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* infragenetic 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*, *Astrophea* 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 forth one remained undefined due to the small number of species classified in it. They also found that the monospecific *Tetrasyllis* 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 transoceanic 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 keramandus*, *Ancistrothrysus* *sp.*, *Barteria* *sp.*, *Deidamia* *sp.*, *Dilkea* *johannesii*, *Mitostemma brevifilis*, *Paropsis brazzeana* and *Paropsis 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’-ATTACATAGAGACAGACT).

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 DYEEnamic™ 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 $3 \times 10^7$ 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 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 alignment for all loci totaled 7,431 nucleotides. The numbers of variable and parsimony informative sites

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

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

![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.](image-url)
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 estimated divergence between Passifloraceae s.s and Turneraceae, suggesting that the non-collapsed branches of the Bayesian tree can be obtained by request from the corresponding author.

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 Gondwanan 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.

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

Muscner et al. 1039
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. *Astroepha*. *Passiflora tryphostemmatoides* is the type species of a ses-}

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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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References


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Internet Resources


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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