Phylogenetic relationships of Amazonian anole lizards (Dactyloa): Taxonomic implications, new insights about phenotypic evolution and the timing of diversification

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A R T I C L E   I N   P R E S S

A B S T R A C T

The ecology and evolution of Caribbean anoles are well described, yet little is known about mainland anole species. Lack of phylogenetic information limits our knowledge about species boundaries, morphological evolution, and the biogeography of anoles in South America. To help fill this gap, we provide an updated molecular phylogeny of the Dactyloa (Dactyloidae), with emphasis on the punctata species group. By sampling understudied Amazonian taxa, we (i) assess the phylogenetic placement of the ‘odd anole’, D. dissimilis; (ii) infer the relationships of the proboscis-bearing D. phyllorhina, testing the hypothesis of independent nasal appendage evolution within the anole radiation; and (iii) examine genetic and dewlap color variation in D. punctata and D. philopunctata. Combining multiple nuclear loci with a review of the fossil record, we also (iv) estimate divergence times within the pleurodont iguanian clade of lizards, including Amazonian representatives of Dactyloa and Norops (Dactyloidae) and of Polychrus (Polychrotidae). We recover the five Dactyloa clades previously referred to as the aequatorialis, heteroderma, latifrons, punctata and roquet species groups, as well as a sixth clade composed of D. dissimilis and the non-Amazonian D. neblinina and D. calimae. We find D. phyllorhina to be nested within the punctata group, suggesting independent evolution of the anole proboscis. We consistently recover D. philopunctata nested within D. punctata, and report limited genetic divergence between distinct dewlap phenotypes. The most recent common ancestor of Dactyloa, Anolis and Norops dates back to the Eocene. Most Amazonian taxa within both Dactyloa and Norops diverged in the Miocene, but some diversification events were as old as the late Eocene and late Oligocene. Amazonian Polychrus diverged in the Pliocene. Our findings have broad implications for anole biogeography, disputing recent suggestions that modern dactyloid genera were present in the Caribbean region during the Cretaceous.

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1. Introduction

Caribbean anole lizards have been central to studies of adaptive radiation and evolutionary ecology for more than half a century (Losos, 2009). However, most of the diversity in the anole family (Dactyloidae) is, in fact, continental (Nicholson et al., 2012). In contrast to the island forms, little is known about the biology and evolution of mainland anoles. Within the species-rich Dactyloa radiation (83 recognized species; Castañeda et al., 2014), unclear phylogenetic affinities limit inferences about species boundaries, morphological evolution, and biogeographic patterns. We help fill this gap by combining morphological and DNA sequence data from four species of previously unsampled or undersampled Amazonian anoles. Based on new information of D. punctata, D. philopunctata, D. phyllorhina, and D. dissimilis, we also provide an updated molecular phylogeny of the Dactyloa.

The Dactyloa radiation is composed of five main clades which define groups of species often referred to as ‘species series’ (Castañeda and de Queiroz, 2013). Most Amazonian Dactyloa anoles are currently assigned to the punctata group, which includes...
Molecular evidence may improve the delimitation of species (Castañeda and de Queiroz, 2013). Dactyloa punctata Daudin, (1802), the ‘spotted’ or ‘green Amazon’ anole (Fig. 1a), is one such example that occurs throughout the Amazon basin, the Andean foothills, the Guiana Shield, and the Atlantic forest of coastal Brazil. 

A previous assessment of population genetic structure in this morphologically conserved lizard was limited to four localities (Glor et al., 2001). To assess the biological and taxonomic implications of genetic variation within D. punctata, we combine observations of dewlap color patterns with new sequence data from a representative portion of the species’ range.

Molecular evidence may improve the delimitation of species within the punctata group, which has often been challenging (Ugueto et al., 2007; Williams, 1982). In this study, we focus particularly on D. philopunctata Rodrigues (1988) (Fig. 1b), a name given to anole populations restricted to central Amazonia that are morphologically distinguishable from D. punctata solely based on the coloration of the dewlap in male specimens. The dewlap of D. punctata presents rows of small whitish scales on an orange background, while that of D. philopunctata has large scattered black spots on a similar dewlap background (Fig. 1). The typical orange dewlap of D. punctata has been described as nearly invariable throughout this species’ distribution (Avila-Pires, 1995; Rodrigues, 1988; Williams, 1982). However, at least one population in southern Brazilian Amazonia presents white dewlaps (Rodrigues et al., 2002). Because there are no records of D. punctata and D. philopunctata occurring in sympathy and females of the two species cannot be distinguished based on their extremely reduced dewlaps, the validity of D. philopunctata has been questioned (Avila-Pires, 1995). Making matters more complex, a recent phylogenetic analysis (Castañeda and de Queiroz, 2013) recovered this species not within the punctata group but closely related to the latifrons group, albeit with low support (Castañeda and de Queiroz, 2013). Using sequence data, we assess the phylogenetic placement of D. philopunctata relative to D. punctata, asking whether dewlap color pattern is a robust diagnostic character in the face of genetic variation within D. punctata.

Limited phylogenetic information also constrains inferences regarding morphological evolution within the anole radiation, as is the case of the poorly understood rostral proboscis observed in D. phyllorhina (Castañeda and de Queiroz, 2013). Recently, D. dissimilis was found in Brazil (Freitas et al., 2013). We found this species to be common in a Southwestern Amazonian site (Melo-Sampaio et al., 2013), where it is associated with clumps of open bamboo forest (Guadua spp.) within dense rainforest. By incorporating D. dissimilis in our analysis, we test for its presumed affinity with the punctata species group.
Finally, to lay the groundwork for forthcoming phylogeographic studies, we combine multi-locus sequence data with a critical review of the available fossil information, providing a new dated phylogeny of the pleurodont iguanian clade that includes representatives of both the *Dactyloa* and *Norops* radiations of anoles, as well as bush anoles (*Polychrus*). By performing improved estimations of divergence times, we provide a temporal framework for Amazonian anole diversification.

Throughout this paper, we follow Nicholson et al.’s (2012) proposal of splitting *Anolis sensu lato*, and recognize *Dactyloa* and *Norops* as genera. We do so for the following reasons. First, we find classification schemes more meaningful when they correlate to, and therefore inform about, aspects of the evolution, biogeography, and morphological variation of a group, and therefore subdivisions may prove instrumental. We also feel that subdivision of Dactyloidae is warranted, as suggested by the widespread use of informal ranks (e.g., ‘species series’, ‘section’) and unranked designations, which sometimes correspond to invalid names (e.g., *Phenacosaurus, Chamaeleolis*) (Castañeda and de Queiroz, 2013; Castañeda et al., 2014; Nicholson, 2002; Poe, 2004). Despite concerns about taxonomic instability resulting from the split of *Anolis* (Poe, 2013), every comprehensive molecular study of the group has recovered the eight clades proposed as genera by Nicholson et al. (2014). We are aware of some of the caveats associated with the taxonomy of Nicholson et al. (2012), as originally proposed (Poe, 2013; Castañeda and de Queiroz, 2013). However, their scheme has been modified to accommodate our yet limited knowledge about the phylogenetic placement of a few problematic taxa (Nicholson et al., 2014).

Our analysis incorporates molecular data generated by a recent phylogenetic assessment of the *Dactyloa* (Castañeda and de Queiroz, 2011), yet we do not reexamine the group’s systematics beyond our target Amazonian taxa. Instead, we refer to the much more extensive work of Castañeda and de Queiroz (2013).

2. Material and methods

2.1. Sampling of molecular data

We generated DNA sequences of six *D. phyllochirina* specimens (three collection sites, Fig. 2), three *D. dissimilis* (one site), two *D. philopunctata* (two sites), 12 *D. punctata* with orange dewlaps (10 Amazonian and two Atlantic Forest sites), one Amazonian *D. punctata* specimen with a white dewlap, one specimen of *D. transversalis*, one of each of four Amazonian species of *Norops* anoles (*N. fuscoauratus, N. ortoni, N. tandai* and *N. trachyderma*), and one individual of the bush anole *Polychrus liogaster* (Polychoridiidae). All samples were collected in Brazil (see online Appendix A for a list of voucher numbers, locality information and GenBank accession numbers). Because the examination of preserved specimens (including the type series of *D. philopunctata*) demonstrated that dewlap patterns remain promptly discernible even after 27 years of fixation (Fig. 3), we only sampled tissues from localities where the dewlap pattern is known, based on direct observation of live or preserved specimens.

For phylogenetic inference within *Dactyloa*, we matched available datasets (Castañeda and de Queiroz, 2011) with new sequences of the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) and the flanking tryptophan transfer RNA (tRNA-Trp) gene, following Jezkova et al. (2009). Yet, sequences obtained in GenBank often included four additional tRNAs flanking the ND2 gene (tRNA-Ala, tRNA-Asx, tRNA-Cys, tRNA-Tyr) (Castañeda and de Queiroz, 2011), which were also used in our final alignments (see Section 2.2). Additionally, we generated sequences of the nuclear recombination-activating gene 1 (RAG1), as per Gartner et al. (2013). For divergence time estimation within pleurodont iguanians, we sampled the nuclear genes *BTB* and *CNC* homology 1 (BACH1), dynein axonemal heavy chain 3 (DNAH3), megakaryoblastic leukemia 1 (MKL1), nerve growth factor beta polypeptide (NGFB) and synuclein alpha interacting protein (SNCAIP), as per Townsend et al. (2008, 2011). Sequences were edited and aligned using Geneious Pro 6 (Biomatters, Auckland).

We used Geneious’ plugin *Find Heterozygotes* with a 0.90 overlap threshold to identify heterozygous positions. For the coalescent-based phylogenetic analyses (see Section 2.2), we ran nuclear sequences through PHASE 2.1.1 (Stephens and Donnelly, 2003) to estimate the haplotypic phase of heterozygotes, after preparation of input files in SeqPHASE (Flot, 2010). We ran PHASE for ten independent times, using a 0.90 probability threshold and a parent-independent mutation model. Models of nucleotide evolution and best-fit partition schemes, including partitions by codon position, were determined with Partition Finder 1.1.1 (Lanfear et al., 2012), implementing PhyML for likelihood estimation (Guindon and Gascuel, 2003) and the Akaike information criterion for model selection (Akaike, 1974). The short length of the five tRNAs markers (~70 bp) prevented Partition Finder from properly estimating substitution parameters for those regions. As a result, we treated all tRNAs (397 base pairs total) as a single partition. A concatenated dataset was generated in Sequence Matrix (Vaidya et al., 2011).

2.2. Inferring phylogenetic relationships

For phylogenetic analyses, we combined our sequences with a subset of the *Dactyloa* molecular dataset generated by Castañeda and de Queiroz (2011). We only included sequences from specimens with traceable voucher numbers. To meet the requirements of the coalescent-based analyses, we only used data for species that had both the mitochondrial and the nuclear fragments sequenced. Also, we did not combine gene fragments of different individuals in chimeric sequences. These criteria ensured that the exact same dataset was used for both coalescent-based and concatenated analyses (see below). Our final dataset was composed of 77 specimens of 33 *Dactyloa* species.

Phylogenetic inference based on multiple loci traditionally involves a concatenation strategy, in which different markers are juxtaposed in a single alignment with the implicit assumption that the different genes have a common genealogy. Yet, gene trees are often discordant and fail to reflect the real relationships among species because stochasticity in the coalescent process may prevent complete fixation and reciprocal monophyly of alleles (Kubatko and Degnan, 2007). By incorporating population parameters such as ancestral population sizes and divergence times, coalescent-based species tree methods account for ancestral polymorphism and incomplete lineage sorting (Edwards, 2009). We implemented a coalescent-based approach using the *BEAST* tool (Heled and Drummond, 2010) of the BEAST 1.8 package (Drummond et al., 2012), applying five independent runs of 100 million generations each and sampling every 10,000 steps. We manually edited the xml files to ensure a single mitochondrial tree while implementing distinct substitution models for ND2 and the tRNA genes, and used phased data for the nuclear RAG1 gene. Because *BEAST* analyses consistently failed to reach stationarity when partitioning protein-coding genes by codon position, suggesting model over-parameterization, we implemented gene partitions only in our coalescent-based analyses. *BEAST* requires a priori assignment of individuals to species; because concatenated analyses and individual gene trees consistently found *D. philopunctata* to be nested within *D. punctata* (see Section 3.1), we treated both as a single species (*D. punctata*) to avoid specifying paraphyletic taxa for coalescent-based phylogenetic inference.
We compared BEAST’s results to trees generated under a concatenated approach, using both maximum likelihood and Bayesian inference. We ran maximum likelihood analyses in Garli 2.0 (Zwickl, 2006), with 100 replicates for tree search and 1000 bootstraps to assess clade support. We summarized bootstraps on the best resulting tree with the SumTrees tool of the DendroPy 3.12 Python package (Sukumaran and Holder, 2010). Additionally, we estimated a Bayesian tree with MrBayes 3.2.1 (Ronquist et al., 2012) through three independent runs and four Markov chains of 20 million generations each, sampling every 1000 steps. In both MrBayes and Garli analyses, we partitioned protein-coding genes by codon position as indicated by Partition Finder.

For the Bayesian analyses (BEAST and MrBayes), we assessed convergence and stationarity of model parameters using Tracer 1.5, combined runs in LogCombiner 1.8 (with a 25% burn-in), and summarized a maximum clade credibility tree in TreeAnnotator 1.8 (Drummond et al., 2012). In all analyses, we unlinked parameters of substitution rates and nucleotide frequencies between partitions. The resulting topologies were visualized in FigTree 1.4 (available from http://tree.bio.ed.ac.uk/software/figtree/).

For descriptive purposes, we also estimated Tamura-Nei corrected pairwise genetic distances (Tamura and Nei, 1993) for the mitochondrial DNA fragment of the newly sampled Dactyloa specimens, using the APE 3.1 package (Paradis et al., 2004) of the R 3.0.2 platform (R Core Team, 2014).

2.3. Divergence time estimation

A recent phylogenetic assessment of the Dactyloidae estimated divergence times for Amazonian anole species (Nicholson et al., 2012), yet with a few caveats. For instance, the phylogenetic
We used three fossils for node calibration: (1) *Cañaheda et al., 2014; de Queiroz et al., 1998; Lazell, 1965*, Moreover, the divergence time estimates presented by Nicholson et al. (2012) were based on a single mitochondrial marker, yet mitochondrial genes are known to mislead deep divergence time estimation due to substitution saturation (Brandley et al., 2011; Lukoschek et al., 2012; Mulcahy et al., 2012), sometimes resulting in 3–10-fold overestimations (Zheng et al., 2011).

To improve estimates of divergence times for Amazonian anoles, we built a dated phylogeny for the pleurodont iguanid clade (sensu Wiens et al., 2012) using five nuclear genes and well-known fossils for calibration points. For that, we combined newly generated sequences of Amazonian lizards in the genera *Dactyloa* and *Norops* (Dactyloidae) and *Polychrus* (Polychorididae) with published sequences of 12 other pleurodont taxa, emphasizing the South American genera (see online Appendix B for taxa, voucher and Genbank accession numbers).

We used three fossils for node calibration: (1) *Saichangurvel davidsoni*, from the late Cretaceous (Conrad and Norell, 2007), calibrated the node defining Pleurodonta, (2) *Suzanniwana* sp. (Cory-tophanidae), from the early Eocene (Smith, 2009, 2011), was placed at the stem of the clade defined by *Corythophanes* and *Basiliscus*, and (3) *Afairiguama avius* (Leiosaureidae), from the early Eocene (Conrad et al., 2007), was placed at the stem of the clade defined by *Leiosaurus* and *Urostrophus*. In the latter case, we conservatively treated *A. avius* as a stem leiosaurid because Conrad et al. (2007) found the relationships between a *A. avius* and extant leiosaurids to be unresolved. Following best practices for divergence time estimation (Parham et al., 2011), we did not incorporate most of the pleurodont fossils used as calibration points to date (e.g. Townsend et al., 2011) due to the lack of phylogenetic analyses or explicit synapomorphies for fossil placement (e.g., Holman, 1972, 1987; Smith, 2006; Yahkola, 1976). In fact, some of those fossils cannot be unambiguously assigned to any iguanian group (Sullivan and Holman, 1996). Because setting maximum age bounds for calibration priors is challenging in the face of the incompleteness and uncertainty of the fossil record (Ho and Phillips, 2009), we conservatively assigned long tails to the probability distribution of lognormally distributed calibration priors. Calibration prior settings, including median values and the 95% highest posterior density intervals, are presented in Table 1.

We performed simultaneous phylogenetic reconstruction and divergence time estimation by implementing an uncorrelated lognormal relaxed clock (Drummond et al., 2006) under a concatenation approach in BEAST. We implemented a uniform prior distribution (interval = 01) to the mean rate of the molecular clock (ucld.mean parameter), while using default settings for the parameters relative to substitution rates, nucleotide frequencies, and the Yule tree prior. Because the use of codon partitions consistently prevented proper Markov chain mixing, we only partitioned the dataset by gene when running the dating analyses. We ran five independent chains of 100 million steps, sampling every 10,000 steps. After assessing stationarity and convergence of model parameters in Tracer, we applied a 25% burn-in, combined the runs, and summarized results into a maximum clade credibility tree as described in Section 2.2.

### 3. Results

#### 3.1. Phylogenetic relationships of Amazonian Dactyloa

Both the coalescent-based (Fig. 4) and the concatenated (Fig. 5) analyses recovered six main clades within *Dactyloa*. Five of them correspond to the previously recognized *aequatorialis*, *heteroderma*, *latifrons*, *punctata* and *roquet* species groups, with nearly the same species composition as listed by Cañaheda and de Queiroz (2013) (exceptions were the phylogenetic placement of *D. fitchi* and *D. philopunctata*, see below). The sixth recovered clade is composed of *D. calimae*, *D. neblina*, and the newly sampled *D. dissimilis*. This clade was highly supported in both the coalescent-based (“BEAST”) and Bayesian concatenated (MrBayes) analyses (posterior probabilities > 98), yet weakly supported in the maximum likelihood (Garli) tree (bootstrap support = 68%). The two concatenated analyses (MrBayes, Garli) recovered the same topology for the entire *Dactyloa* phylogeny.

Relationships among these six major clades were poorly supported and inconsistent across analyses. For instance, the *punctata* species group was inferred as the sister of the remaining *Dactyloa* in the coalescent-based phylogeny (Fig. 4), but not in the trees generated following a concatenated approach (Fig. 5). *Dactyloa fitchi*, the only species assigned to different main clades across our analyses, was recovered with low support as the sister of the clade composed by *D. dissimilis*, *D. neblina*, and *D. calimae* in the coalescent-based phylogeny (Fig. 4). Yet, in the concatenated analyses, *D. fitchi* was placed as closely related to the *aequatorialis* group (Fig. 5), a relationship previously recovered by Cañaheda and de Queiroz (2013).

New sequence data provide important information about relationships within the *Dactyloa punctata* group. All samples of *D. phyllorhina* were deeply nested within this clade, with high support (Fig. 5). This species was recovered as *Dactyloa punctata*’s sister taxon in all analyses, with pairwise corrected genetic distances between *D. phyllorhina* and *D. punctata* ranging 19–25% (see online Appendix C for pairwise genetic distances between all newly sampled *Dactyloa*).

A clade comprised of samples of *D. punctata*, which have orange dewlaps, and of *D. philopunctata*, which have spotted dewlaps, was consistently highly supported (Fig. 5). Although our two samples of *D. philopunctata* composed a clade, the analyses found this species to be nested within *D. punctata*. Corrected pairwise genetic distances between *D. philopunctata* samples and closely related *D. punctata* were relatively small, between 3.4% and 5.1% (Appendix C). By contrast, Amazonian populations of *D. punctata* presenting the orange dewlap pattern often exhibited higher pairwise genetic distances, from 3.4% to 12%. Relationships within the *D. punctata + D. philopunctata* clade were poorly supported. Interestingly, those samples of *D. punctata* collected in the coastal Atlantic Forest (Camacan, Linhares) were nested among Amazonian ones (Fig. 5).

Similar to the *D. philopunctata* individuals, the only sample of *D. punctata* with a white dewlap (MTR967985) was recovered nested among *D. punctata* specimens exhibiting orange dewlaps (Fig. 5).

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### Table 1

Median values and 95% highest posterior density intervals (in millions of years) of lognormally distributed calibration priors applied in dating analyses, based on fossil data. Settings for calibration prior mean, standard deviation and offset are provided. MRCA = most recent common ancestor.

<table>
<thead>
<tr>
<th>Fossil</th>
<th>Calibrated node</th>
<th>Median</th>
<th>95% HPD lower</th>
<th>95% HPD upper</th>
<th>Prior mean</th>
<th>Prior st. dev.</th>
<th>Prior offset</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saichangurvel davidsoni</em></td>
<td>MRCA of Pleurodonta</td>
<td>77.39</td>
<td>71.54</td>
<td>105.4</td>
<td>2</td>
<td>0.8</td>
<td>70</td>
</tr>
<tr>
<td><em>Suzanniwana</em> sp.</td>
<td>Stem of Basiliscus and Corytophanidae</td>
<td>62.39</td>
<td>56.54</td>
<td>90.44</td>
<td>2</td>
<td>0.8</td>
<td>55</td>
</tr>
<tr>
<td><em>Afairiguama avius</em></td>
<td>Stem of Leiosaurus and Urostrophus</td>
<td>57.39</td>
<td>51.54</td>
<td>85.44</td>
<td>2</td>
<td>0.8</td>
<td>50</td>
</tr>
</tbody>
</table>

The lowest genetic distance separating this individual from a sample presenting the typical D. punctata pattern was 6.4%.

3.2. Divergence times between Amazonian lizards

Dating analyses suggest that lineage diversification within the pleurodont iguanian clade dates back to the late Cretaceous (Fig. 6) (see online Appendix D for a complete list of estimated node ages within Pleurodonta). We estimated the most recent common ancestor (MRCA) of sampled pleurodonts to have lived at approximately 82 million years ago (Mya) (median value; 95% of the highest posterior density [HPD] = 71–99 Mya). On the other hand, some of the divergences between Neotropical genera are as recent as the Middle Miocene (e.g., Basiliscus and Corytophanes, Urostrophus and Leiosaurus, Morunasaurus and Enyalioides). Our analyses recovered Dactyloidae as the sister of all other sampled pleurodonts.

Among extant anoles, our analyses suggest that Dactyloa diverged from the MRCA of Anolis and Norops during the middle Eocene (49 Mya, HPD = 38–63 Mya). The MRCA of Amazonian species within Dactyloa, which corresponds to the split between D. dissimilis and the remaining Dactyloa taxa, dated back to the late Eocene (35 Mya, HPD = 25–47). The MRCA of Norops, which corresponds to the divergence of N. tandai from the other sampled Norops, dated back to the late Oligocene (24 Mya, HPD = 16–32 Mya). The remaining diversification events among Amazonian taxa within both Dactyloa and Norops happened during the Miocene (Fig. 6). For instance, the MRCA of D. punctata and D. phyllorhina was dated around 12 Mya (HPD = 7–17 Mya), while the most recent common ancestor of N. fuscocaudatus and N. trachyderma was dated around 12 Mya (HPD = 7–17 Mya). Within Amazonian bush anoles, the dating analyses suggest that Polychrus liogaster and P. marmoratus diverged in the Pliocene (6 Mya, HPD = 3–11 Mya).

4. Discussion

4.1. The phylogenetic affinities of Dactyloa dissimilis

Our analyses suggest that D. dissimilis is closely related to D. neblinina and D. calmae, two species distributed outside the Amazon basin and presenting highly disjunct distributions. While D. dissimilis occurs in southwestern Amazonia, D. calmae is restricted to the western Colombian Andes, and D. neblinina is a narrow endemic within the Guiana Shield. Together, they composed a sixth Dactyloa clade that received low to maximum support in our analyses. Similar to our study, Castaño and de Queiroz (2011) have previously recovered D. neblinina and D. calmae as sister taxa, yet independent of the five main Dactyloa clades. By contrast, Ayala et al. (1983) suggested that D. dissimilis and D. calmae are related to the punctata species group, while Nicholson et al. (2012) proposed D. neblinina to be a member of the heteroderma group.

Due to limited genetic sampling of mainland Dactyloa, it is possible that the closest relatives of these three highly geographically separated taxa have not been included in our investigation. Morphological traits suggest unsampled continental taxa that may be closely related to these species; for instance, D. deltae shares unique tail crests with D. dissimilis, while D. caquetae, D. santamartae and D. deltae exhibit a very large interparietal scale in contact with the semicircles, as seen in D. dissimilis (Williams, 1982). On the other hand, there has been no evident close relative for D. calmae.
To further verify whether the clade composed by *D. dissimilis*, *D. calimae* and *D. neblinina* represents a natural group, it will be key to continue improving the genetic sampling of mainland anoles.

### 4.2. Independent evolution of nasal appendages in anole lizards

We found that *D. phyllorhina* is a member of the punctata species group, and more specifically the sister species of the broadly sympatric *D. punctata*. Interestingly, with the exception of the proboscis and a red dewlap, *D. phyllorhina* differs from *D. punctata* by only a few quantitative morphological traits. The identification of females, which lack both the proboscis and a developed dewlap, is indeed difficult (Rodrigues et al., 2002).

Our data suggest that rostral appendages, found in *D. phyllorhina*, *D. proboscis* and *D. laevis*, have evolved at least twice within the anole radiation. Based on morphological comparisons, Yáñez-Muñoz et al. (2010) also challenged the hypothesis of a close rela...
tionship between D. phyllorhina and D. proboscis. Although no
genetic data are currently available for D. proboscis or D. laevis,
recent phylogenetic studies based on combined molecular and
morphological evidence suggest that D. proboscis is closely related
to the heterodera species group (Castaheda and de Queiroz, 2013;
Nicholson et al., 2012; Poe, 2004; Poe et al., 2012). Morphological
comparisons, in turn, led Williams (1979) to suggest D. laevis to be
closely related to D. heterodera (Williams, 1979). As a result,
the laevis group, a name used to refer to the three anole species
with a rostral proboscis (Williams, 1979), is not supported as a
monophyletic group. Marked structural differences in the probos-
cises of D. phyllorhina, D. proboscis and D. laevis (Williams, 1979)
also support the view that these structures are not homologous
(Yáñez-Muñoz et al., 2010).

4.3. Genetic and phenotypic divergence between D. punctata and D.
philopunctata

Our results indicate paraphyly of D. punctata relative to D. philo-
punctata, which is nested among geographically close populations
of D. punctata from central Amazonia. We recovered short branches
and low genetic distances between individuals with distinct dew-
lap patterns, including those with spotted (D. philopunctata),
orange (D. punctata), and white (D. punctata) dewlaps. Given the
documented role of anole dewlaps in species recognition and pre-
sumably reproductive isolation (e.g., Losos, 1985; Macedo
nia et al., 1994; Macedonia and Stamps, 1994; Sigmund, 1983), these results
may indicate that D. philopunctata is still in its very first stages of
speciation. A vast number of studies have documented paraphyly
among animal species that are phenotypically and often ecologi-
cally distinct (e.g., Brown and Twomey, 2009; Johnson et al.,
2005; McKay and Zink, 2010; Omland et al., 2000), including
anoles (Thorpe and Stenson, 2003). From the perspective of allele
coalescence, the speciation process inherently starts with para-
phly in gene genealogies, and the probability of achieving reciproc-
ally monophyly increases with time since population divergence
(Knowles and Carstens, 2007).

Alternatively, our findings are also consistent with the hypo-
thesis that the names D. philopunctata and D. punctata do not corre-
pond, in fact, to distinct species (Avila-Pires, 1995). The degree of
dewlap dissimilarity that disrupts species recognition and mating
in dactyloids is unclear (Ng and Glor, 2011; Stapley et al., 2011).
In addition to the dewlap, anoles rely on a range of visual signals,
including body coloration and stereotyped head bobbing displays
(Jenssen and Gladson, 1984; Losos, 1985; Macedonia and Stamps,
1994; Muñoz et al., 2013). Other studies have also found low
genetic differentiation between distinct dewlap phenotypes
(D’Angiolella et al., 2011; Lambert et al., 2013; Ng and Glor,
2011; Stapley et al., 2011). Within some anole taxa, divergence
in sexual signals has not prevented high levels of contemporary
gene flow between populations (Muñoz et al., 2013), which might
also be the case of D. punctata and D. philopunctata.

Because of the paraphyly of D. punctata relative to D. philopunc-
tata, and given the low genetic distances among some of their indi-
viduals, we tentatively consider the name D. philopunctata as a
synonym of D. punctata. However, spatially-dense sampling, along
with a comprehensive analysis of morphological and genetic vari-
ation, are needed for a detailed taxonomic evaluation of D. philo-
punctata, and of the often highly genetically divergent populations
within D. punctata.

4.4. The age of Amazonian anoles

We found that most Amazonian taxa within both Dactyloa and
Norops diverged in the Miocene, but some diversification events
were as old as the late Eocene and late Oligocene. Furthermore,
Amazonian Polychrus diverged in the Pliocene. Importantly, our
divergence time estimates contradict the predictions of the Pleisto-
cene Refugia Hypothesis (Haffer, 1969; Vanzolini and Williams,
1970), one of the first models of Amazonian diversification. By
examining distribution patterns and morphological variation
within the Norops chrysolepis species group, Vanzolini and
Williams (1970) suggested that cycles of forest contraction and
expansion during the Quaternary would have triggered repeated
population isolation and divergence, promoting in situ allopatric
speciation. However, our results disagree with the temporal frame-
work implied in that model, since we recovered much older diver-
gences between Amazonian anoles. Glor et al. (2001) also inferred
pre-pleistocene splits between the Amazonian anoles D. punctata,
N. chrysolepis, N. fuscoauratus, N.ortonii, and N. scyphus and N. tandai. A
scenario of Amazonian anole diversification preceding the Quater-
nary is consistent with molecular estimates emerging from a wide
range of Amazonian organisms, because most crown-group ages
date back to the Neogene (reviewed in Antonelli et al., 2010;
Hoorn et al., 2010).

Our inferred divergence times between Amazonian anole spe-
cies are considerably younger than the most recent estimates
(Nicholson et al., 2012), however. Nicholson et al. (2012) recovered
the most recent common ancestor (MRCA) of N. chrysolepis, N. fus-
couaratus, N.ortonii, and N. trachyderma at approximately 51.6 mil-
ion years ago, more than twice our median estimate for the same
node. They also found the MRCA of Dactyloa and Norops to be
around 95 million years old, nearly two times older than our esti-
mate. Our analyses indicate that the lineages leading to modern
Dactyloa and Norops did not diverge until the Eocene, in disagre-
ement with the suggestion that all modern anole genera were pres-
ent in the Caribbean region by the late Cretaceous. Although an
evaluation of dactyloid biogeography is beyond the scope of this
work, our results suggest that existing hypotheses will benefit
from the use of multiple nuclear markers, and of improved fossil
data for node calibration.

4.5. Concluding remarks

Building on the Dactyloa molecular dataset of Cañadella and de
Queiroz (2011), we provide new information about the phyloge-
netic placement of rare Amazonian anoles. The data reveal dawlp
variation among genetically close forms within the punctata spe-
cies group, dispute the monophyly of the laevis group by support-
ing independent evolution of the anole provosics, provide
additional evidence for a sixth main clade within Dactyloa, and
oppose the view that all modern anole genera were present in
the Caribbean region by the late Cretaceous. The findings lead us
to new research questions about the taxonomy, evolution and bio-
geochemistry of dactyloid lizards, which will build from denser sam-
pling of continental species and more extensive molecular
datasets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found,
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