness. The unbalanced sampling can explain the differences observed between species. Allelic richness is specifically drawn to compare samples of different sizes, but it uses the observed allele frequencies that are better estimated for *A. urundeuva* than for the two other species because of a larger sampling size. A second explanation may arise from the use of specific *A. urundeuva* markers. Because microsatellite primers were designed from a unique *A. urundeuva* individual, the former result could primarily indicate some kind of ascertainment effect, according to which microsatellite amplifications are more prone to failure when used on congeneric species (TREUREN, 1998). Accordingly, higher frequencies of null alleles have been found for the most differentiated species *A. fraxinifolium,* whilst for *A. urundeuva* and *A. balansae,* weaker and very similar frequencies were observed. Therefore, significant biases can effectively arise if these *A. urundeuva* specific microsatellites are to be used in intraspecific analyses within other divergent *Astronium* species. This is clearly observed within *A. fraxinifolium,* and might also explain the higher F_{ST} that was found between the two *A. fraxinifolium* populations (0.088), when compared with global F_{ST} obtained for *A. urundeuva* (0.048), whose populations were distributed in a much wider way.

Conclusion

In this study, and contrary to most surveys of genetic variation, the *a priori* information about *Astronium* species based on morphological characters were not used to delineate the clustering of individuals. Individuals were instead grouped together blindly, i.e. with a Bayesian assignment method applied on multiple variable codominant nuclear markers. The morphological determination of each individual done in the field by local botanists was only used as an afterwards confirmation tool. The simple procedure used here proved to be very efficient in the discrimination between *A. urundeuva, A. balansae* and *A. fraxinifolium,* and showed that the leaf morphology represents well the floral characteristics, demonstrating this way the value of this character for the distinction of these species. Only few microsatellite loci were thus sufficient to achieve good assignment accuracy, probably because differentiation between the clusters was quite strong (VÄHÄ & PRIMMER, 2006). Moreover, the high level of interspecific differentiation also justifies the use of Evannos's ΔK method, as it performs better with strong differentiation levels (WAPLES & GAGGIOTTI, 2006).

The recognition of the species based on molecular approaches has been largely criticized by taxonomists, who argue that these new identification methods, such as DNA barcoding (CHASE & al., 2005; KRESS & al., 2005; CHASE & al., 2007; KRESS & ERICKSON, 2007), could result in incorrect species recognition, diminishing the traditional morphology-based approaches. Because of their high specificity, the DNA barcode can not rely on microsatellites and it is instead based on the use of chloroplast markers (CHASE & al., 2007). We demonstrate here that the two chloroplast locus used in this study were unable to differentiate between *A. urundeuva* and *A. balansae,* among which the HA locus that was suggested as a good barcode tool in association with a portion of the coding *rbc*L gene (KRESS & ERICKSON, 2007). HA spacer is indeed known to be one of the most variable plastid region in angiosperms between and within genera (KRESS & al., 2005), which has led to its choice for DNA barcoding (CHASE & al., 2005), although it has been proved to be also variable within species (HAMILTON & al., 2003; INGVARSSON & al., 2003; NACIRI & GAUDEUL, 2007).

The procedure employed in this study differs greatly from the classical phylogenetic reconstruction based on a few gene trees and on a restricted sampling scheme, as it uses population genetic principles based on multilocus data, obtained on a batch of individuals for each species. The increased availability of high polymorphic nuclear markers associated with the development of new powerful statistical methods greatly assists the circumscription of species, and does not compromise the traditional taxonomic approach, and we suggest that cross validation of species identification using different approaches is desirable.

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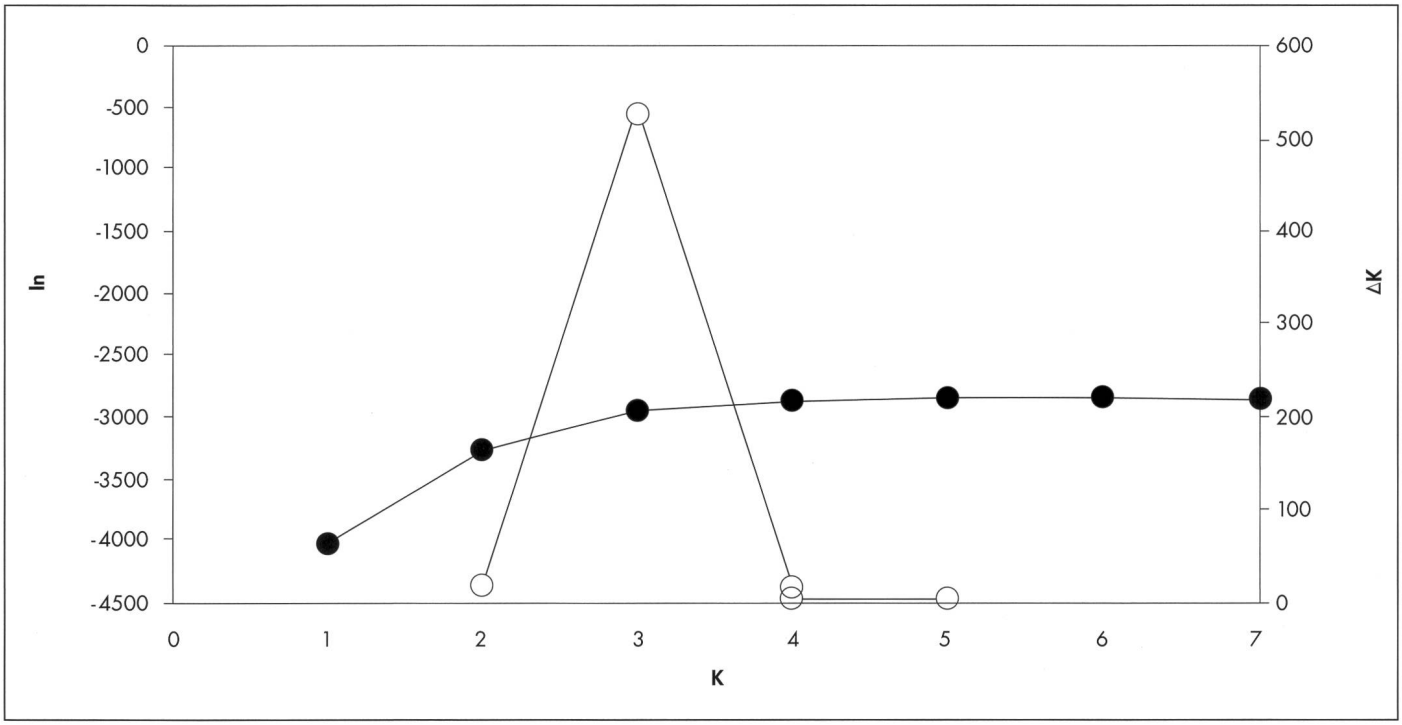
Appendix 1. – Detection of the number of clusters within the seven sampled populations of *Astronium* Jacq. Left y-axis is the mean likelihood over the ten runs as a function of *K* (●), the right y-axis ΔK following EVANNO & al. (2005) as a function of *K* (○).



Appendix 2. – Assignment probabilities obtained for each individual of *Astronium* Jacq. based on six microsatellite loci and chloroplast haplotypes found for *trn-psb*A (HA) and *trnS-trnG* (SG) spacers.

Ind.	Microsatellite data			Chloroplast data	
	Cluster A	Cluster B	Cluster C	HA	SG
Pa_StL_0001	0.994	0.003	0.003	A	A
Pa_StL_0002	0.991	0.006	0.003	B	A
Pa_StL_0003	0.994	0.003	0.003	B	A
Pa_StL_0004	0.994	0.003	0.003	A	A
Pa_StL_0005	0.992	0.005	0.003	A	A
Pa_StL_0006	0.993	0.003	0.004	A	A
Pa_StL_0150	0.993	0.004	0.003	A	A
Pa_StL_0151	0.994	0.003	0.003	A	A
Pa_StL_0153	0.990	0.008	0.002	A	A
Pa_StL_0154	0.983	0.014	0.003	A	A
Pa_StL_0007	0.993	0.004	0.002	A	A
Pa_StL_0008	0.651	0.290	0.059	A	A
Pa_StL_0009	0.988	0.009	0.003	A	A
Pa_StL_0010	0.984	0.013	0.003	A	A
Pa_StL_0011	0.993	0.005	0.002	A	A
Pa_StL_0012	0.909	0.089	0.003	A	A
Pa_StL_0013	0.994	0.004	0.002	A	A
Pa_StL_0014	0.992	0.005	0.003	C	A
Pa_StL_0015	0.993	0.005	0.003	A	A
Pa_StL_0017	0.982	0.015	0.003	A	A
Pa_StL_0018	0.993	0.004	0.002	A	A
Pa_StL_0019	0.990	0.005	0.005	A	A
Pa_StL_0020	0.993	0.005	0.002	C	A
Pa_StL_0021	0.993	0.004	0.003	B	A
Pa_StL_0030	0.992	0.006	0.002	B	A
Pa_StL_0031	0.991	0.006	0.003	A	A
Pa_StL_0032	0.992	0.005	0.003	B	A
Pa_StL_0034	0.993	0.004	0.002	B	A
Pa_StL_0035	0.982	0.015	0.003	B	A
Pa_StL_0036	0.987	0.011	0.003	B	A
Pa_StL_0037	0.993	0.005	0.002	B	A
Pa_StL_0038	0.616	0.381	0.003	B	A
Pa_StL_0039	0.979	0.019	0.002	B	A
Pa_StL_0040	0.988	0.009	0.003	B	A
Pa_StL_0041	0.970	0.004	0.026	B	A
Pa_StL_0042	0.993	0.004	0.002	B	A
Pa_StL_0043	0.917	0.081	0.002	B	A
Pa_StL_0044	0.874	0.122	0.004	B	A
Pa_StL_0045	0.983	0.014	0.003	B	A

Ind.	Microsatellite data			Chloroplast data	
	Cluster A	Cluster B	Cluster C	HA	SG
Pa_StL_0047	0.993	0.005	0.002	B	A
Pa_StL_0048	0.993	0.004	0.003	B	A
Pa_StL_0049	0.993	0.004	0.003	B	A
Pa_StL_0050	0.993	0.005	0.002	B	A
Pa_StL_0051	0.992	0.004	0.005	B	A
Pa_StL_0052	0.992	0.005	0.002	B	A
Pa_StL_0053	0.993	0.004	0.003	B	A
Pa_CLe_0041	0.994	0.003	0.003	B	A
Pa_CLe_0042	0.994	0.004	0.002	A	A
Pa_CLe_0043	0.994	0.004	0.003	A	A
Pa_CLe_0044	0.993	0.005	0.002	A	A
Pa_CLe_0045	0.994	0.004	0.003	A	A
Pa_CLe_0046	0.992	0.005	0.002	A	A
Pa_CLe_0047	0.878	0.120	0.003	A	A
Pa_CLe_0048	0.994	0.003	0.003	A	A
Pa_CLe_0049	0.967	0.031	0.002	A	A
Pa_CLe_0050	0.992	0.006	0.003	A	A
Pa_CLe_0051	0.985	0.012	0.003	A	A
Pa_CLe_0052	0.991	0.006	0.003	A	A
Pa_CLe_0053	0.990	0.007	0.003	A	A
Pa_CLe_0054	0.994	0.004	0.003	A	A
Pa_CLe_0055	0.993	0.005	0.003	A	A
Pa_CLe_0056	0.994	0.002	0.003	A	A
Pa_CLe_0057	0.990	0.007	0.003	A	A
Pa_CLe_0058	0.994	0.003	0.003	A	A
Pa_CLe_0059	0.994	0.003	0.002	A	A
Pa_CLe_0060	0.994	0.003	0.003	A	A
Pa_CLe_0061	0.994	0.004	0.003	A	A
Pa_CLe_0062	0.965	0.031	0.004	A	A
Pa_CLe_0063	0.994	0.004	0.003	A	A
Pa_CLe_0064	0.987	0.008	0.004	A	A
Pa_CLe_0065	0.994	0.003	0.003	A	A
Pa_CLe_0066	0.994	0.002	0.003	A	A
Pa_CLe_0067	0.987	0.010	0.004	A	A
Pa_CLe_0068	0.994	0.003	0.003	A	
APa_CLe_0069	0.987	0.010	0.003	A	A
Pa_CLe_0070	0.990	0.007	0.003	A	A
Pa_CLe_0071	0.966	0.004	0.030	A	A
Pa_CLe_0072	0.986	0.011	0.004	A	A
Pa_CLe_0073	0.994	0.004	0.002	A	A
Pa_Alt_0650	0.989	0.008	0.003	B	A
Pa_Alt_0651	0.959	0.008	0.033	B	A
Pa_Alt_0652	0.983	0.015	0.003	B	A

Ind.	Microsatellite data			Chloroplast data	
	Cluster A	Cluster B	Cluster C	HA	SG
Pa_Alt_0653	0.989	0.008	0.003	B	A
Pa_Alt_0654	0.990	0.007	0.003	B	A
Pa_Alt_0655	0.990	0.008	0.003	B	A
Pa_Alt_0656	0.976	0.021	0.003	B	A
Pa_Alt_0657	0.980	0.017	0.003	B	A
Pa_Alt_0658	0.993	0.004	0.003	B	A
Pa_Alt_0659	0.992	0.004	0.004	B	A
Pa_Alt_0660	0.992	0.005	0.003	B	A
Pa_Alt_0661	0.992	0.005	0.003	B	A
Pa_Alt_0662	0.992	0.005	0.003	B	A
Pa_Alt_0663	0.994	0.004	0.002	B	A
Pa_Cor_0199	0.983	0.014	0.003	B	A
Pa_Cor_0200	0.991	0.006	0.002	B	A
Pa_Cor_0201	0.993	0.004	0.003	B	A
Pa_Cor_0202	0.992	0.005	0.003	B	A
Pa_Cor_0203	0.992	0.005	0.003	B	A
Pa_Cor_0204	0.994	0.003	0.003	B	A
Pa_Cor_0205	0.994	0.003	0.003	B	A
Pa_Cor_0206	0.986	0.012	0.003	B	A
Pa_Cor_0207	0.994	0.004	0.003	B	A
Pa_Cor_0208	0.992	0.005	0.002	B	A
Pa_Cor_0300	0.963	0.005	0.032	B	A
Pa_Cor_0301	0.989	0.008	0.003	B	A
Pa_Cor_0302	0.993	0.003	0.004	B	A
Pa_Cor_0303	0.993	0.005	0.002	B	A
Pa_Cor_0304	0.986	0.012	0.003	B	A
Pa_Cor_0305	0.994	0.004	0.003	B	A
Pa_Cor_0306	0.994	0.003	0.003	B	A
Pa_Cor_0307	0.993	0.005	0.003	B	A
Pa_Cor_0308	0.981	0.016	0.002	B	A
Pa_Cor_0309	0.993	0.005	0.003	B	A
Ag_PsP_0682	0.003	0.994	0.002	B	A
Ag_PsP_0683	0.004	0.994	0.002	B	A
Ag_PsP_0684	0.003	0.994	0.002	B	A
Ag_PsP_0685	0.004	0.993	0.002	B	A
Ag_PsP_0686	0.003	0.994	0.002	B	A
Ag_PsP_0687	0.004	0.994	0.002	B	A
Ag_PsP_0688	0.005	0.992	0.002	B	A
Ag_PsP_0689	0.003	0.995	0.002	B	A
Ag_PsP_0690	0.004	0.993	0.003	B	A
Ag_PsP_0691	0.008	0.990	0.003	B	A
Ag_PsP_0692	0.005	0.993	0.002	B	A
Ag_PsP_0693	0.005	0.993	0.002	B	A

Ind.	Microsatellite data			Chloroplast data	
	Cluster A	Cluster B	Cluster C	HA	SG
Ag_PsP_0694	0.003	0.994	0.002	B	A
Ag_PsP_0695	0.003	0.994	0.002	B	A
Ag_PsP_0696	0.004	0.993	0.003	B	A
Ag_PsP_0697	0.004	0.994	0.002	B	A
Ag_PsP_0698	0.017	0.980	0.003	B	A
Ag_PsP_0699	0.011	0.981	0.008	B	A
Ag_PsP_0700	0.003	0.995	0.002	B	A
Pa_LmP_1001	0.004	0.016	0.981	D	D
Pa_LmP_1002	0.003	0.002	0.995	D	D
Pa_LmP_1003	0.003	0.002	0.995	D	D
Pa_LmP_1004	0.003	0.003	0.994	D	D
Pa_LmP_1005	0.004	0.004	0.991	D	D
Pa_LmP_1006	0.002	0.002	0.995	D	D
Pa_LmP_1007	0.003	0.003	0.994	D	D
Pa_LmP_1008	0.003	0.003	0.994	D	D
Pa_LmP_1009	0.003	0.003	0.994	D	D
Pa_LmP_1010	0.003	0.008	0.989	D	D
Pa_LmP_1011	0.003	0.002	0.995	D	D
Pa_LmP_1012	0.003	0.002	0.995	D	D
Pa_LmP_1013	0.004	0.007	0.989	D	D
Pa_LgC_1014	0.002	0.002	0.995	D	D
Pa_LgC_1015	0.003	0.008	0.989	D	D
Pa_LgC_1016	0.002	0.002	0.995	D	D
Pa_LgC_1017	0.016	0.003	0.981	D	D
Pa_LgC_1018	0.003	0.002	0.994	D	D
Pa_LgC_1019	0.003	0.002	0.995	D	D
Pa_LgC_1020	0.003	0.002	0.995	D	D
Pa_LgC_1021	0.004	0.016	0.981	D	D
Pa_LgC_1022	0.003	0.002	0.995	D	D
Pa_LgC_1023	0.003	0.002	0.995	D	D
Pa_LgC_1024	0.003	0.003	0.994	D	D
Pa_LgC_1025	0.002	0.002	0.995	D	D
Pa_LgC_1026	0.003	0.002	0.995	D	D
Pa_LgC_1027	0.003	0.002	0.995	D	D
Pa_LgC_1028	0.003	0.002	0.995	D	D
Pa_LgC_1029	0.006	0.005	0.989	D	D
Pa_LgC_1030	0.006	0.005	0.989	D	D
Pa_LgC_1031	0.002	0.002	0.995	D	D
Pa_LgC_1032	0.003	0.003	0.994	D	D
Pa_LgC_1033	0.003	0.008	0.989	D	D