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Phylogeny of African Long-Fingered Frogs (Arthroleptidae: *Cardioglossa*) Reveals Recent Allopatric Divergences in Coloration

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The African anuran genus *Cardioglossa* contains 19 described species, most of which are distinguished from one another by striking patterns and colors. We present a well-resolved phylogeny based on analyses of mitochondrial and nuclear loci for 18 species of *Cardioglossa*. This provides the basis for species-delimitation analyses and interpreting historical biogeography in the genus. Whereas much of the diversification within the genus occurred among Central African lineages during the Miocene following the origin of *Cardioglossa* in the latest Oligocene or earliest Miocene, most species-pairs in the genus diverged more recently during the Plio-Pleistocene. The two most geographically peripheral species—*C. cyaneospila* in the Albertine Rift Mountains and *C. occidentalis* in the Upper Guinean Forests of West Africa—both diverged from other lineages during the mid-late Miocene. Because our analyses do not support *C. manengouba* and *C. oreas* as distinct species, we recognize these geographically separate and phenotypically distinct populations as subspecies of *C. oreas* that diverged subsequent to the origin of Mount Manengouba during the past 1.5 million years. In contrast, we find that *C. leucomystax* likely represents two species found in the Lower Guinean and Congolian forests, respectively. We find recent divergences between several allopatric lineages (either species or populations) that differ in coloration and pattern, including in *C. nigromaculata* which varies in color across its range in Central Africa and Bioko Island. These recent divergences among allopatric lineages with distinctive coloration and pattern raise new questions about the significance of these traits in this genus for which little is known of its natural history and biology.

AMONG the more than 800 species of frogs in continental Africa, species of the genus *Cardioglossa* stand out due to their striking patterns and colors (Amiet, 1972a, 1972b; Channing and Rödel, 2019). Most of the 19 species are restricted to the Lower Guinean and Congolian forests of Central Africa that extend from Nigeria to Angola (Gartshore, 1986; Reeder et al., 2011; Ernst et al., 2020), with one other occurring in West Africa (*C. occidentalis*; Blackburn et al., 2008) and two in the Albertine Rift Mountains (*C. cyaneospila*, *C. inornata*; Laurent, 1950; Hirschfeld et al., 2015; Blackburn et al., 2016). Nearly all of these species live in forests and typically are found near streams (Amiet, 1972b; Laurent, 1950; Rödel et al., 2001; Blackburn et al., 2008; Hirschfeld et al., 2015). Eggs are laid on the ground adjacent to streams, and the stream-dwelling tadpoles have dorsoventrally depressed bodies, small eyes, and elongate spiracular tubes (Amiet, 1972b, 1989; Blackburn, 2008a; Channing et al., 2012; Hirschfeld et al., 2012). Most species are rare in museum collections; approximately two-thirds of the 800 specimens in the data aggregator GBIF represent only the two most widespread species, *C. gracilis* and *C. leucomystax* (GBIF, 2020). Many species in the genus are threatened due to habitat loss (IUCN, 2020), including two that are Critically Endangered (*C. manengouba*, *C. trifasciata*), five that are Endangered (*C. alsco*, *C. annulata*, *C. oreas*, *C. pulchra*, *C. venusta*), and two that are Vulnerable (*C. melanogaster*, *C. schioetzi*). Over the past decade, some species in Cameroon have become difficult to locate, suggesting that species such as *C. manengouba* on Mount Manengouba (Hirschfeld et al., 2016), *C. schioetzi* on Mount Mbam (Blackburn, 2006; Tchassem Fokoua et al., 2019a), and *C. pulchra* on Mount Bamboutos (Tchassem F. et al., 2019b)

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may be experiencing population declines. All *Cardioglossa* lack teeth (Laurent, 1973), and the two species studied so far predominantly eat ants and termites (i.e., myrmecophagy; Noble, 1924; Toft, 1982). Early authors noted similarities to the dart-poison frogs of the Dendrobatidae (e.g., Boulenger, 1900; Gadow, 1901), including their striking patterns and lack of teeth. But unlike dendrobatids (e.g., Summers and Clough, 2001), there have been no explicit hypotheses proposed to explain the striking colorations and patterns of this genus.

Cardioglossa is sister to the genus *Arthroleptis*, a widespread clade of terrestrial direct-developing frogs found in forests and savannas across sub-Saharan Africa (Laurent, 1973; Blackburn, 2008b). Males of most species in both genera have elongate third fingers—a unique secondary sexual trait among frogs (Blackburn, 2009) that is used in male–male combat in at least some species of *Arthroleptis* (Amiet, 1989; Bittencourt-Silva et al., 2020). These two genera likely diverged from one another in the latest Oligocene or earliest Miocene (Portik and Blackburn, 2016; Portik et al., 2019; Yuan et al., 2019; Hime et al., 2020). While Pyron and Wiens (2011) did not find *Cardioglossa* to be monophyletic with respect to *Arthroleptis*, the more comprehensive analysis of afrobatrachian relationships by Portik et al. (2019) recovered the monophyly of *Cardioglossa* with high support; Jetz and Pyron (2018) also resolved these two genera as reciprocally monophyletic. Both genera belong to the Arthroleptidae and together form a clade that is sister to the genus *Leptopelis* (Portik and Blackburn, 2016; Feng et al., 2017; Portik et al., 2019; Yuan et al., 2019; Hime et al., 2020). The genus *Cardioglossa* likely began diversifying by the early to middle Miocene (Portik et al., 2019), but divergence times within the genus remain poorly explored. Blackburn (2008b) found a shallow divergence between *C. oreas* and *C. manengouba*, though it remains unknown whether other species-pairs in the genus share similarly recent evolutionary histories.

Taxonomic revisions over the past two decades modified and added to the known diversity of the genus *Cardioglossa*. These include the transfer of *C. aureoli* Schiøtz 1964 to *Arthroleptis* (Blackburn, 2008b), recognition of *Cardioglossa liberiensis* Barbour and Loveridge 1927 as a junior synonym of *Phrynobatrachus fraterculus* (Blackburn, 2005), description of five new species (*C. alsco*, *C. annulata*, *C. congolia*, *C. manengouba*, *C. occidentalis*; Herrmann et al., 2004; Blackburn, 2008a; Blackburn et al., 2008; Hirschfeld et al., 2015), and recognition of *C. nigromaculata inornata* Laurent 1952 as a distinct species (Hirschfeld et al., 2015). There are now 19 recognized species, many of which were not included in previous phylogenetic analyses (e.g., Blackburn, 2008b; Pyron and Wiens, 2011; Portik et al., 2019). Previous authors' interpretations of the diversity of species of *Cardioglossa* and the relationships among them were strongly influenced by variation in coloration and pattern. There are three pairs of species that are believed to be closely related to one another but differ in color and/or pattern: *C. melanogaster* and *C. schioetzi* (Amiet, 1981), *C. alsco* and *C. pulchra* (Herrmann et al., 2004), and *C. manengouba* and *C. oreas* (Blackburn, 2008a). In addition, geographic variation in coloration is documented in *C. nigromaculata*, which can range from orange to lemon yellow to olive green (Parker, 1936; Amiet, 1972c, 1978). A population from Bioko Island that resembles *C. nigromaculata* may be an undescribed species due to differences in coloration and pattern (Oates et al., 2004), though some have recently treated this as *C. nigromaculata* (Channing and Rödel, 2019; Sánchez-Vialas et al., 2020). A species-level phylogeny for the genus is needed to rigorously evaluate species boundaries and explore the extent to which species pairs differ in coloration and pattern.

Amiet (1981) presented the first hypothesis of relationships among species in the genus *Cardioglossa* and recognized four species groups. The first group comprised three lowland (*C. elegans*, *C. gracilis*, *C. leucomystax*) and two montane (*C. melanogaster*, *C. schioetzi*) species that differ from other species in having dull coloration and distinctive hourglass-shaped dorsal markings (Amiet, 1972c, 1981). This first group also included the populations of *C. leucomystax* in the Upper Guinean Forests that are now recognized as *C. occidentalis* (Blackburn et al., 2008). Amiet (1972c, 1981) recognized a group of lowland species (*C. escalerae*, *C. gratiosa*, *C. nigromaculata*) that have finely granular skin, including two species (*C. gratiosa*, *C. nigromaculata*) with whistle-like calls (Amiet, 1973; Amiet and Goutte, 2017) that he believed to be closely related and possibly hybridizing in southern Cameroon (Amiet, 1981, 1987; Amiet and Goutte, 2017). Amiet (1981) also recognized a group of montane species with large and irregular tubercles, bluish ventral coloration, and melodious rolling whistles or series of tinkles (Amiet, 1973) that includes both narrow-range endemics (*C. trifasciata*, *C. venusta*) and the widely distributed species *C. pulchra* (Amiet, 1975). Last, Amiet (1981) placed montane *C. oreas* in its own group distinct from other species based on males lacking both an elongate third finger and small spines along it; Blackburn (2008a) later demonstrated that male *C. oreas* have these spines even though the finger is similar in length to that of females. *Cardioglossa cyaneospila* was not included in Amiet's (1981) groupings, but Blackburn (2008b) hypothesized that it may have a close relationship to montane species in Cameroon such as *C. pulchra* and *C. trifasciata*. Amiet (1983) suggested that diversification of the genus *Cardioglossa* was associated with the uplift of the Cameroon Highlands, but he did not provide a hypothesis for the timing of this diversification or how it might relate to each of his species groups.

In the first molecular phylogenetic analysis of the genus *Cardioglossa*, Blackburn (2008b) sampled ten species that formed two clades, one containing the "long-snouted" forms *C. gracilis*, *C. melanogaster*, and *C. schioetzi*, and the other containing the remaining diversity. In support of Amiet's (1981) groupings, the sampled montane species from Cameroon did not form a clade; *C. melanogaster* and *C. schioetzi* formed a clade with *C. gracilis*, and *C. pulchra* formed a clade with the morphologically similar *C. oreas* and *C. manengouba*. Yet most of the montane diversity was not included in that study, including *C. cyaneospila* from the Albertine Rift Mountains. Recent large-scale phylogenetic analyses (Pyron and Wiens, 2011; Portik et al., 2019) that synthesized available genetic data recovered largely similar patterns of relationships to those reported by Blackburn (2008b). However, the genetic data used by Blackburn (2008b) and Portik et al. (2019) are either exclusively or mostly a single mitochondrial locus, and four species have still not been included in any analysis (*C. congolia*, *C. cyaneospila*, *C. inornata*, *C. venusta*).

In this study, we present a phylogenetic analysis of mitochondrial and nuclear loci of nearly all species of the genus *Cardioglossa*. We do not include *C. inornata* because genetic resources are not available; this species remains known only from specimens collected in 1950 at one locality along the Upper Lubitshako River in what is today the Democratic Republic of Congo (Kabobo Plateau *sensu* Greenbaum and Kusamba, 2012) and of which only one paratype still exists (Hirschfeld et al., 2015). Our time-calibrated phylogeny for the genus provides insights into its historical biogeography, including the timing of divergence of species found only in western Africa or the Albertine Rift. With this phylogenetic framework, we evaluate Amiet's (1975) hypothesis that the diversification of species restricted to the mountains of the Cameroon Volcanic Line was associated with climatic oscillations in the Pleistocene and Holocene. This includes his idea that the divergence among species in the "massifs centraux" (e.g., the Western High Plateau) and "massifs périphériques" (Amiet, 1975) occurred during the "interpluvials," leading to the origin of species endemic to Mount Manengouba such as *C. trifasciata*. This framework also facilitates our evaluation of species boundaries and whether the previously observed recent divergence between *C. manengouba* and *C. oreas* (Blackburn, 2008a) also characterizes other species pairs that differ in coloration and pattern.

MATERIALS AND METHODS

Sampling.—We obtained tissue samples from museum collections and our own field research from 56 specimens (from ten collections in Europe and the United States) that represent 18 recognized species of *Cardioglossa* from nine countries (Fig. 1; Supplemental Table 1; see Data Accessibility). We identified these specimens to species based on available taxonomic literature, including details on coloration, pattern, skin texture, and geographic location; for five species, the included specimens represent the holotype or paratypes (Supplemental Table 1; see Data Accessibility). When possible, we included several samples for each species to capture patterns of genetic variation across their geographic ranges; in some cases, these specimens represent new distributional records such as *C. escalerae* in Republic of Congo. We were unsuccessful in amplifying DNA from type specimens of *C. gratiosa peternageli*, which is known only from the type series (Böhme and Schneider, 1987) and considered a synonym of *C. gratiosa* by Amiet and Goutte (2017). We also included specimens from Bioko Island that are distinctive for their dark red coloration. This population was first photographed during a 1964 research trip by the University College London; R. C. Drewes suggested on the basis of photographs of these specimens (which were subsequently lost) that these represent an undescribed species (in litt. in Oates et al., 2004). More recently, these specimens were referred to simply as *C. nigromaculata* with no information on their uncertain taxonomic history or distinctive coloration (Channing and Rödel, 2019; Sánchez-Vialas et al., 2020).

We isolated genomic DNA from ethanol- and RNAlater®-preserved (95%) liver and/or muscle tissue samples via the Qiagen DNeasy® tissue kit. We used PCR to amplify partial fragments of seven nuclear loci (BDNF, CXCR4, NCX1, POMC, RAG1, RHO, and TYR), and the mitochondrial DNA for 12S and 16S ribosomal RNA and the intervening tRNA for valine (for PCR primers that amplify three overlapping regions, see Supplemental Table 2; see Data Accessibility). For three nuclear loci (CXCR4, NCX1, RAG1), we developed new oligonucleotide primers for PCR. We viewed PCR products via 1.5% agarose gel electrophoresis and, following purification by ethanol precipitation, sequenced the products in the Center for Comparative Genomics at the California Academy of Sciences. The forward and reverse raw sequence reads were edited, assembled, and then aligned using Geneious v.6 (BIOMATTERS; https://www.geneious.com). We also checked the amino-acid translation of protein-coding loci to verify reading frames. Accession numbers for the resulting sequences, as well as the additional sequences from outgroup taxa obtained from GenBank, are listed in Supplemental Table 1 (see Data Accessibility).

Phylogenetic analyses.—We concatenated our multilocus dataset and performed phylogenetic analyses to generate hypotheses of relationships among our ingroup samples. We analyzed the resulting dataset using both Bayesian (MrBayes v.3.2; Ronquist et al., 2012) and maximum likelihood methods (RAxML v.8.2; Stamatakis, 2014) via the CIPRES Science Gateway 3.1 for online phylogenetic analyses (Miller et al., 2010; https://www.phylo.org/index.php). We chose models of sequence evolution for Bayesian analyses and an appropriate partitioning scheme using PartitionFinder 2 (Lanfear et al., 2017), which assessed all possible positions (e.g., each codon position in the nuclear DNA) using the Bayesian information criterion. Final Bayesian analyses ran for 50 million generations with four independent chains, and sampled every 50,000 generations, after which a 25% burn-in was removed, leaving 750 trees for posterior analysis. Maximum likelihood analyses on the concatenated dataset were performed using the default settings for RAxML using the GTRGAMMA model of sequence evolution (Stamatakis, 2014) for 100 bootstrap replicates. We also analyzed a seven-gene dataset containing only nuclear genes ("nuclear-only": BDNF, CXCR4, NCX1, POMC, RAG1, RHO, and TYR) using Bayesian and maximum likelihood approaches, following the methods outlined above.

Divergence dating.—To estimate the crown-group age of *Cardioglossa* and the divergence times among its constituent lineages, we analyzed the nuclear-only dataset constrained to the well-resolved phylogeny from analysis of the full dataset. We did not include mitochondrial data in these analyses because of previously documented effects that they can mislead and overestimate divergence times (Zheng et al., 2011). Analyses were performed in BEAST v.1.8 (Drummond et al., 2012) using a log-normal relaxed molecular clock and a concatenated nuclear alignment limited to one individual per lineage identified by the above phylogenetic analyses (indicated in boldface in Fig. 2). We used three secondary calibrations based on the recent study by Portik and Blackburn (2016) that employed second calibration points based on presults of previous studies (Roelants et al., 2007; Kurabayashi and Sumida, 2013; Loader et al., 2014). Specifically, we used the following as a normally distributed constraint on node age: crown age of Arthroleptidae, 53.7 mya (stdev = 6.0); most recent common ancestor (MRCA) of *Arthroleptis*, 16.2 mya (stdev = 3.0); and MRCA of *Leptopelis*, 10.3 mya (stdev = 2.0). Direct calibration based on fossils was not possible due to the lack of any fossil material confidently attributed to Arthroleptidae. The BEAST analysis ran for 20 million generations, sampling every 20,000 generations,

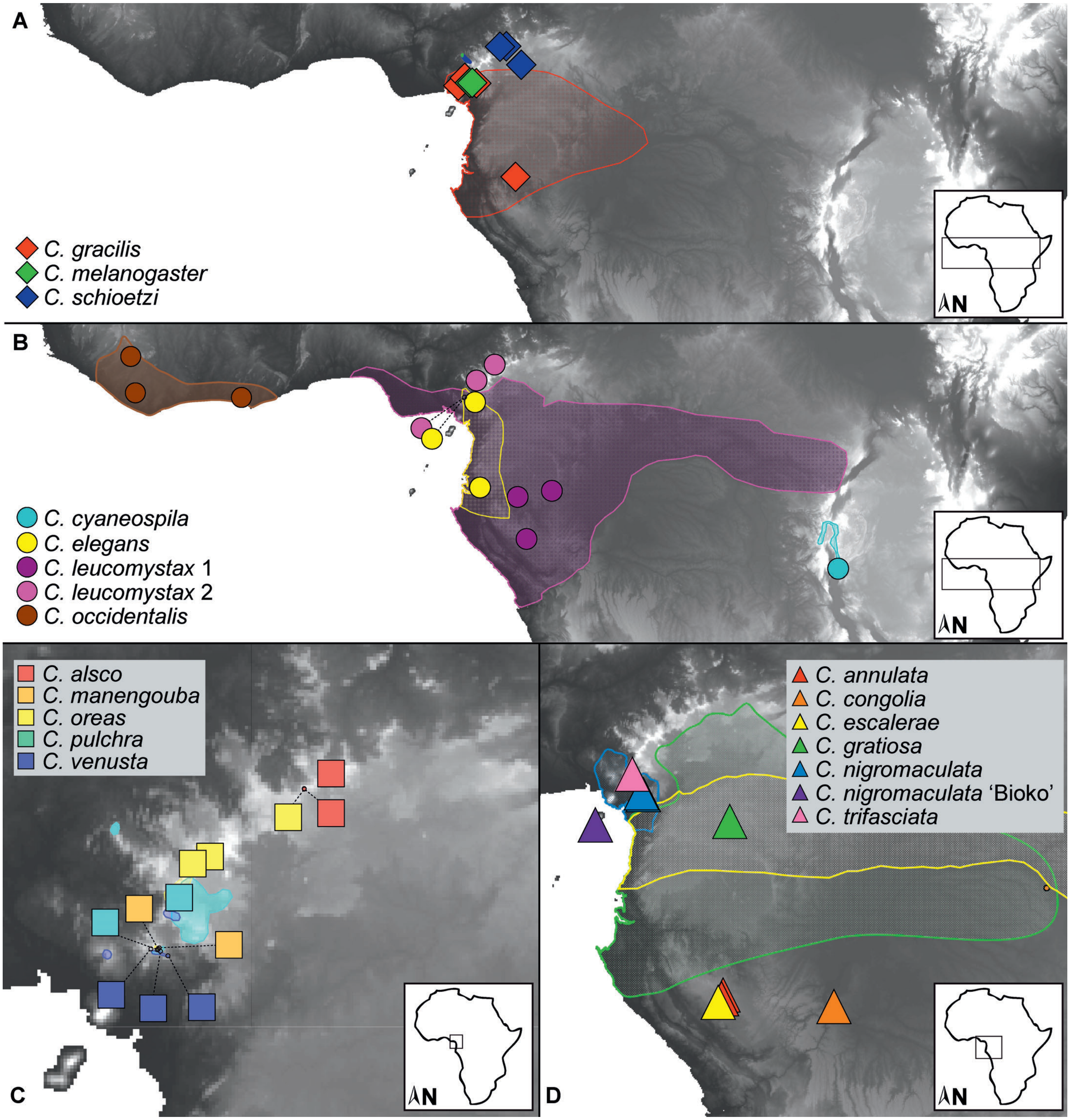

Fig. 1. Map showing the currently recognized distributions (range polygons based on IUCN, 2020) of the 18 species of *Cardioglossa* in this study showing the locations of our samples. For details on sampling, see Supplemental Table 1 (see Data Accessibility).

with a 20% burn-in, and using a Yule prior. Stationarity was reached well before the end of the burn-in. Tracer confirmed that all runs had converged on similar model parameters (estimated sample size >200 for all parameters).

Species delimitation.—Our phylogenetic analyses recovered shallow divergences between and low genetic variation within several described species. Thus, we used multilocus coalescent-based species delimitation analyses using Bayesian Phylogenetics and Phylogeography (BPP v4.0; Flouri et al., 2018) to generate posterior probabilities of described species as well as the two distinct lineages of *C. leucomystax* revealed by our analyses and the population that resembles *C. nigromaculata* on Bioko Island. BPP requires a user-specified guide tree, so we generated a simple newick tree based on the concatenated multilocus phylogenetic analyses. To be conservative, we excluded mitochondrial data and limited the BPP analyses to the seven nuclear loci, phased

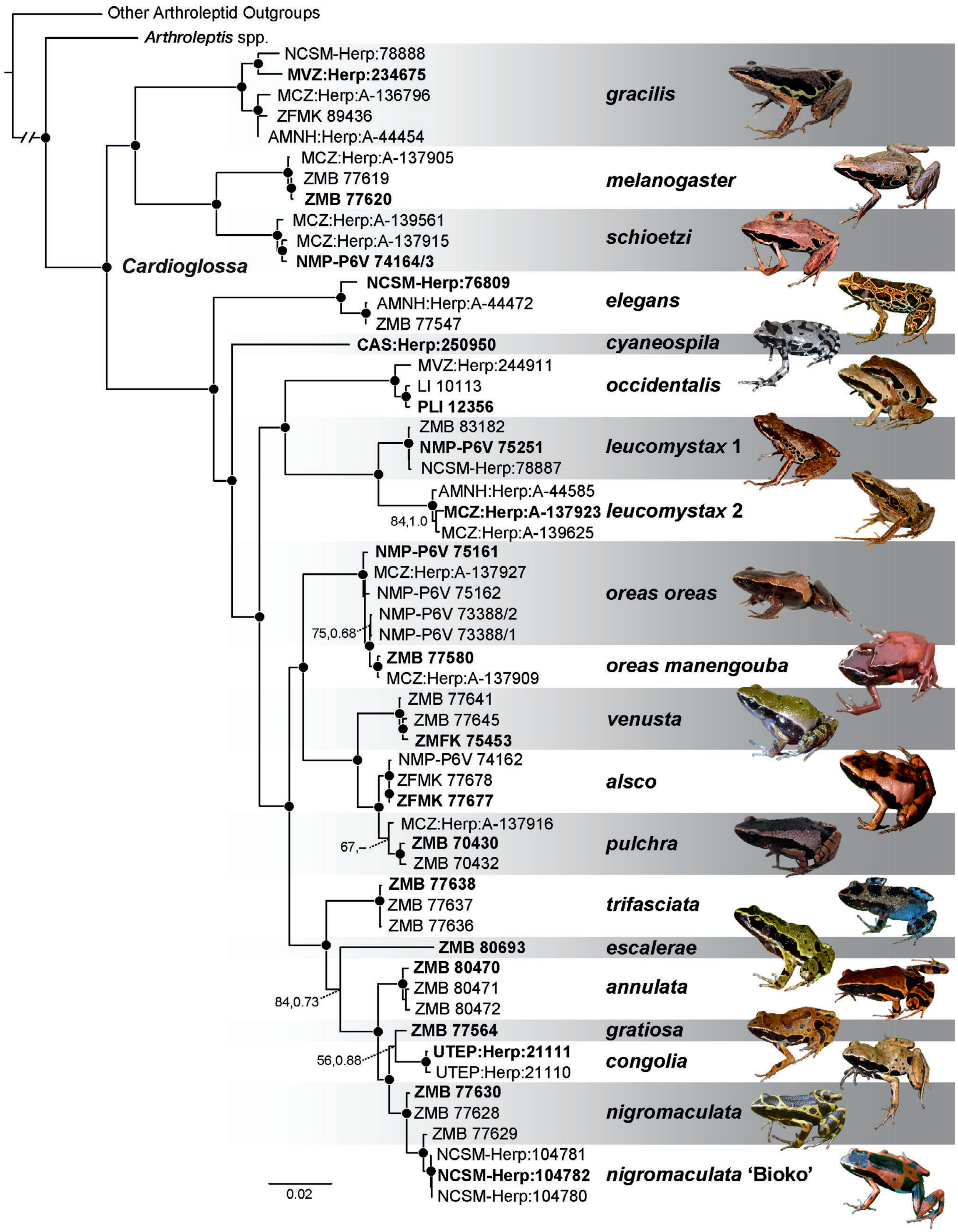

Fig. 2. Maximum likelihood phylogeny of *Cardioglossa* based on the multilocus concatenated, partitioned dataset of mitochondrial and nuclear data. A black dot at each node indicates high support (e.g., Bayesian posterior probability >0.95, maximum likelihood bootstrap >70). Tip labels of individuals used for divergence time analysis are indicated in boldface. Photographs are not scaled to size. (Photo credits: D.C.B.: *C. melanogaster*, *C. oreas manengouba*, *C. pulchra*, *C. schioetzi*; M.B.: *C. annulata*, *C. escalerae*, *C. leucomystax* 1; Brian Freiermuth: *C. leucomystax* 2, *C. oreas oreas*; E.G.: *C. congolia*, *C. cyaneospila*; V.G.: *C. alsco*; M.H.: *C. nigromaculata*, *C. venusta*; Greg Jongsma: *C. gracilis*; P.J.M.: *C. nigromaculata* 'Bioko'; Dan Portik: *C. elegans*, *C. occidentalis*; M.-O.R.: *C. gratiosa*, *C. trifasciata*; images available via https://doi.org/10.7479/3yje-r341). See Data Accessibility for tree file.

using Phase v.2.1.1 (Stephens et al., 2001; Scheet and Stephens, 2006) implemented in DnaSP 6 (Rozas et al., 2017). As prior distributions on the ancestral population size and root age can affect the posterior probabilities for models (Yang and Rannala, 2010), we followed the methods of Leaché and Fujita (2010) by testing different combinations. We used three different configurations of estimated population size (N_e; inverse-gamma $\theta = a, b$) and divergence time (inverse-gamma $\tau = a, b$) priors to begin our parameter estimation. In configuration 1, we assumed large N_e ($\theta = 3, 0.02$) with deep divergence time ($\tau = 3, 0.3$); configuration 2, small N_e ($\theta = 3, 0.0002$) with shallow divergence ($\tau = 3, 0.003$); and finally, configuration 3 was a mixture of priors that assumed large ancestral populations sizes ($\theta = 3, 0.02$) and relatively shallow divergences among species ($\tau = 3, 0.003$). We ran each MCMC chain for 500,000 generations, sampling every 50,000, with a 10% burn-in. We used the 0(e) algorithm with the fine-tuning parameter $\varepsilon = 1$. Analyses were run twice to confirm consistency between runs.

RESULTS

***Phylogeny of* Cardioglossa.**—Our concatenated, multilocus, phylogenetic analyses resulted in a well-supported species-level phylogeny with high support that *Cardioglossa* is monophyletic (bootstrap [bs] 99, posterior probability [pp] 1.0; Fig. 2), and sister to *Arthroleptis* (bs 100, pp 1.0). Within *Cardioglossa*, the diversity is divided into two well-supported clades, each of which typically has high support at all nodes (unless otherwise stated). The first small clade comprises three species: the sister species *C. melanogaster* and *C. schioetzi* that together are sister to *C. gracilis*. The second clade contains the remaining diversity of the genus, including two deeply divergent lineages that form its first two branches (*C. elegans* followed by *C. cyaneospila*). The remaining species of this second large clade are divided into three groups. The first comprises *C. occidentalis* and *C. leucomystax*, the latter of which is a geographically widespread taxon that contains two divergent lineages (indicated as *C. leucomystax* 1 and *C. leucomystax* 2 in Fig. 2). Those two species are sister to a clade comprising, first, a group of five montane species in Cameroon and Nigeria (*C. alsco*, *C. manengouba*, *C. oreas*, *C. pulchra*, *C. venusta*), and, second, five lowland forest species (*C. annulata*, *C. congolia*, *C. escalerae*, *C. gratiosa*, *C. nigromaculata*) plus the montane *C. trifasciata*.

In the clade of five montane species, *C. alsco* and *C. pulchra* are sister taxa, and these in turn are sister to *C. venusta* (Fig. 2). Together, these three species are sister to a strongly supported clade of *C. oreas* and *C. manengouba*, in which the latter renders the former paraphyletic. The only disagreement between the ML and MB analyses of the full dataset relates to the monophyly of *C. pulchra*, which is monophyletic in the RAxML analysis, although subtended with poor support (bs 67). In contrast, in the MB analysis, one sample of *C. pulchra* (MCZ:Herp:A-137916) is sister to the clade containing the samples of *C. alsco*, although again with low support (pp 0.61).

The remaining clade contains five lowland forest species and *C. trifasciata*, which is sister to the others (Fig. 2). *Cardioglossa escalerae* is recovered as sister to the other lowland species, though with low support (bs 84, pp 0.73). There is strong support that *C. annulata* is the sister to a clade containing *C. congolia*, *C. gratiosa*, and *C. nigromaculata*, but there is low support (bs 56, pp 0.88) for the sister relationship between *C. congolia* and *C. gratiosa*. The distinctive population from Bioko Island is embedded within *C. nigromaculata*.

For the deepest nodes in the tree, the nuclear-only analyses result in similar phylogenetic relationships to the combined analyses of mitochondrial and nuclear loci (Supplemental Fig. 1; see Data Accessibility). Because the relationships towards the tips of the phylogeny differ from results from the full dataset, the recovered pattern of relationships in analyses of the full dataset is likely driven by the mitochondrial data. The primary differences between phylogenies resulting from the full and nuclear-only datasets are finding in the latter that (1) *C. cyaneospila* and the clade containing *C. leucomystax* and *C. occidentalis* exchanged positions in the phylogeny, (2) *C. escalerae* is the sister of *C. nigromaculata* (including the population from Bioko) and embedded within the clade of lowland forest species, (3) *C. trifasciata* is sister to the clade containing *C. manengouba* and *C. oreas* and embedded within the clade of montane species, and (4) neither *C. nigromaculata* nor *C. pulchra* is monophyletic. Most of these topological differences between the two datasets receive low support (< 0.95 pp) in the nuclear-only analyses.

Our analyses recover five pairs of sister species, two of which exhibit low genetic divergences between them (Fig. 2; Supplemental Table 3; see Data Accessibility). The most divergent pair of sister species is *C. leucomystax* and *C. occidentalis* (12S–16S *P*-distance: 8–10%), followed by *C. melanogaster* and *C. schioetzi* (12S–16S *P*-distance: 5–7%), and then by *C. congolia* and *C. gratiosa* (16S *P*-distance: 3%). There are much smaller genetic divergences between both *C. manengouba* and *C. oreas* (12S–16S *P*-distance: <1%) and *C. alsco* and *C. pulchra* (12S–16S *P*-distance: 1–3%). Whereas *C. alsco* and *C. pulchra* form reciprocally monophyletic clades (though with low support due to uncertainty in the placement of MCZ:Herp:A-137916), *C. oreas* is paraphyletic with respect to *C. manengouba*.

Two other pairs of lineages exhibit contrasting levels of genetic divergence. Populations of *C. leucomystax* form two well-supported divergent clades (12S–16S *P*-distance: 4%; Figs. 1, 2), one containing specimens from Gabon and the Republic of Congo ("*leucomystax* 1") and the other from Cameroon and Nigeria ("*leucomystax* 2"). Based on the high degree of divergence and the geographic distribution of these two lineages, one of these may represent an undescribed species (see Discussion). In contrast, the genetic divergence between *C. nigromaculata* on the mainland and the population from Bioko Island is small (12S–16S *P*-distance: <1%) and reflects that *C. nigromaculata* is paraphyletic with respect to this island population. Our species delimitation analyses (presented below) further evaluate support for whether these pairs of lineages represent distinct species.

***Origin and diversification of* Cardioglossa.**—The time-calibrated phylogeny resolves the deepest divergence within *Cardioglossa* as occurring in the early Miocene, ~18 mya (95% HPD: 13.8–24.9 mya; Fig. 3). Within the first of the two major subclades, the divergence between *C. melanogaster* and *C. schioetzi* dates to ~6 mya (95% HPD: 2.8–9.1 mya), and the deeper split between these sister taxa and *C. gracilis* is ~13 mya (95% HPD: 8.4–19.2 mya). Within the other larger subclade, many of the divergences date to the Miocene,

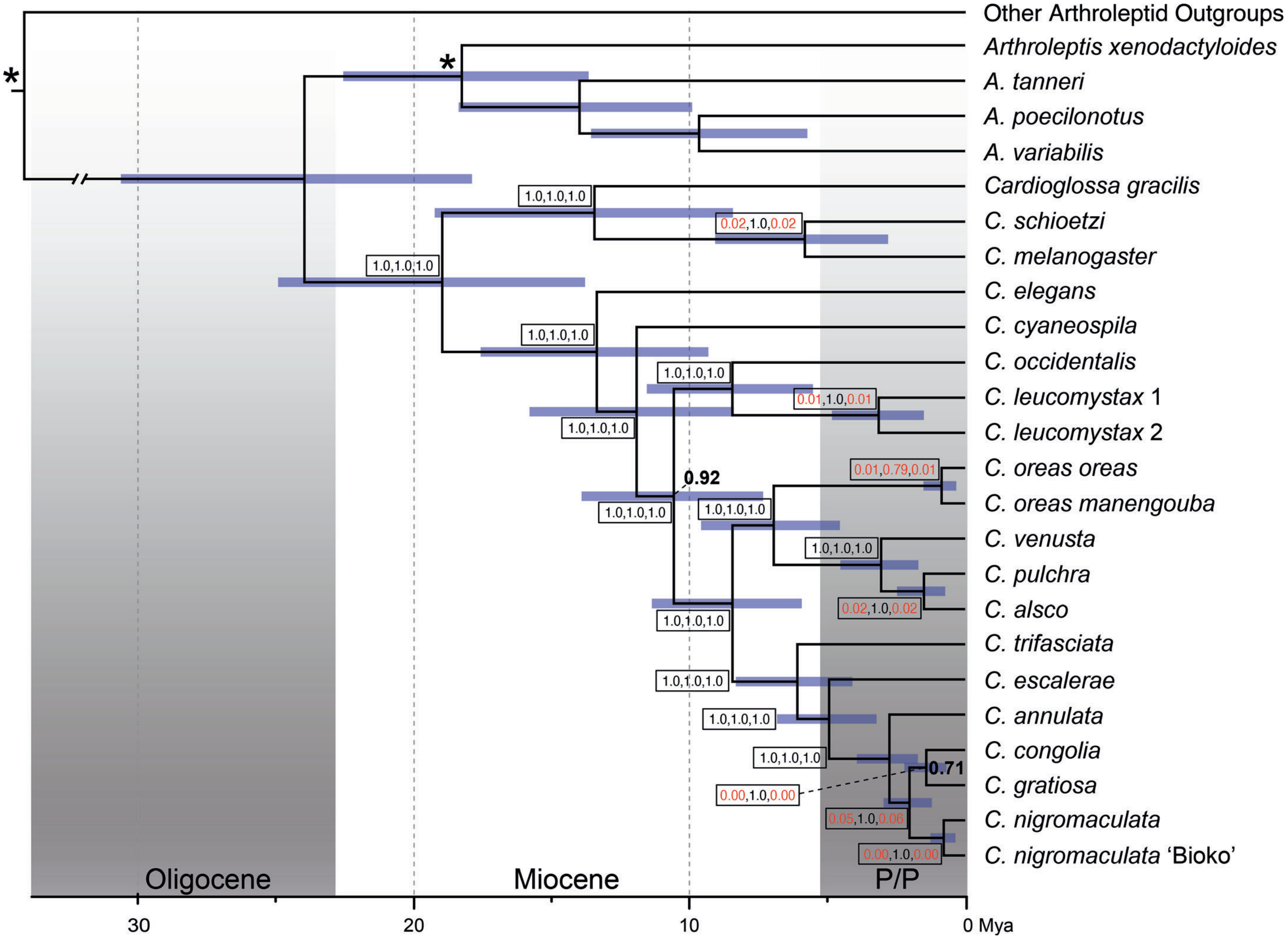

Fig. 3. Chronogram displaying divergence times within *Cardioglossa* and the Arthroleptidae based on the concatenated dataset. Bars at nodes represent 95% highest posterior density estimates and asterisk (*) denotes the nodes anchored with a secondary calibration. All nodes have support values >0.95 unless otherwise noted. In addition, Bayesian species delimitation probabilities are depicted in the box at each node within *Cardioglossa* under three different combinations of priors: left, large population sizes (N_e; $\theta = 3$, 0.02) with deep divergences ($\tau = 3$, 0.3); center, small N_e ($\theta = 3$, 0.0002) with shallow divergences ($\tau = 3$, 0.003); right, large N_e ($\theta = 3$, 0.02) with shallow divergences ($\tau = 3$, 0.003). Support values below 0.95 are colored red. See Data Accessibility for tree file.

including the divergences of *C. elegans* (~13.4 mya, 95% HPD: 9.3–17.6 mya) and *C. cyaneospila* (~11.9 mya, 95% HPD: 8.4–15.8 mya), the divergence of the clade containing *C. leucomystax* and *C. occidentalis* from other species (~10.6 mya, 95% HPD: 7.3–13.9 mya), and the divergences between *C. occidentalis* and *C. leucomystax* (~8.3 mya, 95% HPD: 5.5–11.5 mya). The divergence between the clade containing the montane group from Cameroon and Nigeria and that containing *C. trifasciata* and lowland forest species also occurred in the Miocene (~8.3 mya, 95% HPD: 5.9–11.4 mya).

The remaining divergences occurred in the Pliocene and Pleistocene, including several within the past three million years (Fig. 3). Three of the pairs of sister species diverged in the Pleistocene, including *C. alsco* and *C. pulchra* (1.4 mya, 95% HPD: 0.7–2.5 mya), *C. congolia* and *C. gratiosa* (1.4 mya, 95% HPD: 0.7–2.2 mya), and *C. manengouba* and *C. oreas* (0.8 mya, 95% HPD: 0.3–1.5 mya). The divergence between populations of *C. nigromaculata* on the mainland and on Bioko Island was also during this time (0.7 mya, 95% HPD: 0.4–1.3 mya). In contrast, the divergence between the two lineages of *C. leucomystax* was earlier (3.0 mya, 95% HPD: 1.5–4.8 mya), though not as early as that between *C. melanogaster* and *C. schioetzi* (see above).

Species delimitation.—Specific combinations of priors led to different levels of support for lineages in our Bayesian species delimitation analysis (Fig. 3). *Cardioglossa manengouba* and *C. oreas* are not supported as distinct species in any of the analyses. Two of the three combination of priors, combination 1 (large population sizes with deep divergences) and 3 (a mixture of large population sizes but shallow divergences) produced effectively the same results, suggesting that population size priors outweighed divergence times. Three species pairs—*Cardioglossa melanogaster* and *C. schioetzi*, *C. alsco* and *C. pulchra*, *C. congolia* and *C. gratiosa*—and two pairs of geographically circumscribed lineages, *C. leucomystax* 1 and 2, and *C. nigromaculata* on the mainland and Bioko Island, are only supported in the analysis with small population sizes and shallow divergences.

DISCUSSION

***Phylogeny and evolution of* Cardioglossa.**—Our results provide the first complete understanding of evolutionary relationships among the currently recognized species of the genus *Cardioglossa*. This generally well-resolved phylogenetic hypothesis (Fig. 2) for *Cardioglossa* provides insights into historical biogeography and species-level taxonomy and delimitation in this poorly studied genus, which we discuss below. Similar to recent works based on less sampling (Blackburn, 2008b; Portik et al., 2019), the phylogenetic relationships resolved here support some of the relationships among species proposed by Amiet (1981). Yet there are important differences between our results and Amiet's (1981) species groups.

First, *C. elegans* and *C. leucomystax* do not form a clade with *C. gracilis*, *C. melanogaster*, and *C. schioetzi* as suggested by Amiet (1981). Instead, these species—as well as *C. cyaneospila* and *C. occidentalis*, neither of which were included by Amiet (1981)—form four lineages that branch successively at the base of *Cardioglossa* and within which all other species in the genus are nested (Fig. 2). Species in these four lineages have muted colors of grays and browns (or a bluish gray in *C. cyaneospila*) and have hourglass-shaped markings on their dorsum that are similar to many species in the sister genus *Arthroleptis*. While the coloration and pattern of *C. elegans* is striking, Amiet (1972c) previously noted that it was still most similar to species such as *C. gracilis* and *C. leucomystax*. Our results also support close relationships among Amiet's (1981) group of lowland species (*C. escalerae*, *C. gratiosa*, and *C. nigromaculata*) that have finely granular skin and similar advertisement calls (Amiet, 1972c, 1973; Amiet and Goutte, 2017). The recently described *C. annulata* and *C. congolia* are also part of this clade, which supports patterns of similarity found by Hirschfeld et al. (2015).

There are more dramatic differences, however, between our results and Amiet's (1981) species groups for the remaining montane species in Cameroon and Nigeria. As found by Blackburn (2008b), rather than being sister to all other species, *C. oreas* is nested well within the diversity of the genus and closely related to *C. manengouba* (see below for further discussion of these two taxa). In the nuclear-only analyses, *C. trifasciata* forms a clade with other montane species (*C. alsco*, *C. manengouba*, *C. oreas*, *C. pulchra*, *C. venusta*)—thus supporting Amiet's (1981) grouping—and is most closely related to *C. manengouba* and *C. oreas* (Supplemental Fig. 1; see Data Accessibility). But in our analyses of both nuclear and mitochondrial data, *C. trifasciata* is strongly supported as the sister of the lowland species discussed above (Fig. 2). These two alternative hypotheses of relationships are particularly interesting because *C. trifasciata* has similarities in its advertisement call to both *C. escalerae* and *C. venusta* (Amiet, 1973; Amiet and Goutte, 2017), which we resolve in two different clades. If *C. trifasciata* does not form a clade with the other montane species, then it suggests either another independent colonization of montane habitats or that the clade of lowland species may have evolved from montane ancestors.

Cardioglossa inornata is now the only species in the genus lacking a clear hypothesis of phylogenetic relationships. This taxon was first described as a subspecies of *C. nigromaculata* by Laurent (1950) based on three specimens from a single collecting site along the Upper Lubitshako River. Hirschfeld et al. (2015) elevated it to species-level status based on the coloration revealed in color slides that recently surfaced from Raymond Laurent's field research during 1950. These images reveal a coloration and pattern that are distinct from both *C. nigromaculata* and the other Albertine Rift endemic, *C. cyaneospila* (Hirschfeld et al., 2015). Of the original type specimens (a female holotype and two male paratypes), only one male paratype still exists (Royal Museum for Central Africa A1105-B-66), and it is badly damaged from dehydration. Recent surveys on the Kabobo Plateau did not turn up new records of *C. inornata* (Greenbaum and Kusamba, 2012). Whereas the coloration of the limbs and venter of *C. inornata* are similar to that of *C. cyaneospila*, the dorsal color and pattern are distinctly different, including by lacking prominent large spots. We hypothesize that *C. inornata* is sister to *C. cyaneospila*, and potentially represents yet another case of divergence in coloration and pattern in allopatry.

Discoveries of new species and suggested taxonomic changes.—Our species delimitation analyses provide strong support for most deep nodes across our species-level phylogeny (Fig. 3). Yet, of the five species-pairs in our analysis, only *C. leucomystax* and *C. occidentalis* receive high support as distinct species from all three combinations of priors tested. *Cardioglossa melanogaster* and *C. schioetzi* are not supported as distinct species in the analysis with large population sizes, but it may be unlikely that either of these montane endemics have large population sizes. Two other species pairs (*C. alsco* and *C. pulchra*; *C. congolia* and *C. gratiosa*) are only supported as distinct in species delimitation analyses with small population sizes, though in both cases these may be reasonable assumptions. Amiet and Goutte (2017) considered *C. alsco* to represent a local color variant of *C. pulchra*, though did not formally synonymize these two taxa. Until further information is available, such as from genomic analyses that estimate intraspecific sequence variation in these species, we continue to recognize these four species pairs as distinct species.

Our phylogenetic and species delimitation analyses do not support *C. manengouba* and *C. oreas* as distinct species (Figs. 2, 3). Based on analysis of only mitochondrial DNA sequences, Blackburn (2008b) found that these two species are genetically similar and that *C. oreas* is likely paraphyletic with respect to *C. manengouba*. These appear to represent incompletely separated lineages that are part of a single more inclusive lineage, which we recognize as *C. oreas*. Despite the distinctive coloration, J.-L. Amiet (pers. comm., 2004) suspected initially that the population on Mount Manengouba represented "an extreme form" of *C. oreas*. Blackburn (2008a) recognized this form as a new species, *C. manengouba*, due to its browny red coloration with a pair of golden white lines extending posteriorly from the snout to just over the tympanum—a coloration and pattern that are unique among species of *Cardioglossa*. We have found no evidence of populations of *C. oreas* on Mount Manengouba with contrasting patterns of dark and pale brown with golden yellow lines extending from the snout to inguinal region, which is the pattern typically found in the Bamiléké Highlands. On both Mount Manengouba and in the Bamiléké Highlands, these frogs are found in montane forests; there is also no suitable habitat in the intervening lowland savanna region (the Mbos Plain; Amiet, 1975). Because our analyses do not provide support that these two geographically circumscribed and phenotypically different lineages are completely separated species, we recognize each as subspecies following de Quieroz (2020). *Cardioglossa oreas manengouba* is restricted to the high-elevation forests of Mount Manengouba, whereas *C. oreas oreas* is found in the Bamiléké Highlands with populations in the Bamboutos Mountains, Mount Oku, Mount Lefo, and Gotel Mountains (Amiet, 1972a; Doherty-Bone and Gvoždík, 2017; this study).

Cardioglossa leucomystax was once thought to be the most widespread species of the genus (Rödel et al., 2001). Today, we recognize two distinct species, *C. occidentalis* and *C. leucomystax*, restricted to the Upper Guinean and Central African forests, respectively (Blackburn et al., 2008), which receive high support from our species delimitation analyses. These same analyses suggest that lineages of *C. leucomystax* from the Lower Guinean Forests and Congolian Forests of Central Africa represent two distinct species. These lineages are genetically divergent (12S–16S *P*-distance: 4%), possibly last sharing a common ancestor in the Plio-Pleistocene (Fig. 3) during a time of cyclical change in forest cover in Central Africa (Plana, 2004). Based on the available data, we are unsure of the distributions of these two lineages, whether their distributions ever overlap, and which might correspond to nominotypical *C. leucomystax*. Unfortunately, there are not yet genetic resources for specimens at or near the type locality of *C. leucomystax* in southern coastal mainland Equatorial Guinea (Boulenger, 1903). Future work will need to sample additional specimens from Cameroon, Gabon, and the Republic of Congo as well as from Democratic Republic of Congo and western Uganda where *C. leucomystax* has recently been discovered (https://www.inaturalist.org/observations/48429651). We also do not yet know if these two lineages might differ morphologically or in other aspects of their biology.

The population of *C. nigromaculata* from Bioko Island is not strongly supported to be a distinct species from the populations on the mainland, as previously suspected (Oates et al., 2004). *Cardioglossa nigromaculata* is remarkable among others in the genus for having substantial variation in coloration across populations. Whereas many populations are yellowish orange with black markings, those near Mamfe in western Cameroon are olive green (Parker, 1936; Amiet, 1978), and those closer to the coast near Kumba are yellow (Amiet, 1978). Adding to this variation, our analysis indicates that the red populations on Bioko Island belong to *C. nigromaculata*. Amiet (1981, 1987; see also Amiet and Goutte, 2017) previously suggested that *C. nigromaculata* may hybridize with *C. gratiosa* in southern Cameroon and form "semi-species" or subspecies separated by the Sanaga River in Cameroon. Our analyses provide no obvious evidence of hybridization, but this lack of evidence must be considered preliminary since we were only able to include one sample of *C. gratiosa*. It remains unknown whether hybridization might have an impact on color variation within *C. nigromaculata* (as found in some other frogs; Medina et al., 2013; Akopyan et al., 2020) or whether populations vary substantially in color due to differences in, for example, predator avoidance or advertisement sites (Pröhl and Ostrowski, 2011; Rudh et al., 2011).

***Current taxonomy of* Cardioglossa.**—Based on the analyses presented here, as well as other recent taxonomic work (Hirschfeld et al., 2015), we recognize 18 species of the genus *Cardioglossa*. For clarity, we list those here: *C. alsco*; *C. annulata*; *C. congolia*; *C. cyaneospila*; *C. elegans*; *C. escalerae*; *C. gracilis*; *C. gratiosa*; *C. inornata*; *C. leucomystax*; *C. melanogaster*; *C. nigromaculata*; *C. occidentalis*; *C. oreas oreas*; *C. oreas manengouba*; *C. pulchra*; *C. schioetzi*; *C. trifasciata*; *C. venusta*. The only currently recognized species with a junior synonym is *C. gratiosa* (*C. gratiosa peternageli* Böhme and Schneider, 1987), following Amiet and Goutte (2017).

***Historical biogeography of* Cardioglossa.**—The oldest divergence within the genus *Cardioglossa* occurred in the earliest Miocene (∼18 mya; 95% HPD: 13.8–24.9 mya; Fig. 3), with many of the remaining divergences occurring during the Miocene (5.3–23 mya). These divergence times are broadly similar to those of Portik et al. (2019), though typically those resolved here are somewhat younger. The Miocene divergences include those leading to two geographically peripheral species: *C. cyaneospila* in the Albertine Rift Mountains at the eastern edge of the Congo Basin, and *C. occidentalis* in the Upper Guinean Forests of West Africa. Because these two geographically peripheral species are nested within the diversity of *Cardioglossa*, this suggests that initial diversification took place in the Lower Guinean Forests of Central Africa before the species dispersed to other regions. The analyses do not support the hypothesis of Blackburn (2008a) that *C. cyaneospila* is closely related to montane species in Cameroon and Nigeria. Instead, it seems to be an isolated lineage that invaded montane habitats independent of other montane species in the genus. The deep divergence between *C. occidentalis* and *C. leucomystax*, which for many years were treated as a single species (Blackburn et al., 2008), likely reflects the fragmentation of equatorial forests associated with the expansion of open woodlands and savannas during the Miocene (Jacobs et al., 2010). Similar spatiotemporal patterns of divergence have been found in other African anurans, including torrent frogs (*Petropedetes* and *Arthroleptides*; Barej et al., 2014), toads (*Nimbaphrynoides* and *Didynamipus*; Liedtke et al., 2016), and slippery frogs (*Conraua*; Blackburn et al., 2020).

Oscillations between wetter and drier conditions during the Pliocene and Pleistocene likely played a strong role in the recent evolution of Africa's flora and fauna (deMenocal, 2004; Plana, 2004). Palynological data support an expansion of montane forest taxa into lower elevations in Central Africa during cooler periods of the early Upper Pleistocene (Jahns, 1996; Dupont et al., 2000). Amiet (1975) proposed that the diversity of endemic species of frogs on Mount Manengouba resulted from the expansion and contraction of forests between this isolated mountain and the highlands of the Bamiléké Plateau during the "interpluvials" of the Quaternary. Under this model, during cool humid glacial periods, suitable forested habitats expanded from high elevations into the intervening lowland Mbos Plain (a savanna of 300 km^2), through which montane taxa dispersed. Recent inter- and intraspecific phylogenetic studies of rodents and frogs supports this as a general pattern across the Cameroon Volcanic Line because many of the inferred divergences between montane populations or between montane and lowland populations occurred in the Pleistocene (Taylor et al., 2014; Bell et al., 2017; Charles et al., 2018; Dolinay et al., 2021).

The volcanic activity that formed the mountains of the Cameroon Volcanic Line began in the mid-Cenozoic and continues to the Present (Marzoli et al., 2000; Suh et al., 2003; Wandji et al., 2008), including active uplift and collapse of calderas on the Bamiléké Plateau during the mid- Miocene (Dongmo et al., 2010). Our phylogenetic analysis (Fig. 2) revealed four montane lineages of *Cardioglossa*: *C. melanogaster* and *C. schioetzi*; *C. cyaneospila*; *C. trifasciata*; and the clade containing *C. alsco*, *C. oreas manengouba*, *C. oreas oreas*, *C. pulchra*, and *C. venusta*. Based on our divergence time analysis (Fig. 3), all four of these lineages originated in the middle or late Miocene subsequent to significant volcanic activity such as the formation and collapse of the volcano that represents Mount Bamboutos today (Dongmo et al., 2010). However, most of the divergences within the clade of four montane species occurred in the Plio-Pleistocene, thus providing support to Amiet's (1975) hypothesis that this period of climatic change drove the evolution of new montane species in *Cardioglossa*. Further, the mid-Pleistocene divergence between *C. oreas oreas* in the Bamiléké Plateau and *C. oreas manengouba* on Mount Manengouba is similar to other populations of frogs that colonized Mount Manengouba or the nearby Bakossi Mountains during the Pleistocene, including *Afrixalus paradorsalis* (Charles et al., 2018; *A. manengubensis* in Channing and Rödel, 2019), *Hyperolius dintelmanni* (Bell et al., 2017), *Phrynobatrachus jimzimkusi* (Dolinay et al., 2021), and *P. manengoubensis* (Gvoždík et al., 2020). The recent evolution of distinctive populations on Mount Manengouba reflects the young history of this volcano that arose ~1.5 mya (Pouclet et al., 2014).

***Why are some species of* Cardioglossa *colorful?*—**In two well-studied and charismatic clades of terrestrial frogs, bright colorations are signals to predators of toxicity. The dart-poison frogs in the family Dendrobatidae have warning colorations to deter predators (Saporito et al., 2007; Maan and Cummings, 2012). These colors evolved several times independently (Santos et al., 2003; Wang and Shaffer, 2008) and are associated with specialization on prey such as ants and oribatid mites from which these frogs can sequester toxic alkaloids (Caldwell, 1996; Saporito et al., 2004; Darst et al., 2005, 2006; Heethoff et al., 2016). The Malagasy poison frogs in the family Mantellidae also evolved warning colorations several times independently (Chiari et al., 2004) and also sequester toxic alkaloids that are obtained from mites or ants (Daly et al., 2008; Saporito et al., 2009). Unfortunately, similar natural history and biology remains largely unknown for most species of *Cardioglossa*, which are also not diurnal like colorful dendrobatids and mantellids. The diets of two lowland species with drab coloration, *C. gracilis* and *C. leucomystax*, are dominated by ants and termites (Noble, 1924; Toft, 1982), but we know nothing of the diets of the many colorful species of *Cardioglossa*. Further, there are no investigations of alkaloids or peptides in the skins of these species nor whether their bright coloration and distinctive patterns might be signals to potential predators. There are no records of predation by birds or mammals, but there are records of predation by two venomous Central African snakes, *Causus lichtensteinii* and *C. maculatus*, on *Cardioglossa leucomystax* and some unidentified *Cardioglossa* (Ineich et al., 2006). However, at least in dendrobatids, warning colorations are not visually conspicuous to snakes as they can be for birds and mammals (Maan and Cummings, 2012). Of course, neither being colorful (e.g., species of Hylidae, Rhacophoridae) nor eating ants (e.g., species of Brevicipitidae, Microhylidae) necessitates that a frog produces or sequesters toxins. Regardless, there remain ample opportunities for field-based natural history studies that can contribute to understanding the functional role of coloration in *Cardioglossa*.

Many of the most striking colors, including reds, oranges, yellows, greens, and blues, are found in a clade of ten species that is nested within the diversity of *Cardioglossa*. Our divergence time analyses suggest that many of these colorful lineages—whether recognized as species, subspecies, or just geographically isolated populations—evolved relatively recently (Fig. 3). Pairs of lineages with distinctive coloration and patterns—for example, *C. oreas manengouba* and *C. oreas oreas*; populations of *C. nigromaculata*; *C. alsco* and *C. pulchra*—often appear to have evolved in allopatry, including on isolated mountains and islands. In most cases, non-specialists can easily sort these phenotypes into groups that correspond clearly to the geography of these populations, tempting one to postulate that geography played an important role in the divergence of color and/or pattern in allopatry. However, in other frogs with high levels of color variation across their distribution (e.g., the dendrobatid *Oophaga pumilio*), natural or sexual selection are likely more important than geography in generating this variation (Wang and Summers, 2010; Pröhl and Ostrowski, 2011; Rudh et al., 2011). Perhaps variation in predators across geographic space, including on isolated mountains or islands (such as Mount Manengouba or Bioko Island, respectively), has driven the rapid divergence of coloration among populations. It is also possible that color does not have an important functional role in these nocturnal and often secretive leaf litter frogs.

Many of the montane species of *Cardioglossa* are restricted to riparian forest fragments, and recent declines in these taxa will limit our ability to understand the function of coloration and pattern in these species. While some weak gene flow can continue among forest fragments (Arroyo-Lambaer et al., 2018), increased montane deforestation may lead to the further degradation and loss of these populations. Because many of these species are threatened (IUCN, 2020) and we are likely to lose some or even many distinctive species and populations in the near future, it is imperative to obtain natural history data for these poorly known species of *Cardioglossa*. Hopefully, studies of their natural history will help to inspire an interest from the public and national governments in conserving these remarkable species.

NOTE ADDED IN PROOF

During the generation of proofs for this study, Dubois et al. (2021) published a taxonomic treatment of all lissamphibians, which resulted in many taxonomic changes. The authors state that because "there is no support for the holophyly of *Cardioglossa*, it is synonymized with *Astylosternus*" (p. 222). First, while Dubois et al. (2021) suggest that their results are in contrast to Portik and Blackburn (2016) by stating that ". . . but they [Portik and Blackburn, 2016] obtained holophyletic groups for *Astylosternus*, with *Trichobatrachus* as sister taxon, and *Cardioglossa*, with *Arthroleptis* as sister-taxon" (p. 222), Dubois et al. (2021) also show *Cardioglossa* to be "holophyletic" (p. 415). By "no support," Dubois et al. (2021) presumably meant that the SHL-aLRT support value from Jetz and Pyron (2018) for the monophyly of *Cardioglossa* is less than their criterion of " 'strong' support" of 90% (p. 17). However, it is important to note that Dubois et al. (2021) and Jetz and Pyron (2018) do find *Cardioglossa* to be monophyletic, as found in other studies (Blackburn, 2008b; Portik and Blackburn, 2016; Portik et al., 2019) and notably in contrast to previous work by one of their authors (Pyron and Wiens, 2011). Second, it seems obvious that Dubois et al. (2021) did not mean to say that *Cardioglossa* should be considered a junior synonym of *Astylosternus* because their "complete cladonomy" clearly indicates that they consider *Cardioglossa* to be a junior synonym of *Arthroleptis* (or, to use their terminology, the kyronym of *Cardioglossa* is *Arthroleptis*). This is also indicated in their phylogeny (p. 415) in which all of the species recognized by us as *Cardioglossa*—which form a clade in their phylogeny—have been represented as *Arthroleptis*. Based on previously published studies (Blackburn, 2008b; Portik and Blackburn, 2016; Jetz and Pyron, 2018; Portik et al., 2019), we reject the consideration of *Cardioglossa* as a junior synonym of either *Astylosternus* or *Arthroleptis*.

DATA ACCESSIBILITY

GenBank accession numbers for new sequences from this study: MW623753–MW624206. Supplemental material is available at https://www.ichthyologyandherpetology.org/h2020165. Unless an alternative copyright or statement noting that a figure is reprinted from a previous source is noted in a figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source (American Society of Ichthyologists and Herpetologists, the DOI of the *Ichthyology & Herpetology* article, and any individual image credits listed in the figure caption) in accordance with the Creative Commons Attribution CC BY License.

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