



Phylogeny, biogeography and divergence times in *Passiflora* (Passifloraceae)

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Abstract

As part of a long-term investigation on the evolution of *Passiflora* L., we investigated the divergence ages of the genus and diversification of its subgenera, relating them with biogeographical and/or historical events, and other characteristics of this taxon. The main aim of the present work was to evaluate the biogeographic distribution of this genus to better understand its evolutionary history. This is the first time that representatives from South American and Old World Passifloraceae genera have been studied as a group comprising a total of 106 widely distributed species, with representative samples of the four suggested subgenera. Seven DNA regions were studied, comprising 7,431 nucleotides from plastidial, mitochondrial and nuclear genomes. Divergence time estimates were obtained by using a Bayesian Markov Chain Monte Carlo method and a random local clock model for each partition. Three major subgenera have been shown to be monophyletic and here we are proposing to include another subgenus in the *Passiflora* infrageneric classification. In general, divergence among the four subgenera in *Passiflora* is very ancient, ranging from ~32 to ~38 Mya, and Passifloraceae seems to follow a biogeographic scenario proposed for several plant groups, originating in Africa, crossing to Europe/Asia and arriving in the New World by way of land bridges. Our results indicated that *Passiflora* ancestors arrived in Central America and diversified quickly from there, with many long distance dispersion events.

Keywords: biogeography, molecular phylogenetics analysis, passionflowers, plant evolution, taxonomic classification.

Introduction

Passiflora L. is the largest genus of the Passifloraceae family, and encompasses more than 500 wild species distributed especially in the Neotropical region (Ulmer and MacDougal, 2004). The majority of these are herbaceous, but there are also shrubs and trees among them. Killip (1938) and MacDougal (1994) asserted that among the Angiosperms no other group presents such a high foliar diversity, and its flowers display ample variation in size and color, with the corona and perianth showing diverse orientation and development. Coevolution with insect pollinators has been suggested as an explanation for these features (MacDougal, 1994). Based on morphology only (especially flower structures) Feuillet and MacDougal (2004) proposed a drastic taxonomic reevaluation of the genus that, according to them, would consist of only four subgenera (*Passiflora*, *Decaloba*, *Astropheia* and

Deidamioides), against the 22 or 23 formerly proposed (Killip, 1938; Escobar, 1989).

The first molecular phylogeny of *Passiflora*, published by Muschner *et al.* (2003), included more than 60 species of *Passiflora* studied for plastidial and nuclear genome markers. They found three clearly defined major clades while the fourth one remained undefined due to the small number of species classified in it. They also found that the monospecific *Tetrastyllis* was part of *Passiflora*. The morphological propositions of Feuillet and MacDougal (2004) are mostly in agreement with our molecular phylogeny. Although some attempts to elucidate the phylogeny of the genus did not agree with our molecular results and the proposition by Feuillet and MacDougal (2004) with respect to the number of subgenera and their composition (*e.g.* Yockteng and Nadot, 2004a; Plotze *et al.*, 2005), a study by Hansen *et al.* (2006) with other molecular sequences from other species in *Passiflora* recovered the four subgenera as monophyletic groups.

Inferences regarding the biogeographic history of tropical angiosperms based on morphology were frequently

very poor, given the difficulty of formulating detailed phylogenetic hypotheses and obtaining adequate estimates of divergence times. For example, biogeographical analyses of the tropical flora attribute transtropical disjunctions at high taxonomic levels to the Gondwana breakup (Raven and Axelrod, 1974; Gentry, 1982, 1993; Barlow, 1990; Burnham and Graham, 1999). This interpretation, however, implies divergence times of 100–90 million years ago (Mya) between the African and Neotropical clades, and even higher values for taxa also found in Southeast Asia. In the absence of an adequate fossil record for key areas like South America (Burnham and Graham, 1999), the controversy between Gondwana breakup explanations and those which rely in more recent long-distance dispersion events for the interpretation of present distribution patterns remains unsettled.

Sequences of plastid, mitochondrial and nuclear DNA have been extensively utilized to study plant (especially Angiosperm) phylogenies (*e.g.* Qiu *et al.*, 1999; Kuzoff and Gasser, 2000; Soltis *et al.*, 2002; APG III 2009). The strategy of combining multiple genes with different functions from the three plant genomes should reduce the phylogenetic noise generated by gene function and/or genome specific phenomena, such as heterogeneity of rates of change, GC-content bias, RNA editing and protein structural constraints (Qiu *et al.*, 1999). Rokas *et al.* (2003) showed that as the number of genes increases in a phylogenetic analysis, the better tree reflects the species' phylogeny. The same type of relationship was examined by Rokas and Carroll (2005), who concluded that for phylogenetic precision the number of genes considered is a more important determinant than the number of taxa examined. However, branch representativeness should also be taken into consideration, and when a large number of taxa is being studied, the ideal number of markers should be decided in cost-benefit terms.

The aims of the present work were: (a) to re-examine the *Passiflora* phylogeny combining markers from the three genomes, thus contributing to taxonomic classification, (b) to test monophyly of the genus and its subgenera, (c) to investigate the divergence time between the main clades, and (d) to evaluate the biogeographic distribution, aiming to better understand its evolutionary history. This is the first time that representatives from South American and Old World Passifloraceae genera are included as an outgroup.

Materials and Methods

Taxon sampling

We investigated a total of 106 species distributed in the four subgenera of Feuillet and MacDougal (2004) and representatives from seven other genera of Passifloraceae

(*Adenia isoalensis*, *Adenia keramanthus*, *Ancystrothyrus sp.*, *Barteria sp.*, *Deidamia sp.*, *Dilkea johannesii*, *Mitostemma brevifilis*, *Paropsia brazzeana* and *Paropsia madagascariensis*), one Malesherbiaceae (*Malesherbia linearifolia*) and one Turneraceae (*Turnera subulata*). These species were utilized as outgroups, all being included in Passifloraceae by the Angiosperm Phylogeny Group APG (2003, 2009). More information about DNA sources and GenBank numbers is provided in Table S1 in Supplementary Material.

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh leaves dried in silica gel or obtained from herbarium material, using the method of Roy *et al.* (1992). Eight DNA regions were sequenced: the *rbcL* and *rps4* genes, *trnL* intron and *trnL-trnF* intergenic spacers from the plastid genome, *nad1* b/c and *nad5* d/e introns from the mitochondrial genome and a partial portion of the *26S* gene from the nuclear ribosomal genome. These regions were amplified with primers 1F and 1460R (Savolainen *et al.*, 2000), *rps45* and *rps43* (Souza-Chies *et al.*, 1997), c, d, e and f (Taberlet *et al.*, 1991), *nad1/2* and *nad1/3* (Duminil *et al.*, 2002), *mt3* and *mt6* (Souza *et al.*, 1991), N-nc26S1 and 1229r (Kuzoff *et al.*, 1998). Sequencing primers were used as listed by these authors except for the *nad1* b/c intron, for which we constructed an internal primer specific for *Passiflora* (5'-ATTCACATAGAGACAGACT).

PCR products were purified using the polyethylene glycol/NaCl precipitation method of Dunn and Blattner (1987). Sequencing was performed on a MegaBace 1000 (GE Health Care) automatic sequencer using the DYEnamic™ ET termination cycle sequencing premix kit (GE Health Care) following the manufacturer's protocol. The sequences were deposited in Genbank (Accession numbers are given in Table S1). The sequences were aligned using Clustal W (Thompson *et al.*, 1994) implemented on Mega5 (Tamura *et al.*, 2011). All alignments were manually adjusted. Regions of ambiguous alignment were excluded from the analyses.

Phylogenetic analyses

The phylogenetic analyses were performed for the seven genetic markers with a Bayesian approach using BEAST 1.7.1. Less than 20% of the alignment corresponded to missing data. Two independent runs of 3 x 10⁷ chains were performed, each with sampling at every 3,000 generations. The parameters used were as follows: a single HKY substitution model with eight gamma categories, a Yule tree prior and a random local clock model for each partition, which is highly recommended in *Passiflora*, since different subgenera present different DNA content (Yotoko *et al.*, 2011). Three calibration points were used. A

fossil seed with age 37 Mya assigned to the genus *Passiflora* (Mai, 1967), also used by Hearn (2006) in an *Adenia* (Passifloraceae) divergence time investigation, was used for an exponential prior for the *Passiflora* clade with a mean of 15 Mya and an offset of 37 Mya. A normal prior with a mean of 48 Mya and standard deviation of 10 Mya was used for the Passifloraceae/Turneraceae divergence and for the root of the phylogeny we used a uniform prior of between 70 and 110 Mya (based on Bell *et al.*, 2010). The software Tracer v1.5 was used to check for convergence after the first 10% of generations had been discounted as burn-in. Maximum-clade-credibility trees were estimated using the program TreeAnnotator, which is part of the Beast package. Statistical support for the clades was determined by assessing Bayesian posterior probabilities.

Results and Discussion

The alignment for all loci totaled 7,431 nucleotides. The numbers of variable and parsimony informative sites

Table 1 - Sequences characterization, alignment size, variable and parsimony informative site numbers.

Marker	Total sites	Variable sites	Parsimony informative
<i>rbcL</i>	1345	354	218
<i>rps4</i>	615	231	146
<i>trnL-trnF</i>	411	158	78
<i>trnL</i> intron	681	204	111
<i>nad1</i>	1704	323	120
<i>nad5</i>	1550	210	75
<i>26S</i>	1125	228	122
All	7431	1708	870

for each marker are shown in Table 1. In Figure 1 we present the phylogenetic relationship and divergence times obtained by the Bayesian approach for the main clades. Diversification ages, geological periods and outstanding events for these clades are shown in Table 2. The full

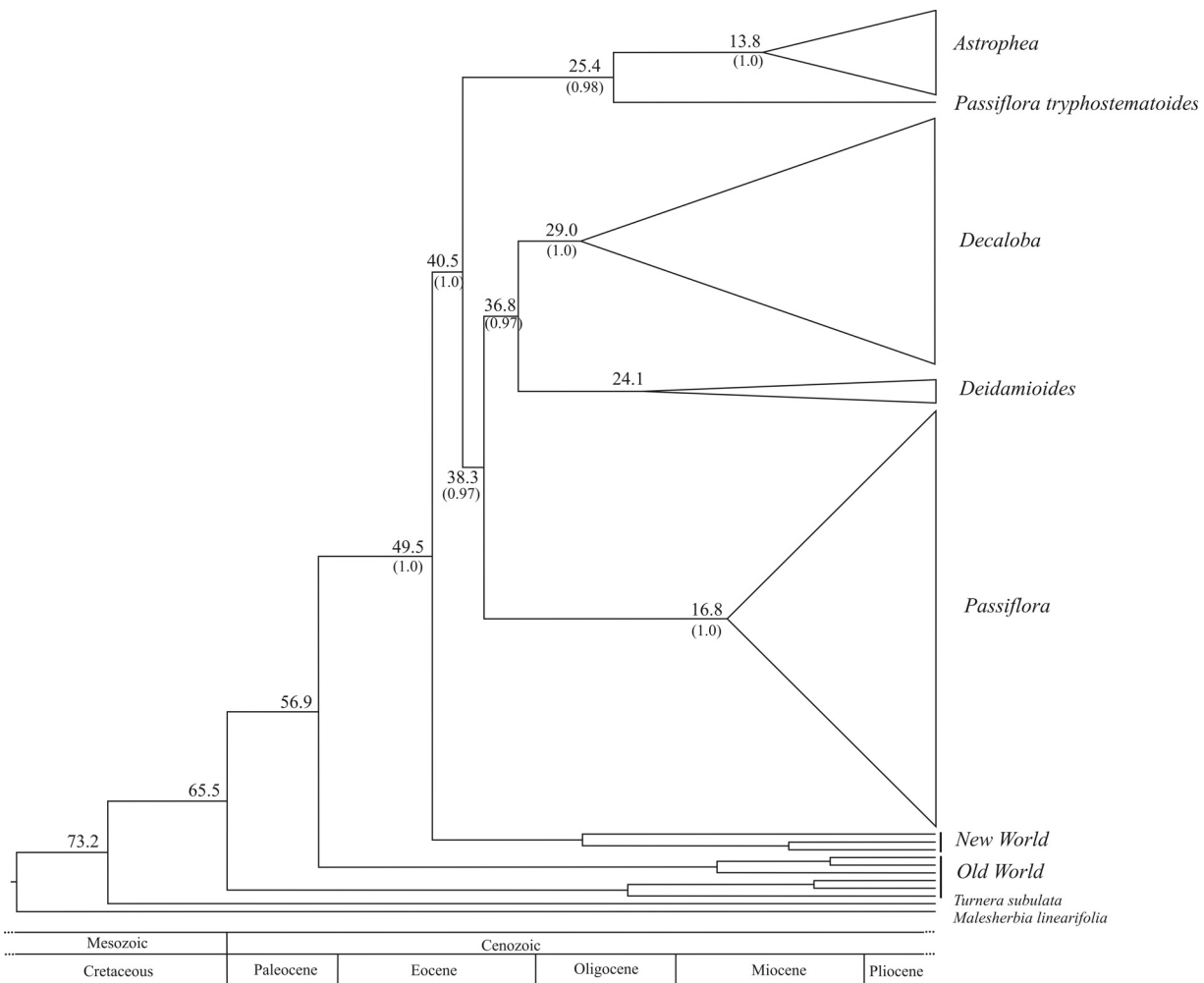


Figure 1 - Phylogenetic relationship and divergence times obtained by the Bayesian approach using seven genetic markers from three plant genomes of *Passiflora* species and related taxa.

non-collapsed branches of the Bayesian tree can be obtained by request from the corresponding author.

In the present analysis, Malesherbiaceae appeared as a sister group of Passifloraceae and Turneraceae, as already proposed by Davis *et al.* (2005) and Krosnick *et al.* (2006), and the divergence time found here (73.2 Mya) is also in agreement with Davis *et al.* (2002). Wikström *et al.* (2001) estimated divergence between Passifloraceae s.s and Turneraceae between 32-36 Mya. We found an older date (65.5 Mya), but note that other authors (*i.e.*, Bremer *et al.*, 2004) also obtained dates older than those reported by Wikström *et al.* (2001) for different groups. A possible explanation for this difference may be that Wikström *et al.* (2001) focused their work on higher taxonomic groups, with a very sparse sample density at lower (below family) taxonomic levels and used a different method to estimate divergence.

Considering that sampling at the molecular level in the present study is similar to other biogeographical analyses (Renner, 2004; Richardson *et al.*, 2004; Bell and Donoghue, 2005; Yuan *et al.*, 2005), the above listed divergence time suggests a post-Gondwanic origin of the Passifloraceae. According to Raven and Axelrod (1974) migration between South America and Africa could have occurred even after the Gondwana breakup at 90-105 Mya. Morley (2003) reviewed the potential world migration routes for the megathermal angiosperms, suggesting that connections between South America and Africa may have existed up to the Oligocene (around 35 Mya). These connections may have been used for stepping stone dispersal across islands of the Rio Grande Rise and the Walvis Ridge, which according to Parrish (1993), were above water southwest of the coast of Africa up until that time, as well as through the Sierra Leone Rise.

Other studies (Wolfe, 1978; Renner *et al.*, 2001; Davis *et al.*, 2002) suggested boreo-tropical migration into southern areas during the Oligocene and Miocene, which

could explain the distribution of plants including Passifloraceae. A possible route to dispersion through Laurasia during the Eocene climatic optimum, which may have supported tropical vegetation, could be the best explanation for many organisms that now have a disjunct distribution in the South American, African, and southeastern Asian tropics (Richardson *et al.*, 2004), such as the Passifloraceae. In this hypothesis, the North Atlantic region was at a thermal maximum between the Eocene/Oligocene (see Wolfe, 1978) and the North Hemisphere was at its warmest period during the Paleocene/Eocene (according to Davies *et al.*, 2004). The land bridges could thus have been warm enough to support plants like Passifloraceae. Given the estimated age of the family, this is a viable route for its migration. As global temperatures dropped during the Oligocene, species might have become extinct in colder regions and expanded their ranges into the warmer south.

Molecular phylogenetic studies have also demonstrated that the role of long-distance dispersals to explain modern distribution patterns may have been underestimated (Renner *et al.*, 2001; Renner, 2004; Yuan *et al.*, 2005). Especially in Passifloraceae, the time frame postulated in the land bridge hypothesis is more plausible than a Gondwana hypothesis because the former allows a larger time window for family evolution and expansion, which would be more favorable for multiple radiations and migrations from South America to Africa and Australia.

Another explanation to the family distribution range is offered by the climate changes that occurred during the late Cretaceous, when the opening of the Tethys Seaway caused a global warming between five and eight degrees (Fluteau, 2003), that would have allowed tropical plants to expand northward. During the following climate cooling, extinctions occurred and many species were restricted to warmer regions in Asia, Africa and the Neotropics. This is congruent with both the Boreo-Tropical and the land bridges hypothesis.

Table 2 - Divergence times, geological periods and outstanding events for the clades presented in Figure 1.

Group	Age	Period	Outstanding events
Malesherbiaceae (Passifloraceae + Turneraceae)	73.2	Cretaceous	Migration from Old to New World through land bridges
Passifloraceae	65.5	Paleocene	
Old and New World genera	49.5	Eocene (E)	Land bridges linking North-Central-South Americas
<i>Passiflora</i> genus	40.5	Eocene (M)	Andes uplifting first stage
(<i>Astrophea</i> + <i>Tryphostematooides</i>) (<i>Passiflora</i> + <i>Decaloba</i> + <i>Deidamioides</i>)	38.3	Eocene (L)	Andes uplifting second stage
(<i>Decaloba</i> + <i>Deidamioides</i>) (<i>Passiflora</i>)	36.8	Eocene (L)	Andes uplifting completed
<i>Decaloba</i> diversification	29.0	Oligocene (L)	Paleo-Orinoco fluvial system
<i>Deidamioides</i> diversification	24.1	Oligocene (L)	
<i>Astrophea</i> diversification	13.8	Miocene (M)	Lake Pebas environment; internal migration/diversification
<i>Passiflora</i> diversification	16.8	Miocene (M)	Lake Pebas environment; internal migration/diversification

The genus *Passiflora* was monophyletic with high support (PP = 1) in this analysis. Three subgenera were equally well supported, but *Passiflora* subg. *Deidamioides* as described by Feuillet and MacDougal (2004) emerged as paraphyletic because *P. tryphostemmatoides* appeared with high support as sister to the *Passiflora* subg. *Astrophea*. *Passiflora tryphostemmatoides* is the type species of a session in the subgenus *Deidamioides* (Feuillet and MacDougal, 2004), but presents unique morphological traits in that group, such that Killip (1938) and Escobar (1989) considered it as the type species of a new subgenus. Although the positioning of *P. tryphostemmatoides* in a separate group was also obtained by Yockteng and Nadot (2004b), our results must be considered with caution because we included only one species of Session Tryphostemmatoides. Yockteng and Nadot (2004b) proposed a different infrageneric classification to *Passiflora*, including eight subgenera. Our results did not support the three extra subgenera (in addition to the four subgenera above plus a clade with *P. tryphostemmatoides*). We therefore suggest a review of the infrageneric classification, including the well supported *Tryphostemmatoides* as a new subgenus.

In general, divergence among the four subgenera in *Passiflora* is very ancient, ranging from ~33 to ~38 Mya (Table 1 and Figure 1). The first divergence event in the *Passiflora* occurred as a split up between the clade *Tryphostemmatoides*+*Astrophea* and the clade *Passiflora*+*Decaloba*+*Deidamioides* (38.3 Mya). In the latter, the two major subclades (*Deidamioides*+*Decaloba* and *Passiflora*) split 36.8 Mya. The subgenera *Deidamioides* and *Decaloba* diverged around 33.5 Mya and *Tryphostemmatoides* and *Astrophea* 25.4 Mya.

The very ancient (~40 Mya) separation of *Astrophea* from the clade *Passiflora*+*Decaloba*+*Deidamioides* could help to explain why the former encompasses species that present the most unusual morphological traits within *Passiflora*, some do not even look much like passionflowers. In *Astrophea* there are species that present tree, shrub or woody vine habits. Similarly, the older divergence of the *Passiflora* subgenus in relation to *Decaloba*+*Deidamioides* is intriguing since many authors have suggested that within the genus the former present ancestral traits in relation to morphology (see Ulmer and MacDougal, 2004, for a review of species description and characteristics) and genetics (Melo and Guerra, 2003 for cytogenetics; Muschner *et al.*, 2006 for organelar inheritance; Yotoko *et al.*, 2011 for genome size evolution). However, the diversification age within *Passiflora* (~16.8 Mya) was much more recent than diversification in *Decaloba* (~29 Mya).

Differences in evolutionary rates between taxa are widespread in plants (Muse, 2000), and can be ascribed to factors intrinsic to each genome type (plastidial, mitochondrial and nuclear) and to extrinsic factors like speciation

dynamics, population size and life history (Bousquet *et al.*, 1992; Muse, 2000; Andreasen and Baldwin, 2001; Barraclough and Savolainen, 2001; Smith and Donoghue, 2009). The *Decaloba* and *Deidamioides* subgenera have longer branches (see Fig. S1) than those of the *Passiflora* and *Astrophea* subgenera, indicating a pattern of accelerated molecular evolution. The mechanisms that could lead to high evolutionary rates in the former subgenera are a generation time that is shorter in *Decaloba* and *Deidamioides* than those in the others, and that they comprise most of the self-compatible species described so far in *Passiflora* (Benson *et al.*, 1975; Ulmer and MacDougal, 2004).

This is the first study that considers *Passiflora* diversification times in detail. Just a few species grow in North America, mainly in Mexico, which could be attributed to the presently unfavorable climate for these species that prefer warmer and moister conditions. Passifloraceae in general seems to follow a biogeographic scenario proposed by other authors for several plant groups (see Antonelli *et al.*, 2009 and Antonelli and Sanmartín, 2011, for more details), with an origin in Africa, crossing to Europe/Asia and arrival in the New World by way of land bridges. Our results indicate that *Passiflora* ancestors arrived in Central America and diversified quickly from there. *Passiflora* subgenera divergence times show the gradual colonization of Americas from north to south. Below we present a more detailed account of this biogeographic scenario.

An alternative hypothesis explaining the disjunct distribution of *Passiflora* (see below) could be by Trans-Pacific dispersion, as suggested for other groups (Sanmartín and Ronquist, 2004). The relationship [(South America, New Zealand) Australia] is the most frequently observed in the flora and fauna of the South Hemisphere and is in conflict with the geologically predicted vicariance patterns (Renner *et al.*, 2000; Winkworth *et al.*, 2002). Sanmartín and Ronquist (2004) documented highly asymmetrical, westward long-distance plant dispersal from South America to New Zealand, against the prevailing wind and oceanic currents (Winkworth *et al.*, 2002). Instead of direct jumps, the dispersal could have occurred in a stepping stone manner along the Antarctic coastline (Renner *et al.*, 2000). This hypothesis is supported by the presence of temperate forests in this area until at least the Pliocene (Swenson and Bremer, 1997; Sanmartín and Ronquist, 2004). This dispersal could have been mediated by the west-flowing East Wind Drift, which runs close to the Antarctic coast, or could have followed the West Wind Drift around Antarctica, involving dispersal first to the sub-Antarctic islands (and/or Australia) and from there to New Zealand (Swenson and Bremer, 1997). Large birds could have contributed to this dispersion, as suggested by Winkworth *et al.* (2002) and Ulmer and MacDougal (2004). Such processes have been proposed for species with characteristics very

similar to those of the *Decaloba* subgenus (Renner *et al.*, 2001; Knapp *et al.*, 2005). This type of dispersion could therefore explain the presence in southeast Asia and Australia of species of a monophyletic session of the *Decaloba* subgenus, as found by Krosnick and Freudenstein (2005).

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Internet Resources

Tracer v1.5 software, <http://beast.bio.ed.ac.uk/Tracer> (accessed in October 22, 2012)

Supplementary Material

The following online material is available for this article:

- Table S1 - DNA sources and GenBank numbers of the *Passiflora* species included in the analysis.

This material is available as part of the online article from <http://www.scielo.br/gmb>.

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