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Evidence of transoceanic dispersion of the genus *Vanilla* based on plastid DNA phylogenetic analysis

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ABSTRACT

The phylogeny and the biogeographical history of the genus *Vanilla* was investigated using four chloroplastic genes (*psb*B, *psb*C; *psa*B and *rbc*L), on 47 accessions of *Vanilla* chosen from the *ex situ* CIRAD collection maintained in Reunion Island and additional sequences from GenBank. Bayesian methods provided a fairly well supported reconstruction of the phylogeny of the Vanilloideae sub-family and more particularly of the genus *Vanilla*. Three major phylogenetic groups in the genus *Vanilla* were differentiated, which is in disagreement with the actual classification in two sections (Foliosae and Aphyllae) based on morphological traits. Recent Bayesian relaxed molecular clock methods allowed to test the two main hypotheses of the phylogeography of the genus *Vanilla*. Early radiation of the *Vanilla* genus and diversification by vicariance consecutive to the break-up of Gondwana, 95 million years ago (Mya), was incompatible with the admitted age of origin of Angiosperm. Based on the Vanilloideae age recently estimated to 71 million years ago (Mya), we conclude that the genus *Vanilla* would have appeared ~34 Mya in South America, when continents were already separated. Nevertheless, whatever the two extreme scenarios tested, at least three long distance migration events are needed to explain the present distribution of *Vanilla* species in tropical areas. These transoceanic dispersions could have occurred via transoceanic passageway such as the Rio Grande Ridge and the involvement of floating vegetation mats and migratory birds.

1. Introduction

The genus *Vanilla* Plumier ex Miller is one of the most familiar of all orchids due notably to *V. planifolia* G. Jackson which is the principal source of the famous natural vanilla flavoring. The genus contains as many as 110 different species distributed throughout the New and Old World tropics, but absent from Australia. Most (52) of the species are found in tropical America, 31 species are found in south-east Asia and New Guinea, 14 in Africa, 10 in the Indian Ocean islands, and 3 in the Pacific islands (Bory et al., 2008b; Portères, 1954).

The phylogenetic position of *Vanilla* among Orchidaceae is now clearer thanks mostly to molecular phylogenetic studies based on combinations or individual matrices of plastid *rbc*L (Cameron et al., 1999; Soto Arenas, 2003), *psa*B (Cameron, 2004), or *psb*B and *psb*C (Cameron and Molina, 2006) gene sequences with good sampling of Vanilloideae. Plastid genes present a real interest in phylogenetics due to unilateral inheritance, numerous copies per cell, ease of amplification and sequencing and minimized risk of contamination by fungi (Clegg and Zurawski, 1992; Olmstead and Palmer, 1994; Palmer et al., 1988). Other studies have also used *nad*1b-c mitochondrial gene sequences (Freudenstein et al., 2004) or nuclear 18S gene sequences (Cameron and Chase, 2000), to discriminate the Orchidaceae family. Thus, taxonomists now agree that *Vanilla* belongs to the sub-tribe Vanillinae, in the tribe Vanilleae, which forms with the tribe Pogonieae, a primitive monophyletic orchid lineage which is now recognized as a distinct subfamily Vanilloideae and comprises approximately 14 other genera (Cameron, 2004, 2009; Cameron et al., 1999; Cameron and Molina, 2006; Soto Arenas, 2003).

However, the taxonomy and the phylogeography of the genus *Vanilla* still remain uncertain. Rolfe (1896) was the first author to

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propose a classification of the genus from morphological observations of flowers, leaves and stems. He recognized two sections: Foliosae and Aphyllae, respectively, leafy and leafless. Portères (1954) further divided the Foliosae section into three subsections: Papillosae (thick leaves and labellum with more or less fleshy hairs), Lamellosae (thick leaves and labellum with scaly lamellae) and Membranaceae (thin membraneous to sub-membraneous leaves). More recently, Soto Arenas (2003) invalidated this classification by stressing that subsections are heterogeneous and incomplete. However, as no revision of the taxonomy has been proposed so far, Portères' classification stays the most complete and is still used.

On the basis of the discriminant floral characters, Portères (1954) suggested that the primary diversification center of genus *Vanilla* could be Indo-Malaysian. This Indo-Malaysian stock would have subsequently diversified and evolved in two groups: on one hand in Madagascar, Mascarene islands and Africa, and on the other hand in Oriental Asia and Occidental Pacific islands, with subsequent migration towards America from the Pacific during the Tertiary (65.5–2.5 million years ago (Mya)) (Portères, 1954). Molecular phylogenetic studies invalidated this hypothesis and it is now established that the sub-tribe Vanillinae originated from South America (Cameron, 2000; Cameron and Chase, 1999). Nevertheless, previous molecular studies were based on a limited number of *Vanilla* samples therefore the fine phylogeny of the genus *Vanilla* still remains uncertain.

Following the 1960s general acceptance of the theory of continental drift, vicariance became the predominant hypothesis in historical biogeography (Cuenca et al., 2008). However, an increasing number of phylogenetic studies of tropical angiosperms evolving in both the Old and New World already suggested the spread of taxa either from the Old World to the New World for Meliaceae (Muellner et al., 2005), Lauraceae (Von Balthazar et al., 2007) and Annonaceae (Doyle and Thomas, 1997), or in the opposite direction for Melastomataceae (Morley and Dick, 2003) and Malpighiaceae (Davis et al., 2001) by transoceanic passageway.

Those two main hypotheses are relevant with regards to the dispersion history of the genus *Vanilla*. The first proposed a migration of the genus before the break-up of Gondwana from South America to Africa (~160–120 Mya), then, from Africa to Asia (Cameron, 2005). However, the recent discovery in the Dominican Republic of well preserved orchid pollinarium (of *Meliorchis caribea*) attached to an extinct stingless bee (*Problebeia dominica*), recovered in Miocene amber has allowed to estimate that the common ancestor of present day orchids lived in the late Cretaceous (76–84 Mya), at least 20 Mya after the break-up of Gondwana (Ramirez et al., 2007). This finding is therefore inconsistent with the hypothesis of transcontinental dispersion. Thus, transoceanic migration would seem to better explain the present dispersion pattern of the genus *Vanilla*.

Here, we present a generic-level phylogeny of vanilloids including an extended set of *Vanilla* species using four chloroplastic genes: *psb*B, *psb*C; *psa*B and *rbc*L, coding, respectively, for subunits of proteins P680 (i.e. photosystem II), P700 (i.e. photosystem I) and Rubisco (i.e. Calvin cycle). We used this phylogeny to address two objectives. Firstly, the obtained phylogenetic data are compared with previous hypotheses generated with morphological (Portères, 1954) and molecular data (Cameron, 2004; Cameron et al., 1999; Cameron and Molina, 2006; Soto Arenas, 2003) in order to clarify the phylogeny of the genus. Secondly, relative divergence times of the two main phylogeographical hypotheses are estimated using recent Bayesian relaxed molecular clock methods, in order to obtain a better understanding of the temporal pattern of diversification of the genus between the New World (Neotropical America) and the Old World (Africa and Asia).

2. Materials and methods

2.1. Plant material

Forty-seven accessions of *Vanilla*, representing 23 specimen identified on the basis of floral characters, three hybrids and 21 unidentified species, were chosen from the *ex situ* collection maintained at the CRB VATEL (CIRAD) in Reunion Island (Grisoni et al., 2007), to represent maximum variability within the genus on the basis of previous AFLP and microsatellite characterization (Bory et al., 2008a,c). Twenty-eight accessions originate from America, 18 from Africa or Asia and one is of unknown origin. Thirty-eight accessions belong to the Foliosae section, of which sixteen are from of the Lamellosae subsection, one from the Membranaceae subsection and two from the Papillosae subsection. The remaining nine *Vanilla* accessions belong to the Aphyllae section (Portères, 1954) (Table 1).

2.2. PCR and sequencing reactions

DNA from leaf or stem material was extracted as described previously (Bory et al., 2008c). No further purification of the DNA before PCR amplification was necessary. Two primer pairs were used to amplify each of four chloroplastic genes, *psa*B, *psb*B, *psb*C and *rbc*L, in two overlapping DNA fragments, each with a length inferior to 900 bp in order to permit sequencing reactions (Table 2). Primers were designed using primer 3 software (Rozen and Skaletsky, 2000) based on the sequences of four chloroplastic genes, *psa*B, *psb*B, *psb*C and *rbc*L from seven *Vanilla* species (Table 3) deposited in GenBank (Cameron, 2004; Cameron et al., 1999; Cameron and Molina, 2006). PCR reactions were conducted on a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 μL containing 50 ng DNA, 200 μM dNTPs (New England Biolabs, Ipswich, MA, USA), 1.5 mM $MgCl_2$ (Eurogentec, Liège, Belgium), 1× PCR buffer, 0.2 μM of each primer and 2 U *Taq* DAP GoldStar™ (Eurogentec, Liège, Belgium). Cycling parameters were 94 °C for 4 min, then 35 cycles of 95 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min and a final elongation at 72 °C for 7 min. The integrity of target loci was checked using electrophoresis on a 1.5% agarose gel. Then, PCR products were purified and sequenced reverse and forward using amplification primers, by Macrogen Inc. (South Korea).

2.3. Sequence analysis

Sequences were checked and cleaned by editing reverse and forward electrophoregrams using BioEdit Sequence Alignment Editor v.5.0.6 (Hall, 1999) and MEGA 3.1 (Kumar et al., 2004) software. They have been deposited in GenBank under the accession numbers FN545387 to FN545433 (*psa*B), FN545434 to FN545479 (*psb*B), FN545480 to FN545527 (*psb*C) and FN545528 to FN545574 (*rbc*L). After alignment with ClustalW (Thompson et al., 1994) implemented in Bioedit, the Reading Frame (RF) was determined and a preliminary analysis of the polymorphism of the DNA matrices was carried out using DNaSP 4.0 software (Rozas et al., 2003). The four independent gene matrices showed comparable features, notably concerning the level of polymorphism and the number of haplotypes differentiated (data not shown) suggesting they were submitted to similar evolution forces.

The DNA sequences previously published (Cameron, 2004; Cameron and Chase, 1999; Cameron and Molina, 2006) for *psa*B, *psb*B, *psb*C and *rbc*L genes for six *Vanilla* species, 17 others Vanilloideae (eight other Vanilleae and nine Pogoniae) and one superior Epidendroïdeae have been added to the analysis to complete the sequence dataset. To root the phylogenetic trees *psa*B, *psb*B, *psb*C