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Evidence of a possible double hybrid origin for two Malagasy species of Piper L. (Piperaceae)

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Abstract: two new species of genus *Piper* L. from Madagascar: *Piper malgassicum* and *Piper tsarasotrae*, were analyzed to investigate their phylogenetic position and evolutionary history. Both plastidial and nuclear markers were used for sequencing.

The plastidial markers (*ndh*F and the *trn*L intron) showed a close relationship between the two species with respect to the other species of *Piper*. Both species appeared phylogenetically related to the African *P. guineense* and the Malagasian/Mascarenhas endemic *P. borbonense*. The nuclear marker (*G3PDH*) amplification produced two separate sets of sequences: "long" sequences, that could be easily translated into an amino acid chain, and "short" sequences, characterized by deletions that did not allowed to translate them correctly to an amino acid sequence.

Analyzing together the nuclear sequences, we observed that the "long" sequence of *P. tsarasotrae* had a stricter relationship to the African accessions of *P. guineense*, while the accession of *P. malgassicum* was more strictly related to *P. borbonense*. On the contrary both "short" sequences of *Piper malgassicum* and *Piper tsaratsotrae* resulted phylogenetically related to Asian accessions and more distantly related to the formerly cited species.

This unexpected result was tentatively explained with a more ancient hybridization event between an ancestor of *P. malgassicum* and *P. tsarasotrae* (and possibly *P. borbonense*) and an Asian species of Piper. The Asian contribution would have produced the ancestors of the "short" sequences that would eventually have lost functionality by deletions, becoming paralogs. A more recent hybridization event would have led to the separation of *Piper malgassicum* from *Piper tsarasotrae* with an African pollen-derived genome contribution from *P. guineense* or, more probably, an ancestor thereof, to an ancestor of *P. tsarasotrae*.

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The chromosome numbers of *P. tsarasotrae* (2n = about 38) and *P. malgassicum* (2n = about 46), were more like the Asian species than to the American species. Unfortunately, no chromosome number of the African species *P. guineense* is currently available, to analyze eventual chromosomal connections.

Keywords: *Piper malgassicum*, *Piper tsarasotrae*, Piperaceae, chromosomes, hybridization, DNA sequences, *G3PDH*, *trn*L, *ndh*F, Malagasy biodiversity

1. Introduction

Genus Piper L. (Piperaceae) is one of the largest genera of Angiosperms, with more than 2000 species [1]. Piper L. and hence family Piperaceae, were considered belonging to a basal group of angiosperms, the so called "paleoherbs" [2].

Piper is a pantropical genus developing highly variable growth forms [3], with the highest biodiversity in the American continent with a number of species ranging from 500 [4] [5], to 1100 [6], later increased to more than 1800 [7], many of them with a small distribution areal [1].

The separation of species is often tricky, due to the small dimension of the floral parts and hence the number of synonyms may be high [8], while other species tend to get naturalized [9]. While only two species are known as native of the African continent, *P. guineense* and *P. capense*, more species are known of Madagascar, even if some of them are known only for a single herbarium sample. The currently recognized species in Madagascar are *P. heimii* C. DC, *P. pachyphyllum* Baker and possibly *P. borbonense* (Miq.) C. DC., described for the island Île Bourbon, nowadays La Reunion [10], belonging to Mascarenhas Islands. However, its presence in Madagascar was affirmed by De Candolle [11] [12]. The fact that *P. borbonense* is cultivated makes more complex to understand its real distribution areal [13].

Piper malgassicum Papini, Palchetti, Gori, Rota Nodari and *Piper tsarasotrae* Papini, Palchetti, Gori, Rota Nodari, were recently described as new Malagasy species [13] and are of economic interest, since their dried fruits are often mixed with *P. borbonense* to produce the typical Malagasy spice called in local language "voatsiperifery" pepper.

2. Materials and Methods

The plants were collected by E. Palchetti and N. Gandolfi in two different areas of the Ambositra region in Madagascar: the first group belonging to the *P. malgassicum* type was collected into the tropical rainy forest of Vohiday and the second group, belonging to the *P. tsarasotrae* type in the semi-dry area of Tsaratsotra village. These plants were compared with the samples of *P. tsarasotrae* and *P. malgassicum* which have been used for the previous research [13]. Samples were conserved either in ethanol 96% either as herbarium sample by the ET (Tropical Herbarium of

Florence, CSET, https://www.bio.unifi.it). Some seeds were also germinated in Florence for karyotyping. The DNA used for this work was extracted from tissue conserved in ethanol 96% [14] [15].

DNA was extracted from 40 mg of the ethanol preserved leaves after drying under vacuum. The starting material was inserted in 2m L tube, together with tungsten carbide beads, frozen in liquid nitrogen and finely ground in a tissue homogenizer (Tissue Lyser ®, Qiagen). DNA was extracted using Invisorb Spin Plant Mini kit (Stratec molecular®).

Amplification of the trnL (UAA) intron (trnL) and the low copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) followed respectively the protocols by Taberlet et al. [16] and Strand et al. [17]. Two new primer pairs were designed using the chloroplast genome sequence of Piper kadsura (GenBank: KT223569.1) as template to cover the entire NADH dehydrogenase F (ndhF) plastid gene: ndhF-F3_forward 5′-AGGTTCTTATCGAGCCGCTT-3′ and reverse 5′-GTAAGAAGAAATGCGCCCCC-3′ and ndhF-F10_forward 5′-CTTCGCCGTATGTGGGCTTT-3′ and reverse 5′-TCGACCAAAAGCAAGCAAGAG-3′. The amplicons have been directly and bi-directionally sequenced by using the corresponding primers for each amplified sequence. Since direct sequencing of G3PDH showed fragments of extra peaked sequencing data, we proceeded with cloning with InsTAclone PCR Cloning Kit (Thermo Scientific®) of the G3PDH amplification products. Several colonies for each cloned sample were amplified using T7 and SP6 primers whose sites are located at the boundaries of the cloning vector. PCR products were purified using the QIAquick PCR Purification Kit (Quiagen) and sent to the University of Florence internal sequencing service CIBIACI (www.cibiaci.unifi.it). Manual correction and assembly of the sequences was performed using the software programmes Multalin [18] and MEGA7 [19]. Unexpectedly, two DNA sequences were obtained, after removing the cloning vector fragments, showing a different size: 965bp and 1058bp which were named “short” and “long” sequences respectively.

The sequences used during our investigation are available in Genbank: Piper tsarasotrae G3pdH long sequence (MH234634), G3pdh short sequence (MT793801), trnL (MH234638), ndhF (MH234636) and Piper malgassicum: G3pdH long sequence (MH234633), G3pdh short sequence (MT793800), trnL (MH234637), ndhF (MH234635).

2.1. Phylogenetic analysis

The DNA sequences were aligned with CLUSTALX 2.0 [20] and checked by eye for manual adjustment. The plastidial and the nuclear sequences were aligned separately to produce matrices that were later combined with the software combinex2_0.py (Python version 2.6.4; Biopython 1.57), by A. Papini, released under GPL license and available at www.unifi.it/caryologia/PapiniPrograms.html as implemented in Bandara et al. 2013 [21] and in Simeone et al. 2016 [22].

The phylogenetic analysis was executed on both cpDNA (ndhF and trnL) and nuclear sequences (G3PDH). Maximum parsimony analysis was performed with PAUP* 4.0b1 [23] [24]). The genbank sequences of *P. humistratum* Görts & K. U. were used as outgroups both in the nuclear and the plastid genes matrix, following the previous phylogenetic analysis by Smith et al. [9]: see (Fig. 1B). This species resulted in the sister clade with respect to the clade containing the species more relevant to the African species and the related clades. All characters had equal weight and unordered state transitions. Gaps were coded with the "simple indel coding" model [25], with the software Gapcoder [26] and added to the final matrix after the DNA sequences as in Papini et al. [27].

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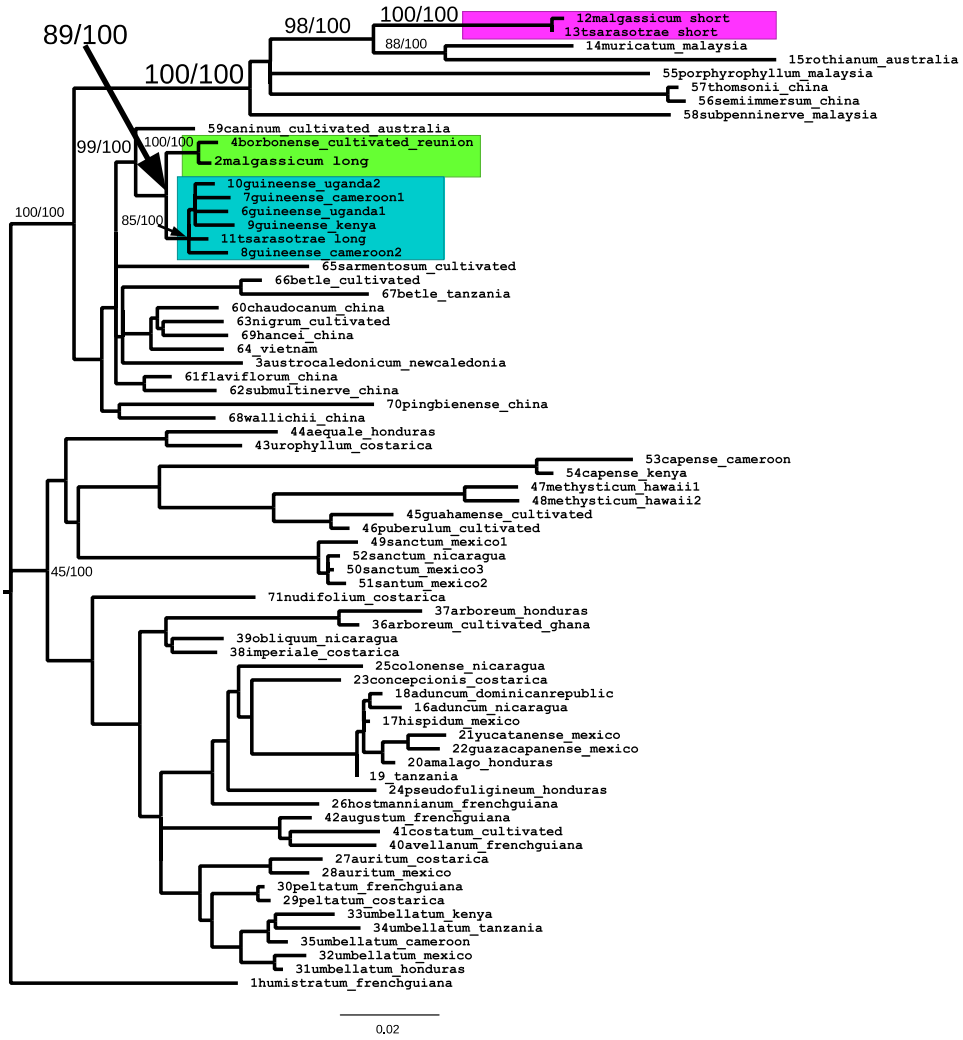

Figure 1. Maximum likelihood tree produced by RAXML with nuclear sequences. The supports above or below the branches are, respectively, the bootstrap resampling support with maximum likelihood criterion produced by RAXML, and the bayesian support calculated including the information derived from indels. In case the bayesian support is lower than 50, it is not indicated on the figure.

The evolutionary model implemented in Mrbayes for treating gaps was the same as that proposed by Lewis et al. [28] for treating morphological data, the Mk model.

We used MrMODELTEST 2.0 [29] to choose the best evolutionary model of DNA sequences on the basis of the Akaike information criterion [30]. The best model was used as settings with MrBayes 3.2.7 [31] for Bayesian Inference. A maximum likelihood (ML) phylogenetic analysis was carried out with RaxML [32] and the resulting trees were edited with Figtree [33]. We mapped the support on the tree branches with the results of the Bayesian phylogenetic analysis after removing the first trees with low likelihood values as "burn-in", as in Papini et al. [34], [35]. The remaining trees were used to produce a 50% majority-rule consensus tree in which the percentage indicated on branches was used as a measure of the Bayesian posterior probability.

2.2. Karyological analysis

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Chromosomes images were obtained from somatic mitoses recorded from root tips of only one plant living in a pot. The procedure was the same as in Mosti et al. [36] and Mousavi et al. [37], with a pretreatment in 8-hydroxichinoline and fixation in Carnoy. Then the material was hydrolyzed in Hcl and then stained with Lacto-propionic-orceine.

We observed metaphase plates of meristematic cells, with the technique of fresh squashes of root tips. Chromosome counts were made during direct observations with the microscope, and later recounted on enlarged digital images. Images were recorded with a microscope Leica DM RB Fluo.

3. Results

Amplification of the plastid fragments ndhF and trnL intron was carried on and the amplicons correctly sequenced producing reads of 1860 bp and 920 bp, respectively. Cloning of the amplicon of the nuclear gene G3pdhG3PDH of *P. malgassicum* and *P. tsarasotrae* allowed to isolate two haplotypes, which were named after their size of 1058 bp "long" and 965 bp "short", even if the difference in length was little (93bp). We used a total of 71 sequences, both for the G3PDH and the plastid sequences matrix. The total alignment of the G3PDH region was 1127 nucleotides long including gaps. The final parts of the sequences were very variable and hence the alignment was ambiguous. For this reason, we excluded the characters from position 957 to 1127. The rest of the alignment was used for indels (gap) coding (with the software gapcoder), resulting in further 99 characters that were inserted after the nucleotide sequences. The plastid genes ndHF and trnL were inserted one after the other in the sequence, producing an aligned matrix of 2016 characters. The coding of indels resulted in further 115 characters.

RAxML applied on the nuclear G3PDH matrix (indels coding excluded) produced a maximum likelihood tree with bootstrap support obtained with 1000 replicates (Fig. 1). The support on branches corresponds to maximum likelihood bootstrap support (left) and Bayesian support with gaps (on the right).

The same method was using for the plastid matrix (Fig. 2).

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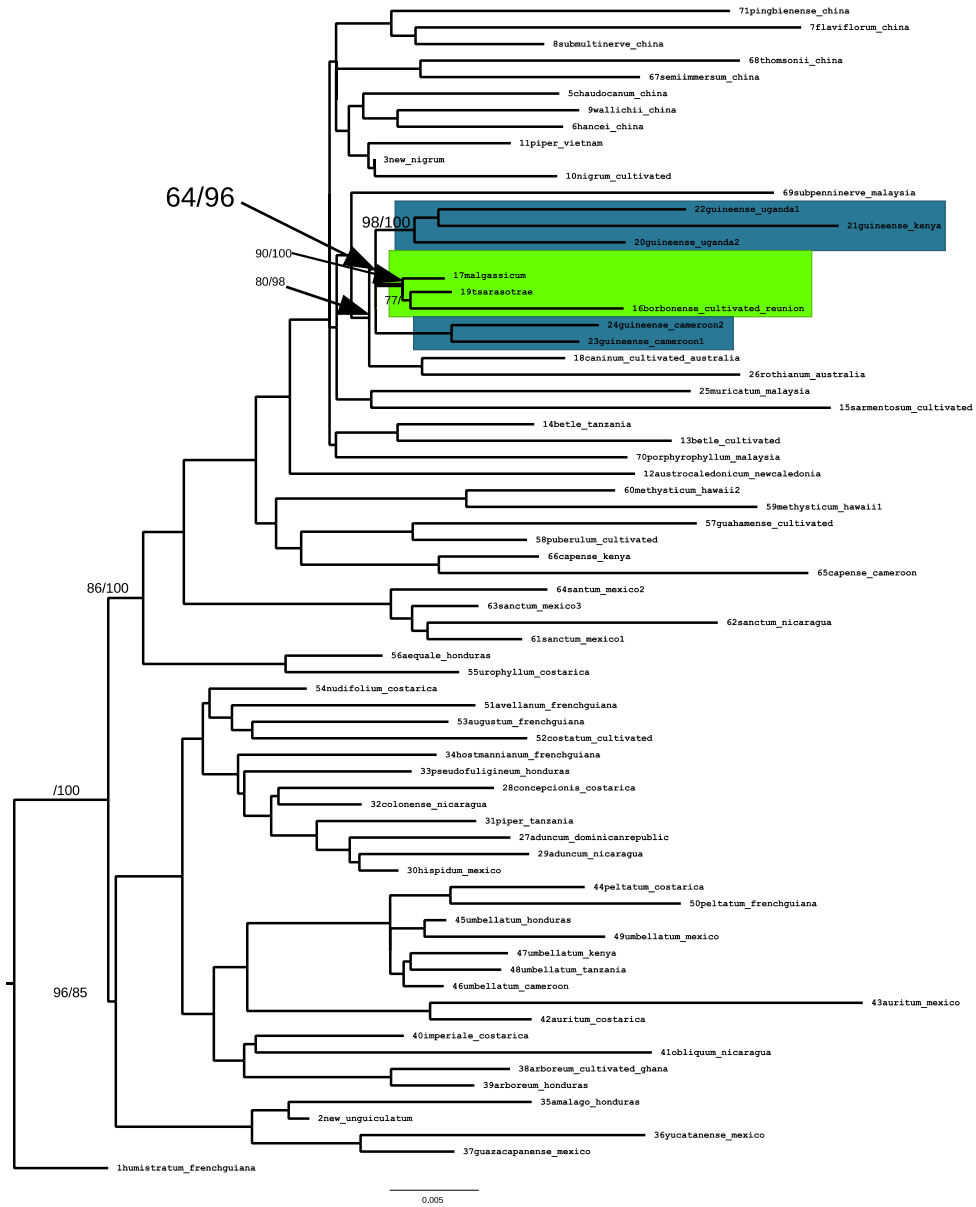

Figure 2. Maximum likelihood tree produced by RAXML with chloroplast sequences. The support indexes indicated on the tree are the same as in Fig. 1 (maximum likelihood bootstrap and bayesian support).

Comparing the two maximum likelihood trees, the one based on nuclear DNA data (G3PDH sequences) and that obtained with plastid markers, we could observe that in the first case the two entries corresponding to two different populations of *P. malgassicum*, clustered together and as sister group of *P. borbonense* (Fig. 1), another species from the La Reunion Island, which lies relatively close to Madagascar. This relationship is corroborated by 100% maximum likelihood bootstrap (MLS) and bayesian (BS) support. The other Malagasy species, *P. tsarasotrae*, typical of arid forest, was more strictly related to the entries of the African species *P. guineense*, with

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100% MLS and 100% BS. All these species formed a well characterized clade with 89% MLS and 100% BS and their closest species appeared to be Asian species *P. caninum*, (Fig. 1). The BS without considering gaps coding gave the same support in this clade (Fig 1 suppl. mat.).

The "short" sequences of G3PDH of both *P. tsarasotrae* and *P. malgassicum* clustered together within a group of Asian species, mainly originating from Malaysia and Australia with 98% MLS and 100% BS (Fig. 1).

The (phylogenetic) story told by the data obtained from chloroplast genome sequences was quite different: the Malagasian species *P. tsarasotrae* and *P. malgassicum* clustered together with the phytogeopraphically close *P. borbonense* with 90% MLS and 100% BS, while the 5 accessions of the African *P. guineense* were in a more external condition with respect to the former group and separated in two groups, one from Cameroon (NW Africa) and one from Uganda/Kenya (Central-East Africa). All these species together formed a monophyletic group with 64% MLS and 96% BS (95% bayesian support in the analysis without gaps). Also in this case *P. caninum*, together with *P. rothianum*, was the outgroup to the African + Malagasy species (Fig. 2) with 80% MLS and 98% BS (99% without gaps).

The counted chromosome numbers varied from 2n=46±2 in *P. malgassicum* (Fig. 3A) to 2n=36± 2 in *P. tsarasotrae* (Fig. 3B). The uncertainty in the counts, that should be taken only as preliminary result, derived from the small dimension of the chromosomes (many of them less than 1 μm of length), the low amount of metaphases in the root tips of the plants cultivated in Florence and the apparently small dimension of the mitotic spindle, leading to partial overlapping of many of the small chromosomes.

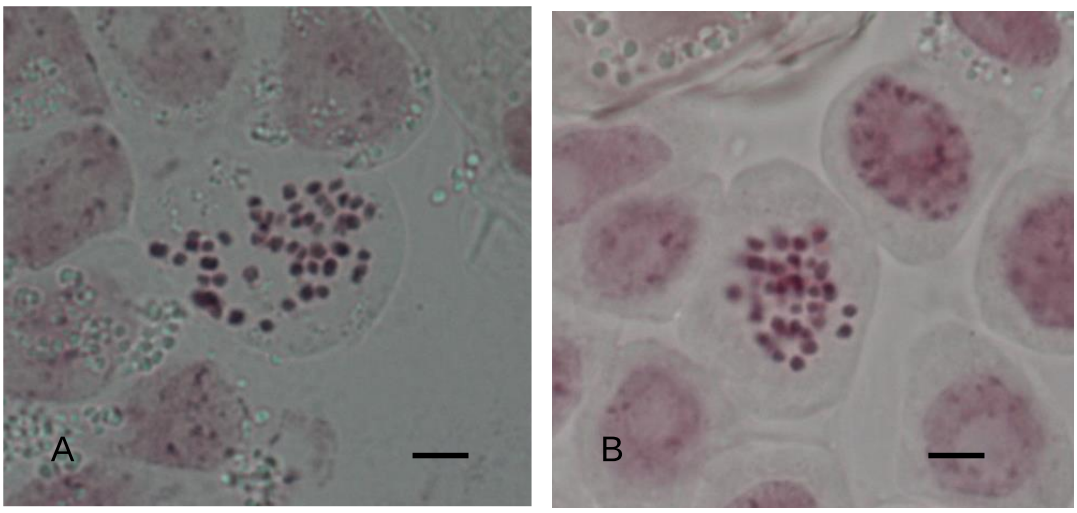

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Figure 3. Chromosomes. A) P. malgassicum number of chromosomes: about 2n = about 46. Bar = 5 µm; B) P. tsarasotrae: 2n = about 38. Bar = 5 µm.

4. Discussion

The fact that the phylogenetic history based on the chloroplast markers told a different tale with respect to the tree produced with nuclear markers may be explained with a possible ancient hybridization/introgression event with pollen coming from an ancestor of the African *P. guineense* and reaching the ancestor of *P. tsarasotrae*, that would hence share some part of the nuclear genome with the African species. The only species of Piperaceae analyzed under the point of view of the type of plastid inheritance was a species of Peperomia, resulted only with maternal plastidial inheritance [38]. The presence of the short G3PDH sequences among the nuclear DNA sequences may be related to a still more ancient hybridization event involving the ancestor of the Malagasy species and some ancestor of Asian origin, also in this case probably with Asian pollen entering in contact with the ancestor´s stigma of the Malagasian species. As a matter of fact the closest relatives to the African species *sensu lato* (including the Malagasy and the Reunion species) are Asian, with the closest species (among those here sampled) apparently from Malaysia (see Figg. 1 and 2).

The preliminary results about the chromosome numbers scored about 2n=46+-2 in *P. malgassicum* and 2n=36+-2 in *P. tsarasotrae*. The uncertainty in the counts was due to the small dimension of the chromosomes that were observed in most of the species of the genus, together with stickiness [39], the low amount of metaphases in the root tips of the plants cultivated in vitro and the apparently small dimension of the mitotic fuse, leading to partial overlapping of many of the small chromosomes. The mitotic spindle can reach dimensions up to 60 µm [40]; [41], while in *P. malgassicum* and *P. tsarasotrae* it was about 15-20 µm (see Fig. 3).

Apparently interspecific hybrids can be obtained in genus Piper also experimentally [42], while the hybrid origin of several Andean species was already proposed by Quijano-Abril et al. [1].

The chromosome numbers in genus Piper are very variable, ranging from 2n=26 to 2n=104, with some species apparently able to possess several possible chromosome numbers [39]. Most new world species show a karyotype of 2n=26, while in Asia tetraploids 2n=52 would prevail [39], while no data was available for African and Malagasy species up to the here presented results. However, the clear difference in karyotype between *P. tsarasotrae* and *P. malgassicum*, two species otherwise strictly related may confirm a possible hybridization/introgression event with a species with a different chromosome number with respect to the ancestor of the Malagasian species.

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5. Conclusions

The surprising discrepancy between the nuclear and the plastid phylogeny could be explained with an ancestral introgression event due probably to pollen contribution from an ancestor of the African mainland *P. guineense* towards the ancestor of *P. tsarasotrae*. The presence of paralogs of the nuclear gene G3PDH, clustering together with more distantly related Asian species lead to the hypothesis that a second more ancient hybridation/introgression event would have occurred between south Asian species and the ancestor of the Malagasian species. The chromosome numbers observed in the Malagasian species would confirm different evolutionary history.

Further studies about the karyotypes of the Malagasy species, *P. guineense* and *P. borbonense* will be necessary together with the investigation of the possible presence of short sequences in *P. borbonense*.

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