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CALIBRATION OF MOLECULAR CLOCKS AND THE BIOGEOGRAPHIC HISTORY OF CRYPTERONIACEAE: A REPLY TO MOYLE

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To test the molecular dating results and biogeographic interpretations reported by Conti et al. (2002), R. G. Moyle reanalyzed our published dataset of 13 *rbc*L sequences representing Melastomataceae and five small taxa: the Southeast Asian Crypteroniaceae (the C clade) and their western Gondwanan sister clade, formed by the South American *Alzatea* and the African *Rhynchocalyx*, Oliniaceae, and Penaeaceae (the AROP clade). Using a single calibration point and nonparametric rate smoothing (NPRS; Sanderson 2003), Moyle (2004) estimated an age of 68 million years ago (mya; ± 10.6 mya) for the split between Crypteroniaceae and the AROP clade, which contrasts with our published age of 116 mya (± 24 mya), obtained with fossil calibration and penalized likelihood (PL; Sanderson 2003), and an age range of 50 to 151 mya, obtained by using three different calibration points and three different dating methods. Moyle (2004) concludes that his estimated age for the origin of the Crypteroniaceae stem lineage is ''not congruent with a strict Gondwanan vicariance hypothesis for the distribution of Crypteroniaceae and nearest relatives'' and that the differences in calibration ''explain most of the differences in results.''

Although Moyle's comment is timely by focusing on one of the most problematic issues in molecular dating analyses—namely, calibration—we would like to highlight some weaknesses in his chosen analytical procedure, along with factual inaccuracies and misrepresentations of our original article. At the same time, we offer some general reflections on the controversial issue of calibration in molecular dating analyses.

The criticism posed by Moyle that is most readily addressed concerns the phylogenetic placement of the South American *Alzatea*. Moyle's (2004) maximum likelihood (ML) analyses supported the placement of the South American *Alzatea* within the African clade, rather than as sister to the African clade as reported in Conti et al. (2002). He proposed that this discrepancy may be explained by the use of different models of nucleotide substitution in the two analyses, adding that low statistical support for the position of *Alzatea* suggests that its relationships remain unresolved. However, we would like to note that Moyle used outgroups (*Hauya*, Onagraceae and *Quisqualis*, Combretaceae) that are phylogenetically more distant from the CAROP/Melastomataceae clade than the outgroup we used (*Heteropyxis*, representing the sister clade of the CAROP/Melastomataceae clade; see Conti et al. 1996). After rerunning ML analyses with different combinations of outgroups and substitution models, we have concluded that instability in the position of *Alzatea* is caused primarily by different outgroup choices.

A second point of disagreement concerns the properties and inferential value of the estimated age ranges. Moyle (2004) states: ''Because of the wide range of age estimates produced by the different calibration points and molecular dating procedures, I re-examined the biogeographic history of Crypteroniaceae with particular attention to calibration procedure.'' He then elaborates on results based on a single dating method (NPRS) and a single calibration point (an age of 23 mya assigned to node E). This methodological approach will tend to provide a narrower range of estimated ages than would be obtained by using a number of different methods, but such a superficially precise result may not be indicative of increased accuracy. Indeed, from a strictly analytical perspective, the choice of NPRS as the single dating method is questionable, since NPRS tends to overfit the data, especially when rates of molecular evolution change abruptly (Sanderson 2002).

A more critical issue, however, is the way in which fossils are used to calibrate trees. Moyle used a single calibration point based on seeds that are dated at 23–26 mya and characterized by the large testa tubercles arranged in rows. These seeds have been assigned confidently to Melastomeae, which were monophyletic in recent analyses (Renner et al. 2001; Renner and Meyer 2001; see also Collinson and Pingen 1992). Yet, most likely due to scarce sampling, the three representatives of Melastomeae (*Tibouchina*, *Osbeckia*, and *Rhexia*) included in our *rbcL* analysis did *not* form a monophyletic group, but were members of a clade that also included *Medinilla* from the Dissochaeteae/Sonerileae (Conti et al. 2002, fig. 3). It was for this reason that we refrained from using this fossil as a calibration point. Moyle (see his fig. 1), in contrast, used the age of 23 mya to constrain node E, which subtends a clade formed by members of Melastomeae *and* Dissochaeteae/Sonerileae. Since node E necessarily predates the origin of the Melastomeae, to which the fossil seeds may be assigned (see also Renner et al. 2001, fig. 1; Renner and Meyer 2001, fig. 3), Moyle's improper calibration procedure automatically produces an underestimation of all nodal ages.

A further source of disagreement between Moyle's and our analyses concerns the nodal assignment of fossil leaves (dated at 53 mya) that are characterized by acrodromous venation, a synapomorphy exclusive for Melastomataceae among the sampled taxa (Renner et al. 2001). Moyle criticizes our decision to use these fossil leaves to constrain the base of the Melastomataceae crown group (corresponding to node E in his fig. 1), instead of the base of its stem lineage (corresponding to node D in his fig. 1). Although we agree with

Moyle that the inclusion of basal lineages (e.g., *Pternandra*) would have been desirable (hence we are including them in ongoing analyses), we disagree with his conclusion that these fossil leaves should be assigned to the base of the Melastomataceae stem lineage. The fossil leaves most closely resemble the leaves of extant Miconieae and Merianieae (Hickey 1977), suggesting that they might be better assigned to shallower nodes within Melastomataceae. This observation, coupled with the fact that acrodromous venation is shared by all Melastomataceae whereas their sister group (Memecylaceae) is characterized by brochidodromous venation, led us to conclude that, in the absence of additional information, the fossil leaves are more reasonably used to provide a minimal age for the crown rather than stem node of Melastomataceae (as done also by Renner et al. 2001; Renner 2004).

Some of the temporal uncertainties inherent to nodal assignment of fossils in a molecular phylogeny stem from the fact that the time of first appearance of distinctive synapomorphies in the fossil record postdates the origin of the group to which the fossils are assigned. Thus, when those fossils are used to provide minimal ages of the subtending stem lineages, and if those minimal ages are interpreted as estimates of actual ages, then one inevitably obtains a systematic underestimation of divergence times. The extent of the temporal gap between time of first appearance in the fossil record and origin of a group depends on several factors, including the fossil's probability of preservation, which in turn is influenced by properties inherent to the fossilized structures; the changing abundance through time of the structures being preserved in the fossil record; taphonomic idiosyncrasies (Morley and Dick 2003); and the geologic characteristics of the stratigraphic layer where the fossil is retrieved (for a review of calibration problems in molecular dating see Magallón 2004; see also Graur and Martin 2004). Ideally, it would be possible to estimate the difference between the observed age of the fossil and the ''real'' age of the group. Recently developed methods that attempt to achieve this goal make use of multiple lines of evidence, including the density and distribution of gaps in the fossil record, the number of extant species, the mean species lifetime, and clade diversification models. To our knowledge, these methods have been applied primarily to mammals (Foote et al. 1999; Tavaré et al. 2002).

In our original paper we also explored the results of assuming a correspondence between certain nodes and well-dated geologic events, specifically equating the phylogenetic split between the South American *Alzatea* and its African sister clade with the formation of the South Atlantic. Moyle criticizes our use of geologic calibration as an example of circular reasoning. It is true that we did use geologic calibration to constrain this node, but always in conjunction with fossil calibrations (Conti et al. 2002), subscribing to the practice of using as many calibration points as possible and then comparing and discussing the results (Sanderson and Doyle 2001; Thorne and Kishino 2002; Yang and Yoder 2003; Graur and Martin 2004; Magallón 2004). Furthermore, given his criticism, it is ironic that Moyle claimed support for his estimated age of the Crypteroniaceae stem lineage by noting its correspondence with the 68 mya age inferred (sic!) by Morley and Dick (2003). However, the latter authors did not estimate that divergence at 68 mya, but used it as a geologic constraint, marking the separation of India from Madagascar, for their dating analyses (see fig. 1 in Morley and Dick 2003).

A further point of contention concerns how age ranges are used to reconstruct possible biogeographic scenarios. For example, Moyle criticizes the choice of using only the older portion (106–141 mya) of the inferred 50–151 mya range for our biogeographic deductions (Conti et al. 2002). However, the 106–141 range corresponded to the ages estimated by a variable-rate method (PL), whereas the younger portion of the age range included the ages estimated by two constant-rate methods (ML with clock enforced and Langley-Fitch; Sanderson 2003). Because the likelihood ratio test had rejected rate constancy, it seemed dubious to use clock-based age ranges for our biogeographic inferences.

Irrespective of the discrepancies produced by different dating and calibration procedures, we would like to highlight some of Moyle's misrepresentations of our biogeographic conclusions. First, Moyle fails to explain the general context of our analyses: we used molecular dating estimates to test competing hypotheses on the Laurasian (Raven and Axelrod 1974) versus Gondwanan (Tobe and Raven 1984) origin of Crypteroniaceae. Our results supported a Gondwanan origin for the family, a conclusion to which Moyle also subscribes. Then Moyle states that our dating estimates are incongruent with a strict Gondwanan vicariance scenario, while conforming to the hypothesis that the Crypteroniaceae stem lineage dispersed from Africa to India as India drifted northward during the Late Cretaceous to Early Tertiary. We were surprised to see that Moyle proposed this conclusion as a novel interpretation, because we had already highlighted India's likely role in expanding the range of Crypteroniaceae from Africa to Asia during its northbound movement along the African coast (Conti et al. 2002; see also Lieberman 2003). While we remain open to the possibility that the use of different calibration points might affect age estimates for the origin of Crypteroniaceae, we also note that Moyle's and our results both confirm a Cretaceous origin for this taxon and India's crucial role in shaping its biogeographic history, as stated in the title of our original paper.

Moyle concludes that the main differences between his and our age estimates stem essentially from differences in calibration, seemingly implying that his calibration procedure is right and ours is wrong. However, the complex issues revolving around nodal assignment of fossils cannot be easily reduced to a Manichaean view of scientific inference that relies on fixed categories of ''right'' and ''wrong''. The only kind of paleobotanical record that would unquestionably support the Gondwanan origin of Crypteroniaceae would be the retrieval of pre-Tertiary fossils attributable to Crypteroniaceae from Africa, Madagascar, or India. Barring that, we believe that biogeographic deductions should be based on multiple lines of evidence, drawn from both phylogenetic patterns and molecular dating, in combination with paleogeologic and paleoclimatic reconstructions and the evaluation of the potential for long-distance dispersal of the propagules. We pursued this integrative, multi-faceted approach in our original and subsequent papers (Rutschmann et al. 2004).

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