Molecular Phylogenetics and Evolution 55 (2010) 699–710

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogeny and biogeography of Philippine bent-toed geckos (Gekkonidae: *Cyrtodactylus*) contradict a prevailing model of Pleistocene diversification

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ARTICLE INFO

Article history:
Received 27 August 2009
Revised 26 January 2010
Accepted 27 January 2010
Available online 2 February 2010

Keywords:
AMOVA
Biogeography
Diversification
Gene tree conflict
Islands
Pleistocene aggregate island diversification model
Sea-level fluctuations
Species trees

ABSTRACT

In the Philippines, Pleistocene sea level oscillations repeatedly connected and isolated neighboring islands. Hence, an understanding of the island platforms adjoined during periods of low sea level has led biologists to a suite of expectations that, taken together, represent a paradigm for the process of recent diversification in southeast Asia. We employ statistical tests of phylogenetic topology and population genetic analyses of widespread species of bent-toed geckos (*Cyrtodactylus*) to ascertain whether patterns of inter- and intra-specific diversity can be explained by a Pleistocene aggregate island model of diversification. Contrary to many classic studies of Philippine vertebrates, we find complex patterns that are only partially explained by past island connectivity. In particular, we determine that some populations inhabiting previously united island groups show substantial genetic divergence and are inferred to be polyphyletic. Additionally, greater genetic diversity is found within islands, than between them. Among the topological patterns inconsistent with the Pleistocene model, we note some similarities with other lineages, but no obviously shared causal mechanisms are apparent. Finally, we infer well-supported discordance between the gene trees inferred from mitochondrial and nuclear DNA sequences of two species, which we suspect is the result of incomplete lineage sorting. This study contributes to a nascent body of literature suggesting that the current paradigm for Philippine biogeography is an oversimplification requiring revision.

1. Introduction

Owing to its complex geological history and exceptionally variable geography, insular Southeast Asia serves as an excellent model for testing the effects of geographical change on phylogenetic processes (Brown and Guttman, 2002; Esselstyn et al., 2009; Evans et al., 2003; Jansa et al., 2006). The Philippines spans the area between the Sundaland-Eurasian and Philippines sea plates (Rangin, 1990; Yumul et al., 2003, 2009; Dimalanta and Yumul, 2004). Beginning in the Mesozoic, various geological processes shaped the distribution of land, including the collision of sea plates, strike-slip fault formation, and volcanic activity (Rangin, 1990; Yumul et al., 2003, 2009; Dimalanta and Yumul, 2004).

In addition to the geological processes that created the island arc system, Pleistocene sea-level fluctuations have caused repeated and dramatic changes in the size and connectivity of islands (Heaney, 1986; Hall, 1996, 1998, 2001; Rohling et al., 1998; Karns et al., 2000; Siddall et al., 2003). During periods of lower sea levels (100–140 m below current levels), nearby islands became connected into island platforms known as Pleistocene Aggregate Island Complexes (PAICs: Brown and Diesmos, 2002, 2009). In the Philippines, landmass amalgamation resulted in the removal of marine dispersal barriers between nearby islands, potentially allowing for biotic exchange between islands of today. As sea levels subsequently rose and inundated these larger islands, aggregate islands were fragmented and marine barriers re-established. This was then followed by later lowering sea levels, aggregate island reformation, and periods of relaxed dispersal barriers (Heaney, 1986; Heaney et al., 1998; Brown and Diesmos, 2009). This cyclic process of aggregate island formation and fragmentation is believed to have generated terrestrial biodiversity and also maintained it in distinct faunistic subprovinces.

The repeated formation and fragmentation of PAICs has served as an elegant explanatory model and heuristic tool for understanding evolutionary processes that may have contributed to the accumulation of biodiversity across the Philippines and Indo-Malayan Archipelago (Brown and Guttman, 2002; Catibog-Sinha and Heaney, 2006; Dickerson, 1928; Dickinson et al., 1991; Delacour and Mayr, 1946; Diesmos et al., 2002; Esselstyn and Brown, 2009; Heaney and Regalado, 1998; Inger and Voris, 2001; Inger, 1954; Kloss, 1929). Four large PAICs are consistently recognized as the major faunal demarcations within the country: the Greater Luzon,

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1055-7903/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ympev.2010.01.027

Greater Mindanao, Greater Palawan, and Greater Negros–Panay Faunal Regions (Heaney, 1985, 1986; Heaney et al., 2005). There also exist many isolated islands surrounded by deep-ocean channels that never shared land-bridge connections to other islands. These include large islands like Mindoro and small islands like the Babuyan and Batanes archipelagos, Dinagat, Siargao, Siquijor, Lubang, Camiguin Sur, and Sibuyan (Fig. 1; Brown and Diesmos, 2002, 2009; Brown et al., 2008). Islands separated by deep-ocean channels tend to hold restricted range endemic species (Brown and Diesmos, 2002; Dickerson, 1928; Heaney et al., 1998; Steppan et al., 2003).

Previous studies involving many vertebrate taxa (e.g., Heaney et al., 2005; Jones and Kennedy, 2008; Roberts, 2006a,b) have explained faunal affinity by evoking geological history, with suites of species said to be endemic to particular groups of historically connected islands (Heaney et al., 1998; Kennedy et al., 2000; Brown and Diesmos, 2002; Brown and Guttman, 2002; Evans et al., 2003; Esselstyn et al., 2004). This pattern of diversity partitioned by historically connected island groups can be assumed to vary with a species' ability to disperse across terrestrial landscapes and oceanic barriers (Roberts, 2006a,b). Expectations derived from the PAIC model include testable a priori hypotheses that address the predicted distribution of taxonomic diversity (Esselstyn and Brown, 2009): (1) populations from a given island should be more closely related to each other than to populations on different islands within the same PAIC; (2) intra-PAIC populations are expected to be more closely related to each other than inter-PAIC populations; and (3) monophyletic lineages are expected to be found within PAICs, not across PAICs, though incomplete lineage sorting, variation in relative dispersal abilities, and the timing of island colonization could obscure these patterns.

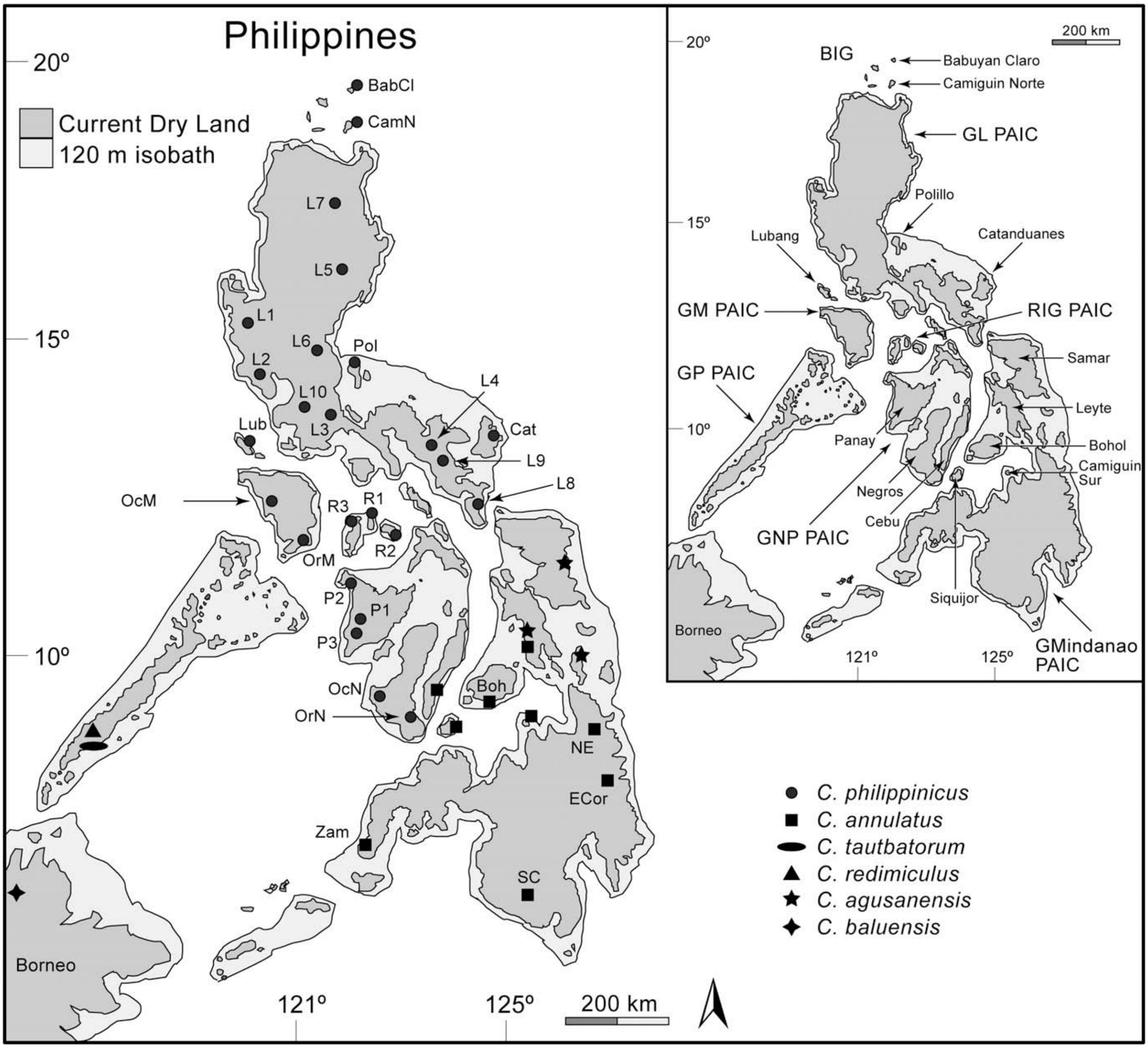

Fig. 1. Distribution of *Cyrtodactylus* samples from the Philippines (see species key at lower right). Locality codes on larger map indicate sampling localities, corresponding to terminals and clades in Figs. 3–5 and Table 3. The inset shows the five recognized major Pleistocene aggregate island complexes (PAICs) and additional deep-water islands. Abbreviated PAIC names are defined in Table 3. Current islands in the Philippines are shown in medium grey; light gray areas enclosed in black 120 m bathymetric contours indicate the hypothesized maximum extent of land during the mid- to late Pleistocene.

Recently, studies have revealed phylogenetic patterns inconsistent with the pure PAIC model of diversification (Jones and Kennedy, 2008; McGuire and Kiew, 2001; Evans et al., 2003; Steppan et al., 2003; Alfaro et al., 2004; Brown and Diesmos, 2009). In a study of *Cerberus* water snakes, Alfaro et al. (2004) found strong support for Luzon populations of *C. rhynchops* to be sister to a clade of western Visayan populations of *C. rhynchops* and *C. microlepis* from Luzon. McGuire and Kiew (2001) found strong support for a Mindoro + Sibuyan (Romblon Province) clade within *Draco quadrasi*, support for the polyphyly of Luzon *D. spilopterus*, and support for a Luzon + Western Visayan clade within *D. spilopterus*. Jones and Kennedy (2008) showed support for Luzon + Mindoro clades in several passerines (*Eumyias panayensis*, *Ficedula hyperythra, Phylloscopus trivirgatus*, and *Zosterops montanus*), as well as support for polyphyly of Greater Luzon populations. Evans et al. (2003) demonstrated that some clades of fanged frogs defy PAIC boundaries and that when older and more recent invasions of the Philippines are compared, older clades span more PAICs. Finally, Steppan et al. (2003) showed support for a rodent species of the genus *Apomys* to possess a Sibuyan + Negros distribution; however, without genetic samples available from other central Philippine islands, this relationship remains poorly tested. Additional exceptions are discussed by Brown and Diesmos (2009).

Phylogenies of Philippine vertebrates inconsistent with the PAIC model of diversification highlight the potential risk associated with delineating taxonomic units according to PAIC geography (Esselstyn and Brown, 2009). Currently there is a need for rigorous evaluation of the PAIC paradigm, including summary of patterns that contradict the model's expectations. However, few studies exist that have done so (but see Esselstyn and Brown, 2009; Brown and Guttman, 2002; Evans et al., 2003; Heaney et al., 2005; Roberts, 2006a,b). An appropriate system for testing the PAIC model would be a well-sampled, widespread lineage with a geographic range spanning multiple PAICs and deep-water islands. In this regard, Philippine bent-toed geckos of the genus *Cyrtodactylus* are an excellent model system.

Philippine *Cyrtodactylus* include two widespread species (*C. annulatus* and *C. philippinicus*), three restricted-range endemics (*C. agusanensis*, *C. redimiculus*, and *C. tautbatorum*), and one undescribed, restricted-range species from southwestern Mindanao (Welton et al., in press). The known distribution of *C. philippinicus* spans the Luzon, Mindoro, and Visayan PAICs, as well as deep-water, oceanic islands of Lubang and the Romblon and Babuyan island groups (Brown and Alcala, 1978; Fig. 1).

In this study we investigate the biogeography of Philippine *Cyrtodactylus* from a phylogenetic and population-genetic perspective, testing whether patterns of extant diversity are consistent with predictions derived from the PAIC model. Our data reveal patterns largely inconsistent with the PAIC model of diversification and we conclude that this model of diversification provides a useful heuristic and generates a suite of testable predictions, but is likely an oversimplification of the evolutionary history of *Cyrtodactylus*.

2. Materials and methods

2.1. Taxon sampling and data collection

Ingroup sampling included 229 individuals collected from 40 localities, with all five currently recognized Philippine *Cyrtodactylus* represented (Fig. 1; Appendix 1). To assess the monophyly of the Philippine species as well as investigate appropriate outgroup taxa, a broad sampling of *Cyrtodactylus* species from the Asian mainland, Borneo, and Sulawesi, were included (Appendix 1). For all 260 samples, the mitochondrial gene NADH Dehydrogenase Subunit 2 (ND2) and components of six flanking transfer RNA genes (tRNA^{met}, tRNA^{trp}, tRNA^{ala}, tRNA^{asn}, tRNA^{cys}, tRNA^{tyr}) were sequenced (Table 1). For 214 of these samples, the second intron of the nuclear ribosomal protein gene L35 (RPL35) was sequenced (Table 1). Primers for the nuclear locus were developed from a cDNA library (M. Fujita, unpublished data). All sequences were deposited in GenBank (ND2 Accession Nos. GU366079–366104, GU550709–550942; RPL35 Accession Nos. GU458034–458247).

Genomic DNA was extracted from liver tissues stored in 95–100% ethanol following a guanidine thiocyanate protocol (Esselstyn et al., 2008). We used a combination of published and newly developed primers (Table 1), with cycle sequencing reactions for ND2 performed with combinations of nested and internal primers. We used the following thermal profiles for ND2: 4 min at 94°, followed by 35 cycles of 94° for 30 s, 52–53° for 30 s, and 72° for 1 min 30 s, and a final extension phase at 72° for 7 min. and RPL35: 4 min at 94°, followed by 35 cycles of 94° for 30 s, 58° for 30 s, and 72° for 1 min 30 s, and a final extension phase at 72° for 7 min. Amplified products were visualized on 1.5% agarose gels. PCR products were purified with 1 μL of a 20% dilution of ExoSAP-IT (US78201, Amersham Biosciences, Piscataway, NJ) on the following thermal profile: 31 min at 37°, followed by 15 min at 80°. Cycle sequencing reactions were run using ABI Prism BigDye Terminator chemistry (Ver. 3.1; Applied Biosystems, Foster City, CA), and purified with Sephadex Medium (NC9406038, Amersham Biosciences, Piscataway, NJ) in Centri-Sep 96 spin plates (CS-961, Princeton Separations, Princeton, NJ). Purified product was analyzed with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Gene sequence contigs were assembled and edited using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI).

2.2. Sequence alignment and phylogenetic analyses

Initial alignments were produced in Muscle (Edgar, 2004), and manual adjustments made in MacClade 4.08 (Maddison and Maddison, 2005). To assess phylogenetic congruence between the mitochondrial and nuclear data, we inferred the phylogeny for each subset independently using likelihood and Bayesian analyses, and performed a partition homogeneity test in PAUP 4.0b 10 (Swofford, 1999) with 100 replicates to assess set congruence. Following the observation of statistically significant incongruence between datasets, we decided to conduct separate analyses on each locus, as

Table 1
Summary of primers and annealing temperatures used in this study.

Locus	Primer name	Sequence	Location	Annealing temperatures	Primer source
NADH 2	Metf6	5′-AAGCTTTCGGGCCCATACC-3′	External	52–53°	Macey et al. (1999)
	CO1H	5′-AGRGTGCCAATGTCTTTGTGRTT-3′	External	52–53°	Macey et al. (1999)
	CyrtInt.F1	5′-TAGCCYTCTCYTCYATYGCCC-3′	Internal	52–53°	This study
	CyrtInt.F2	5′-CRCACAACCACYTAAACCMHRCC-3′	Internal	52–53°	This study
	CyrtInt.R1	5′-ATTGTKAGDGTRGCYAGGSTKGG-3′	Internal	52–53°	This study
	CyrtInt.R2	5′-TRTGGGCRATRGARGAGARGGC-3′	Internal	52–53°	This study
	CyrtNest.F2	5′-ATAAARYCHCAYCAYCCHCGHCG-3′	Nested	52–53°	This study
	CyrtNest.R1	5′-ARTGCAAATTTGGAGACGYACCC-3′	Nested	52–53°	This study
Rpl35, Intron 2	N66.F	5′-GCTAAACAAGCACAGAGTTGATCC-3′	External	58°	M. Fujita, (unpublished data)
Rpl35, Intron 2	N67.R	5′-TCAGGCTCAGAAAGRACTATTATGG-3′	External	58°	M. Fujita, (unpublished data)

well as the combined data. Exploratory analyses of the combined dataset of 260 individuals (including 46 lacking RPL35 sequences) and a reduced dataset of 214 individuals (no missing data) supported identical relationships; we therefore chose to include all available data (260 individuals) for subsequent analyses of the concatenated ND2 + RPL35 dataset, except for statistical hypothesis testing. Alignments and resulting topologies were deposited in TreeBase (SN4900).

Parsimony analyses were conducted in PAUP* 4.0b 10 (Swofford, 1999) for the mitochondrial, nuclear, and combined datasets, with gaps treated as missing data and all characters weighted equally. Most parsimonious trees were estimated using heuristic searches with 1000 random addition-sequence replicates and tree bisection and reconnection (TBR) branch swapping. To assess clade confidence, nonparametric bootstrapping was conducted using 1000 bootstrap replicates, each with 100 random addition-sequence replicates and TBR branch swapping.

Partitioned Bayesian analyses were conducted in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) for the mitochondrial, nuclear, and combined datasets. The mitochondrial dataset was partitioned by codon position for the protein-coding region of ND2, and the six flanking tRNAs (tRNA^{met}, tRNA^{trp}, tRNA^{ala}, tRNA^{asn}, tRNA^{cys}, tRNA^{tyr}) were analyzed as a single subset. The Akaike information criterion (AIC), as implemented in Modeltest v3.7 (Posada and Crandall, 1998), was used to select the best model of nucleotide substitution for each subset (Table 2). The best-fit model for each of the four subsets of mitochondrial data was the general time reversible (GTR) model with a proportion of invariable sites (I) and gamma distributed rate variation among sites (Γ). The best-fit model for the nuclear data was GTR + Γ. A rate multiplier model was used to allow substitution rates to vary among subsets, and default priors were used for all model parameters. We ran four independent Metropolis-coupled MCMC analyses, each with four chains and the default heating scheme (temp = 0.2). All analyses were run for 10 million generations, sampling every 5000 generations. To assess stationarity, all sampled parameter values and log-likelihood scores from the cold Markov chain were plotted against generation time and compared among independent runs using Tracer v1.4 (Rambaut and Drummond, 2007). Furthermore, we plotted the cumulative and non-overlapping split frequencies of the 20 most variable nodes, and compared split frequencies among independent runs using Are We There Yet? [AWTY (Wilgenbusch et al., 2004)]. All samples showed patterns consistent with stationarity after 5 million generations, hence the first 50% of samples were discarded as burn-in for all three analyses.

In preliminary Bayesian analyses of the combined dataset, the independent runs failed to converge. We tried (1) replacing all GTR + I + G models with GTR + G models, and (2) removing the taxon responsible for topological conflict between the mitochondrial and nuclear data (*C. redimiculus*). Neither of these strategies yielded convergence. However, after lowering the incremental heating temperature to 0.02 the chains appeared to converge.

Table 2
Models of evolution selected by AIC and applied for partitioned, model-based phylogenetic analyses.

Partition	AIC model	Model applied	Number of characters
NADH 2, 1st codon position	GTR + I + G	GTR + I + G	346
NADH 2, 2nd codon position	GTR + I + G	GTR + I + G	346
NADH 2, 3rd codon position	GTR + I + G	GTR + I + G	346
tRNAs Met, Trp, Ala, Asn, Cys, Tyr	GTR + I + G	GTR + I + G	385
N66	GTR + G	GTR + G*/ GTR + I + G**	555

* GTR + G was applied for this data partition for Bayesian analyses.
** GTR + I + G was applied for this data partition in partitioned ML analyses.

Partitioned maximum likelihood (ML) analyses were conducted in RAxMLHPC v7.0 (Stamatakis, 2006) for all three datasets under the same partitioning strategy as for Bayesian analysis. The more complex model (GTR + I + Γ) was used for all subsets, and 100 replicate ML inferences were performed for each analysis. Each inference was initiated with a random starting tree, and employed the rapid hill-climbing algorithm (Stamatakis et al., 2007). Clade confidence was assessed with 1000 bootstrap pseudoreplicates employing the rapid hill-climbing algorithm (Stamatakis et al., 2008).

2.3. Topology tests and AMOVAs

We asked two general questions concerning the patterns of Philippine *Cyrtodactylus* diversity: (1) Does the PAIC model of diversification explain the patterns of genetic diversity found in Philippine *Cyrtodactylus*? (2) Are the patterns of genetic diversity in Philippine *Cyrtodactylus* similar to those noted in other lineages (Jones and Kennedy, 2008; McGuire and Kiew, 2001; Steppan et al., 2003; Alfaro et al., 2004)? We evaluated each question using the approximately unbiased (AU) test (Shimodaira and Hasegawa, 2001; Shimodaira, 2002). The constraints of monophyly for these questions are outlined in Fig. 2, with hypotheses 1 and 2 derived from the PAIC predictions; the remaining hypotheses have been observed in other taxa and are, in part, derived from expectations based on geological history and/or island proximity.

Although significant topological incongruence was observed between the mitochondrial and nuclear loci, exploration of the conflict revealed that it had no observable impact on the relationships within the widespread *Cyrtodactylus philippinicus*. Although topological differences existed between analyses with and without individuals lacking nuclear data, we conservatively chose to use the reduced, and combined (ND2 + RPL35) dataset of 214 individuals for all topology tests. Using the same settings as the RAxML analyses described above, 100 ML searches were performed under each of the eight monophyly constraints. All 900 trees produced by RAxML (100 from the unconstrained analysis and 100 from each of the eight constrained analyses), were filtered in PAUP to remove identical topologies. All 900 trees were unique. A modified version of RAxML (provided by Alexandros Stamatakis) allowed the per-site likelihoods to be estimated for each tree under a partitioned model. An AU test was then performed on the per-site likelihoods from all 900 using CONSEL v0.1i (Shimodaira and Hasegawa, 2001). The *P*-value reported for a given hypothesis is the largest *P*-value of all the trees inferred under that constraint.

Analyses of molecular variation (AMOVAs) were conducted on sequence data for *C. philippinicus*, with both broad- and fine-scale analyses to investigate the amount of genetic variation explained at different spatial scales. All analyses were run with 1000 permutations in Arlequin 3.1 (Excoffier et al., 2005). Broad-scale analyses were conducted to elucidate the amount of genetic variation that could be explained among PAICs, among islands within PAICs, and within islands. For the two PAICs where *C. philippinicus* was sampled across multiple islands within the PAIC (Greater Luzon and Greater Negros–Panay), fine-scale analyses were conducted to estimate the amount of genetic variation that could be explained among islands within the PAIC, among populations within islands, and within populations.

3. Results

3.1. Taxon sampling, data collection, and sequence alignment

The complete, aligned matrices contain 31 and 27 samples of non-Philippine species for the mitochondrial and nuclear data-

Table 3
Summary of sampling localities and sample sizes for *Cyrtodactylus philippinicus*. Locality codes correspond to those provided in Figs. 1 and 3–5.

Species	Island	PAIC or island group (code)	Locality	Locality code	Sample size
Cyrtodactylus philippinicus	Luzon	Greater Luzon (GL)	Zambales Province	L1	2
			Subic Bay	L2	2
			Quezon National Park	L3	2
			Mt. Isarog	L4	2
			Mt. Palali	L5	4
			Aurora Province	L6	2
			Apaya	L7	11
			Bulusan Province	L8	2
			Mt. Malinao	L9	6
			Mt. Banahao	L10	3
	Polillo	Greater Luzon (GL)		Pol	14
	Catanduanes	Greater Luzon (GL)	Gigmoto	Cat	4
	Babuyan Claro	Babuyan Island Group (BIG)		BabCl	3
	Camiguin Norte	Babuyan Island Group (BIG)		CamN	10
	Lubang	Isolated, deep-water Island		Lub	10
	Romblon	Romblon Island Group (RIG)		R1	1
	Sibuyan	Romblon Island Group (RIG)		R2	4
	Tablas	Romblon Island Group (RIG)		R3	5
	Mindoro	Greater Mindoro (GM)	Mindoro Oriental Province	OrM	3
			Mindoro Occidental Province	OcM	10
	Negros	Greater Negros–Panay (GNP)	Negros Oriental Province	OrN	3
			Negros Occidental Province	OcN	3
	Panay	Greater Negros–Panay (GNP)	Sibalom	P1	4
			Pandan	P2	9
			Valderamma	P3	1

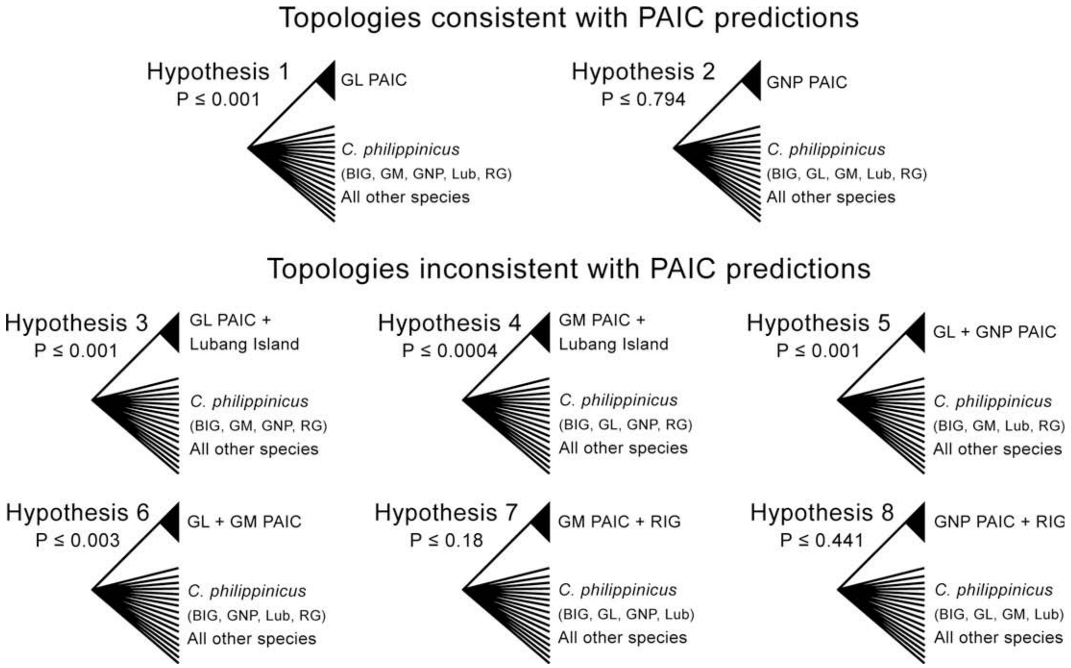

Fig. 2. Eight topological hypotheses derived from predictions of the Pleistocene aggregate island (PAIC) model and topological patterns noted in other taxa. Each hypothesis is illustrated by constraint trees used in AU tests. The highest *P*-values recovered from each test are shown.

sets, respectively. Following initial unrooted analyses, and assuming the root of the tree does not lie within Philippine *Cyrtodactylus*, we polarized the tree using samples of *C. intermedius* from Thailand. We reduced the mitochondrial matrix to match the nuclear dataset for all topology tests. Within the mtDNA gene sequences, 81 bp from the tRNA genes could not be confidently aligned and were excluded from analyses. Within each dataset, variable and parsimony-informative characters were observed as follows: 869 and 835 out of 1423 for the mitochondrial data and 226 and 171 out of 555 for the nuclear data.

3.2. Phylogenetic analyses

Analyses of the mitochondrial (ND2), nuclear (RPL35), and combined data (ND2 + RPL35) resulted in topologies with high bootstrap support (MP and ML) and posterior probabilities (Figs. 3–5). Topologies were congruent across these analyses, with the exception of the placement of *C. redimiculus* (Fig. 3). All analyses of the mitochondrial data supported the monophyly of Philippine *Cyrtodactylus*, with *C. baluensis* from Borneo sister to this clade (Fig. 3). However, all analyses of nuclear data recovered a strongly

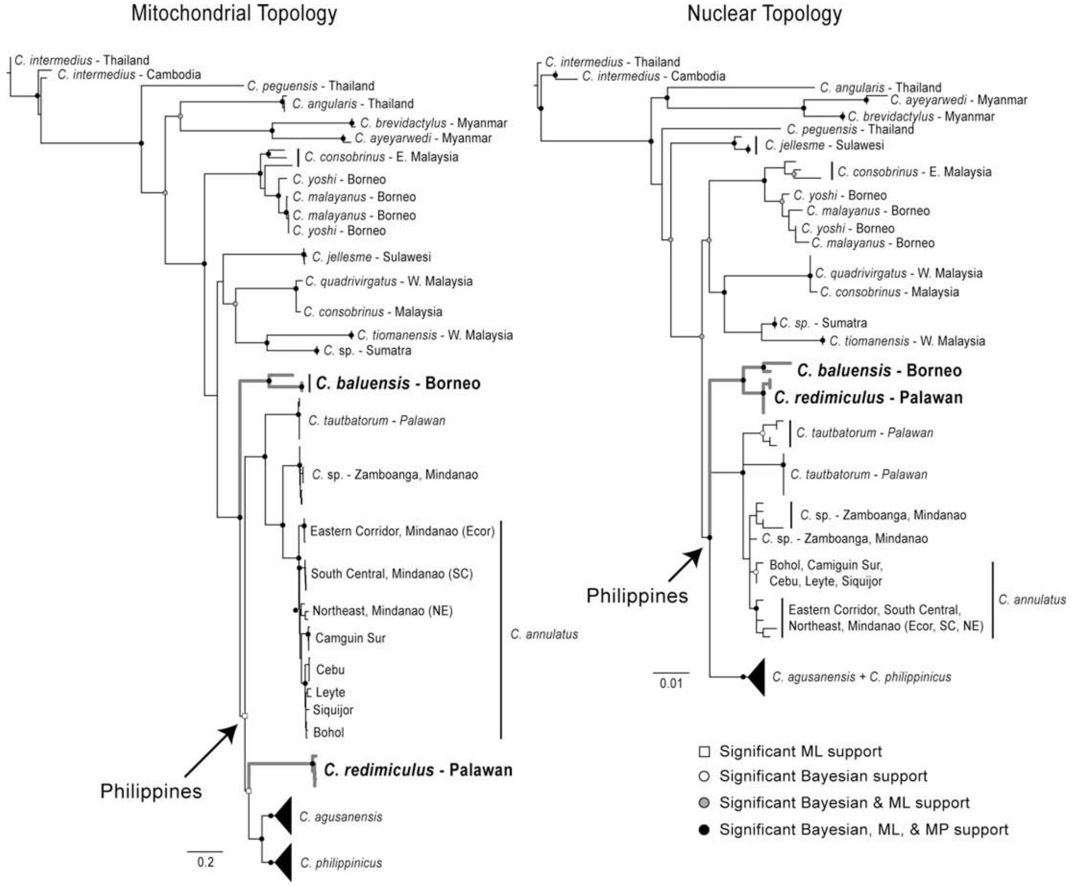

Fig. 3. Hypothesized relationships of Philippine *Cyrtodactylus*, illustrated by ML estimates (mitochondrial ND2, −ln L 26168.842888; nuclear N66, −ln L 3040.700786). Nodes supported by ⩾95% Bayesian PP, ⩾70% MLBP, and ⩾70% MPBP were considered significantly supported. Terminals are labeled with abbreviated taxonomic names, followed by sampling localities (codes presented in Fig. 1 and Table 3). Black triangles designate *C. agusanensis* and *C. philippinicus* clades (Fig. 4). Strongly supported mito-nuclear discordance is emphasized by bold, black branches.

supported clade of *C. baluensis* + *C. redimiculus* from Palawan (Fig. 3). To assess the effect of this discordance on the overall topology, three pruned datasets were created and analyzed for the independent and combined datasets: all taxa minus *C. baluensis*, all taxa minus *C. redimiculus*, and all taxa minus both *C. baluensis* and *C. redimiculus*. Removal of these taxa had no impact on the relationships recovered (results not shown), and we therefore, elected to include both taxa in analyses of the combined ND2 + RPL35 dataset. Weakly supported differences between the two loci were also observed in the outgroup taxa (Fig. 3). The position of sampled individuals of *Cyrtodactylus peguensis* and *C. jellesme* varied between analyses and between the two sampled loci, with most analyses providing only weak support for their placement in the phylogeny (Fig. 3). All analyses of individual and combined datasets strongly support both the monophyly of *C. baluensis* and all Philippine species and the relationships among the remaining Philippine species of *Cyrtodactylus,* regardless of the observed mito-nuclear discordance (Figs. 3–5).

Whereas the nuclear RPL35 locus provided less resolution for intra-species relationships, the resulting topologies remained consistent with the strongly supported relationships recovered from analyses of the mitochondrial data. The number of most parsimonious trees and consistency indices resulting from MP analyses of the three datasets were 35 trees/CI = 0.289 for the mitochondrial dataset, 33 trees/CI = 0.744 for the nuclear dataset, and 37 trees/CI = 0.312 for the combined dataset.

Paraphyletic relationships within two clades of outgroup species were strongly supported in all analyses (Fig. 5, Clades A, B). Samples of *C. consobrinus* are found in each clade. In addition to *C. consobrinus*, Clade A consists of samples of *C. yoshi* and *C. malayanus*, and both species appear to be paraphyletic. Clade B consists of more samples of *C. consobrinus* and samples of *C. quadrivirgatus*. Given the possibility that the chosen root for analyses likely influenced outgroup relationships, additional data for these taxa should be analyzed under different rooting schemes before definitive conclusions may be drawn.

Analyses of the combined dataset mirrored those of the nuclear dataset in their support of a monophyletic *C. baluensis* + *C. redimiculus* (Fig. 5, Clade C). The clade's phylogenetic position in the tree, however, is weakly supported. The remaining Philippine species of *Cyrtodactylus* (as currently recognized) make up three large clades (Fig. 5, Clades D–F), each consisting of several well-supported subclades. Clade D includes *Cyrtodactylus tautbatorum* from Palawan Island (Welton et al., 2009) sister to an undescribed species (Welton et al., in press) from southwestern Mindanao Island and true *C. annulatus* sampled from its known distribution, including the type locality. In all analyses, Clades E and F were consistently supported to be reciprocally monophyletic (Fig. 5). Clade E consists of three genetically distinct and strongly supported subclades of *C. agusanensis* sampled from Dinagat, Samar, and Leyte Islands respectively (Fig. 5). The highly structured Clade F consists of *C. philippinicus* sampled throughout its known distribution in the Philippines (Fig. 5). Taking a conservative approach, at least four subclades (Fig. 5) were consistently recovered and strongly supported as follows: (F.1) consists of samples from southwest Luzon Island and the deep-water oceanic island of Lubang; (F.2) consists

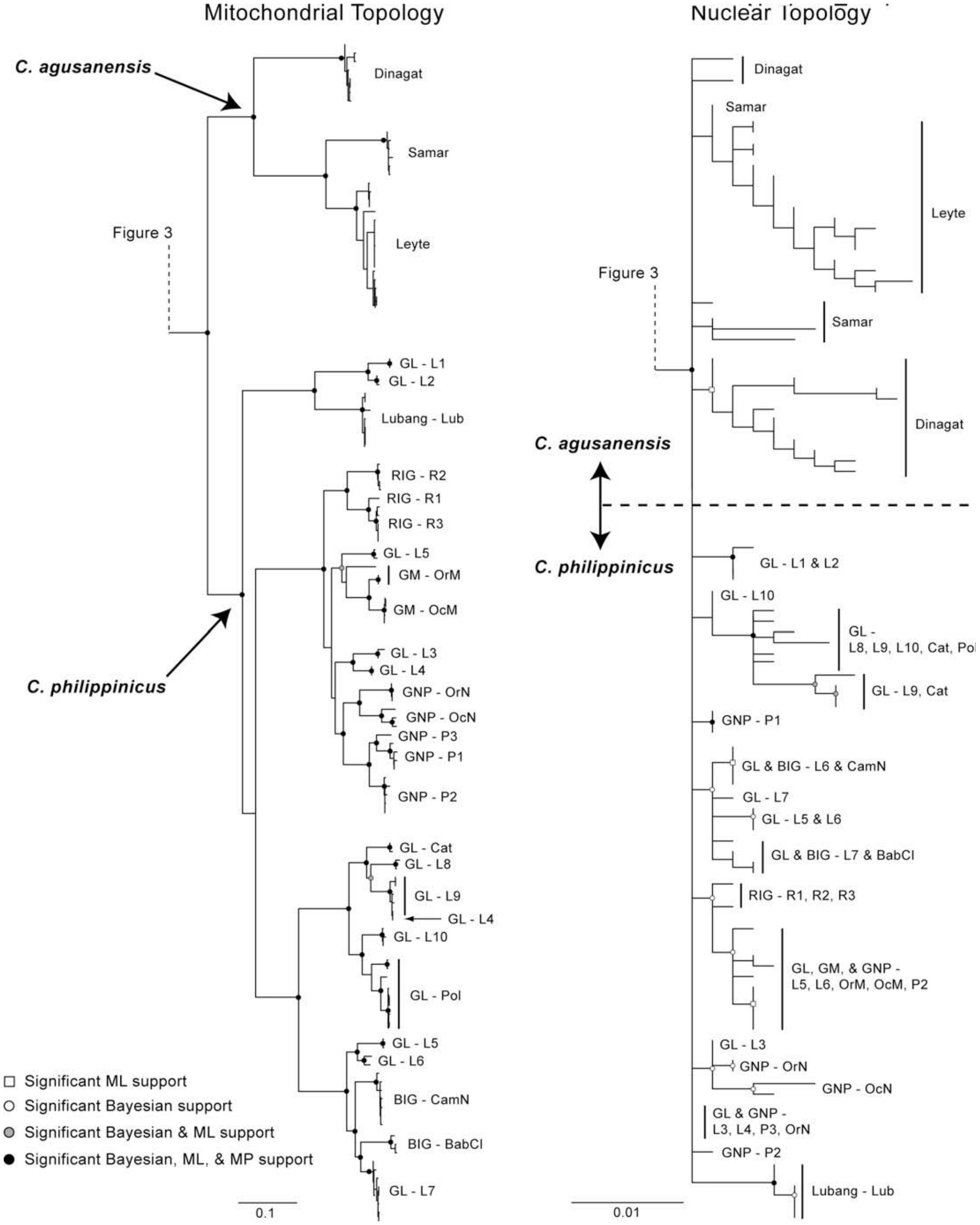

Fig. 4. Maximum likelihood estimate of *C. agusanensis* and *C. philippinicus* relationships (continued from Fig. 3). Nodes supported by ⩾95% Bayesian PP, ⩾70% MLBP, and ⩾70% MPBP were considered significantly supported. Terminals are labeled with codes denoting Pleistocene aggregate island complexes and sampling localities (defined in Fig. 1 and Table 3).

of samples from the islands of Luzon, Mindoro, Negros, Panay, Romblon, Sibuyan, and Tablas; (F.3) consists of samples from the islands of Catanduanes, Luzon, and Polillo; and (F.4) consists of samples from the islands of Babuyan Claro, Camiguin Norte, and Luzon.

3.3. Topology tests and AMOVAs

The AU tests resulted in mixed support for topologies consistent with PAIC predictions as well as those observed in other taxa. For the hypotheses derived from PAIC predictions, the Greater Luzon PAIC hypothesis was rejected, but we failed to reject the Greater Negros–Panay PAIC hypothesis. All but two of the hypotheses derived from topologies observed in other taxa were rejected (Fig. 2). The two hypotheses we failed to reject were #7, which predicts a monophyletic relationship between all populations from the Greater Mindoro PAIC and the Romblon Island Group, and #8, which predicts a monophyletic relationship between all populations from the Greater Negros–Panay PAIC and the Romblon Island Group (Fig. 2).

Analyses of molecular variation within *C. philippinicus* samples revealed significant ($P < 0.001$) portions of genetic variation explained by each level of the AMOVA (Fig. 7). The among PAICs and among islands within PAICs categories explained relatively equal proportions of genetic variation, while the within island category explained the greatest proportion of genetic diversity (Fig. 7A).

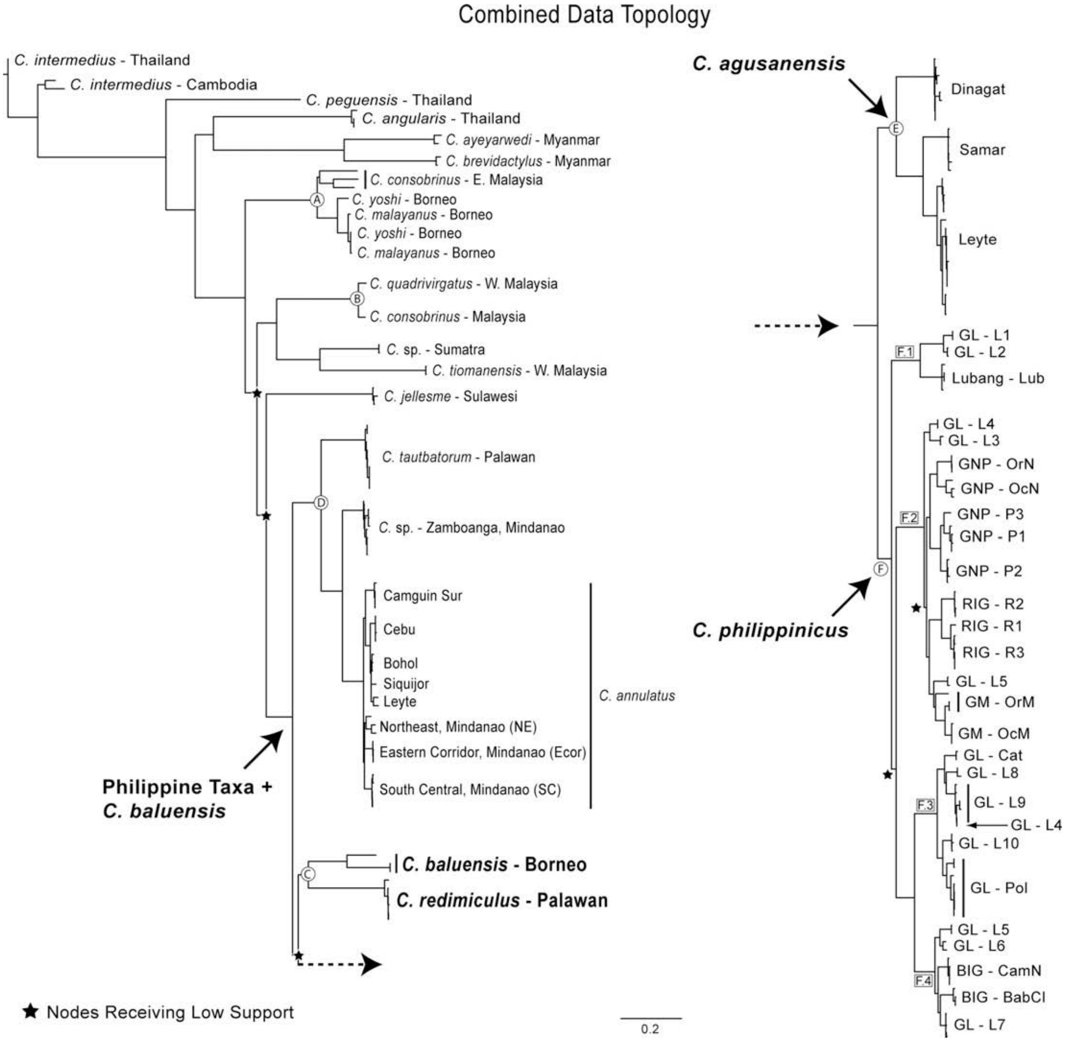

Fig. 5. Maximum likelihood estimate of combined data for Philippine and selected Southeast Asian *Cyrtodactylus* (preferred ML tree, −ln L 30107.924842; ND2 + N66 genes). Nodes with low phylogenetic support in all analyses indicated by black stars, all other nodes supported by ⩾95% Bayesian PP, ⩾70% MLBP, and ⩾70% MPBP. Terminals are labeled with abbreviated PAIC codes (Fig. 1, Table 3), taxonomic names, and/or sampling localities. Taxonomic labels for lineages affected by mito-nuclear discordance highlighted in bold (*C. baluensis* and *C. redimiculus*). Alpha and alpha-numerical labels correspond to clades referred to in Sections 3 and 4.

Samples were available from multiple islands within the Greater Luzon and Greater Negros–Panay PAICs, and the fine-scale AMOVAs within each of these PAICs revealed the majority of genetic variation to be explained among populations within islands (Fig. 7B). For analyses of both Greater Luzon and Greater Negros–Panay samples, all proportions of genetic variation were significant except for the within populations category (Fig. 7B). Although the inter-population diversity appears to account for the majority of genetic diversity among PAICs, differences were observed between the Greater Luzon and Greater Negros–Panay PAIC (Fig. 7B). These could be the result of the number of islands sampled and/or the size of the islands within each PAIC, with Luzon being significantly larger with much greater topographical relief than any islands within the Greater Negros–Panay PAIC.

4. Discussion

4.1. Taxon sampling

Our widespread sampling of individuals across the range of most Philippines species allowed for fine-scale resolution of phylogenetic relationships and inter-population genetic diversity. However, the relationship of *C. redimiculus* from Palawan to all other Philippine species remains ambiguous. Although our attempt to widely sample outgroup taxa resulted in some well-supported relationships, the most closely related species to *C. baluensis* and the sister lineage to all Philippine *Cyrtodactylus* is not clear. Additional outgroup sampling and the incorporation of more quickly evolving nuclear loci would aid resolution of these relationships.

4.2. Mito-nuclear discordance

Discordant mitochondrial and nuclear gene trees are well documented in the literature (reviewed by Buckley et al., 2006). For example, several evolutionary processes potentially giving rise to conflict among gene trees, including incomplete lineage sorting, genetic polymorphism, hybridization, and introgression (Buckley et al., 2006; Gompert et al., 2008; Bossu and Near, 2009; Leaché et al., 2009). Although new methods have become available for distinguishing among processes, evolutionary mechanisms giving rise to observed mito-nuclear discordance are not easily ascertained, and ideal data sets should include many, unlinked loci (Holder et al., 2001; Buckley et al., 2006). For SE Asian *Cyrtodactylus*, additional data are needed before proper tests can be conducted; however, we suggest preliminary alternative explanations.

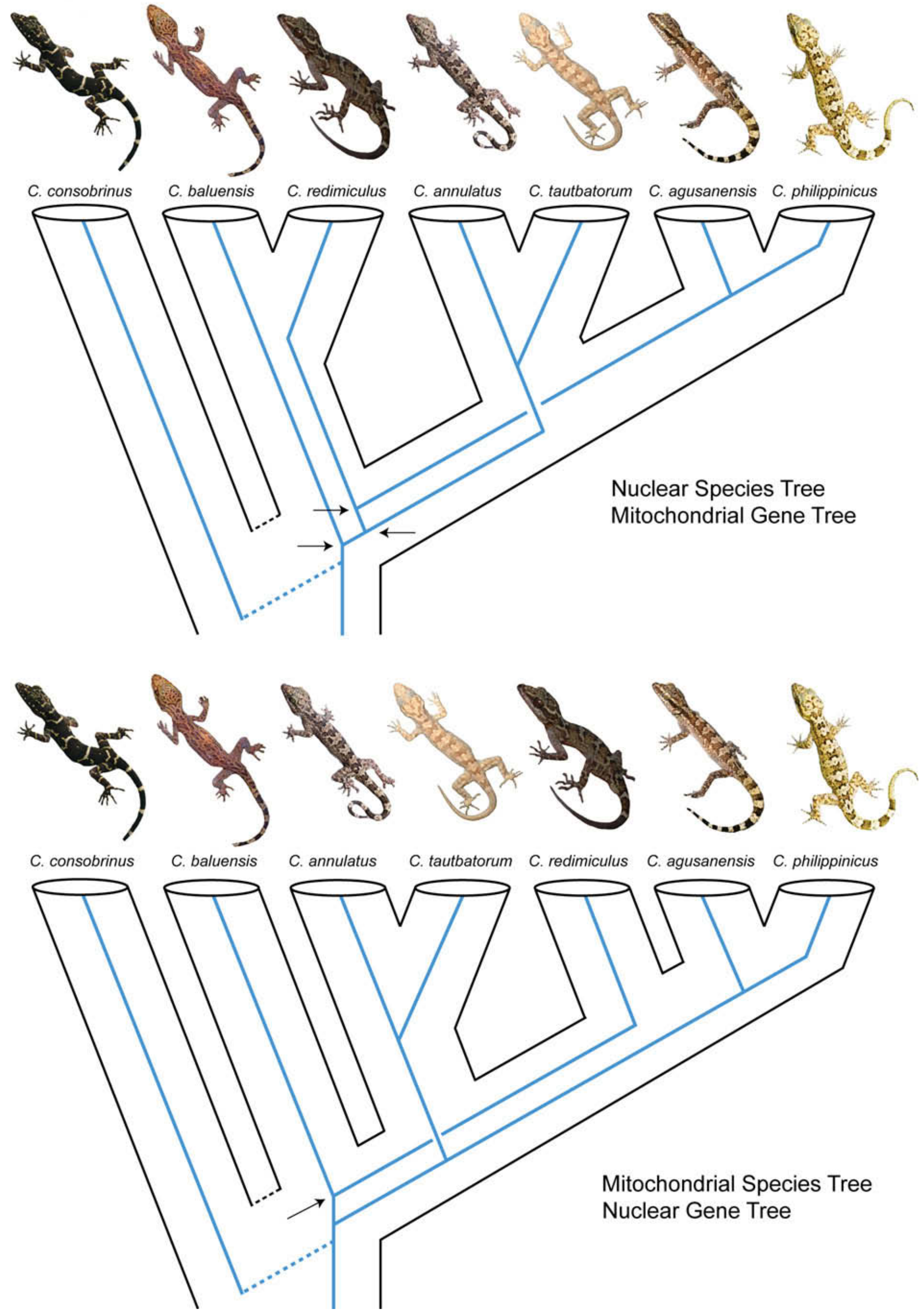

Fig. 6. Diagramatic summary of possible gene trees and species trees, showing gene trees (blue lines) traced within species trees (tubes). The outgroup species, *C. consobrinus*, is included to emphasize its morphological similarity with *C. redimiculus*. The top figure illustrates a species tree enclosing a mitochondrial gene tree with three deep coalescent events (indicated by arrows). The lower figure shows a possible species tree and nuclear gene tree, with one deep coalescent event required to reconstruct the geneology (indicated by arrow). Photographs by Lee Grismer, Chan Kin Onn, RMB, and CDS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hybridization between *C. baluensis* and *C. redimiculus* is possible, however, for several reasons, we doubt this is the cause of the observed mito-nuclear discordance. First, although Palawan and Borneo are geographically close to one another, *C. baluensis* and *C. redimiculus* have never been documented to possess overlapping distributions. Second, all Philippine species except *C. redimiculus* share a dorsal banding pattern and coloration with *C. baluensis*. *Cyrtodactylus redimiculus* possesses dorsal bands and the head and body pattern is dominated by a white-on-black reticulum (Brown and Alcala, 1978; Welton et al., 2009, in press), reminiscent of the color pattern of Bornean and Peninsular Malaysian *C. consubrinus*. In fact, newborn and juvenile specimens of *C. redimiculus* are nearly identical to specimens of *C. consobrinus* (RMB and ACD, personal observation). In cases of mitochondrial introgression, which can be viewed as a special case of hybridization, analyses of the mitochondrial data alone tend to suggest common ancestry (Linnen and Farrell, 2008)—the opposite of the pattern we find here (Fig. 6). In our analyses, it was the nuclear gene tree that showed a sister relationship between *C. baluensis* and *C. redimiculus*, contrary to what we would expect in a case of mtDNA introgression or a misleading signal derived from deep coalescence of mtDNA gene lineages.

Given the non-overlapping distributions, disparate morphologies, and observed patterns of mito-nuclear discordance atypical

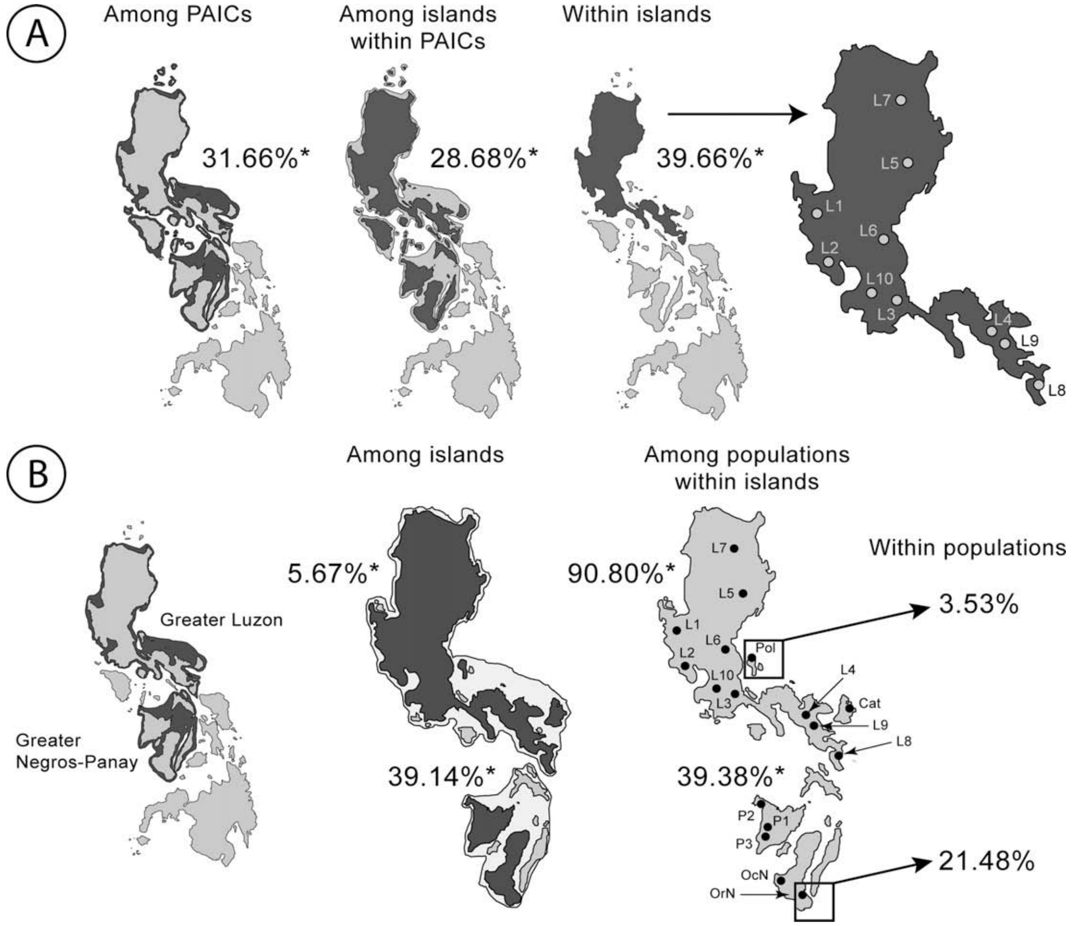

Fig. 7. Maps of the Philippine Islands, with dark highlights illustrating the geographical scale of interest for each level of analyses of molecular variation (AMOVA). Percentages indicate the proportion of genetic variation explained by each level of the three-way AMOVAs conducted for *C. philippinicus*. (A) Analysis of broad scale geographic variation. (B) Analysis of fine-scale variation for the two PAICs where samples were available for multiple islands.

for processes of hybridization and introgression (Fig. 3), we suspect that the gene tree conflicts are likely the result of incomplete lineage sorting in the nuclear marker. If this is assumed to be the process that has given rise to the strongly supported discordance, which topology represents the true species tree?

If the discordance has arisen from incomplete lineage sorting, the more rapidly evolving mitochondrial locus would be expected to better represent the species tree (Birky et al., 1989; Moore, 1995; Palumbi et al., 2001; Bonaccorso, 2009; Bossu and Near, 2009). Assuming the topologies recovered from analyses of the mitochondrial marker are representative of the species tree, one deep coalescent event is necessary to trace the nuclear gene tree on the species tree (Fig. 6). In contrast, if the topology recovered from analyses of the nuclear marker best represents species tree relationships, three deep coalescent events are necessary to trace the mitochondrial gene tree on the species tree (Fig. 6).

4.3. Phylogeny and cryptic genetic diversity

Although we focus on diversity within Philippine bent-toed geckos, our results provide clear evidence that a broad phylogenetic study of the genus *Cyrtodactylus* is needed to accurately estimate the group's species-level diversity. Outgroup sampling resulted in analyses showing undocumented species diversity from Sumatra and the paraphyletic relationships of *C. consobrinus* and *C. yoshi*, suggesting a need for taxonomic revision (Figs. 3 and 5). Analyses of the combined mitochondrial and nuclear datasets revealed strong support for most relationships among outgroup samples; however, low support was continually observed for several short internodes among Malaysian, Bornean, and Philippine species (Fig. 5). Additionally, the low support for higher-level relationships among clades of species from Borneo, Malaysia, Indonesia, and the Philippines, seems to suggest that rapid dispersal and diversification of *Cyrtodactylus* has taken place across this region (Figs. 3 and 5). The routes of dispersal into the Philippines, and the source populations giving rise to the Philippine radiation remain unclear; however, the topology (Figs. 3 and 5) implies the probable reinvasion of the mainland from the islands. A higher-level study of *Cyrtodactylus*, incorporating analyses of morphology and a more substantial multi-locus dataset, would provide additional insights into biogeographic patterns, levels of undocumented species diversity, and the prevalence of gene tree discordance within the group.

If our supposition that the mito-nuclear discordance between *C. baluensis* and *C. redimiculus* is the result of incomplete lineage sorting is correct, then *C. baluensis* is the sister species to Philippine taxa. Very short internodes between *C. baluensis*, *C. redimiculus*, the clade containing *C. annulatus* + *C. tautbatorum* + an undescribed species from SW Mindanao, and the clade containing *C. agusanensis* + *C. philippinicus* indicate the possible rapid radiation of bent-toed geckos across Borneo and the Philippines (Figs. 3 and 5). For some Philippine species, intra-island geography appears to have played a larger role in the diversification of island endemic taxa (Figs. 3 and 5). Other Philippine species appear to have experienced recent and rapid dispersal across historically separate deep-water oceanic barriers. For example, true *C. annul-*

atus appears to have dispersed across much of the Greater Mindanao PAIC, into the Greater Negros–Panay PAIC (Cebu Island populations), and has reached the deep-ocean islands of Camiguin Sur and Siquijor (Figs. 3 and 5). In contrast, some populations that were once considered extensions of the range of *C. annulatus* now appear to be island endemic species (Figs. 3 and 5: Welton et al., 2009, in press).

Cryptic diversity has been documented as a global phenomenon (Pfenninger and Schwenk, 2007). We now suspect the phenomenon to also be common among Southeast Asian gekkonids (Brown et al., 2008, 2009; Brown and Diesmos, 2009). Dense population sampling across the range of *C. annulatus, C. agusanensis*, and *C. philippinicus* revealed significant genetic structure and genetically distinct clades of previously undocumented diversity (but see Welton et al., 2009, in press). *Cyrtodactylus agusanensis* samples make up three unique clades, each from a distinct island in the Greater Mindanao PAIC (Figs. 4 and 5). A strange pattern of population genetic diversity is observed for *C. philippinicus*, where, conservatively, four highly supported clades partition the diversity of samples across the species' range (Figs. 4 and 5). A cursory inspection of specimens from the full range of *C. philippinicus* reveals only slight morphological variation (CDS and RMB, personal observation). However, upon closer inspection of populations from distinct genetic clades (Figs. 4 and 5), several diagnostic morphological character differences are apparent. Thus, what appeared to be a "widely distributed" species may actually represent a complex of cryptic species (CDS and RMB, unpublished data). Taxonomic studies of other Philippine gekkonids (Gaulke et al., 2007; Brown et al., 2008, 2009, in press) suggest that examination of non-traditional characters may be most useful for resolving cryptic species boundaries in Philippine gekkonids.

4.4. Diversification and population-level patterns

Although we rejected some topologies predicted from a PAIC-based model, and most hypotheses derived from patterns observed in other taxa, we failed to reject some patterns in both categories. The failure to reject the monophyly of both the Greater Mindoro and Greater Negros–Panay PAICs with the Romblon Island Group supports observations made in previous studies (McGuire and Kiew, 2001; Steppan et al., 2003). Although the isolated, deep-ocean islands of the Romblon Island Group possess many endemic taxa, the degree of historical dispersal to and from this area may be underestimated and deserving of more detailed investigation.

The AMOVAs revealed that the largest percentage of genetic diversity within *C. philippinicus* is explained by inter-population differences (Fig. 7A and B), not by PAICs. Although significant levels of diversity are still explained by PAICs (Fig. 7A), the results are similar to patterns noted by Esselstyn and Brown (2009), where modern islands, as opposed to paleo-islands, best explained genetic diversity for Philippine shrews. However, this study, that of Esselstyn and Brown (2009), and all others that have examined predictions derived from a PAIC model (e.g., Heaney et al., 2005; Peterson and Heaney, 1993; Roberts, 2006a,b), have suffered from a paucity of available samples, with most authors only able to sample broadly within 1 or 2 PAICs. This places severe limits on researchers' capacity for testing the PAIC paradigm and for developing alternative models. However, based on available data, it seems likely that several factors, including sea-level fluctuations, island proximity, geological uplift, topography on large islands, and clade age, probably have played roles in determining the diversification trajectories of lineages. Combining all of these factors into a single predictive model would be challenging, but testing models based solely on any one of these factors remains possible and useful. The emerging pattern from such studies is that PAICs have indeed influenced patterns of biodiversity partitioning, but they are not the only explanation for the diversification of terrestrial Philippine biodiversity.

Without time-calibrated phylogenies, and a general paucity of closely related fossil calibrations, it is difficult to say whether the observed divergences occurred during the Pleistocene. Given our results, and those of other studies that explicitly test the PAIC model of diversification (Evans et al., 2003; Esselstyn and Brown, 2009; Roberts, 2006a,b), we are left with many unanswered questions. If the PAIC model does not suffice, roughly what proportion of Philippine biodiversity has been generated by PAIC-relevant processes? If the last century's paradigm does not suffice, how "wrong" is it? These and other synthetic questions that address the processes of evolutionary diversification must be assessed with a comparative, multi-taxon, multi-locus approach.

Acknowledgments

We thank the Protected Areas and Wildlife Bureau (PAWB) of the Philippine Department of Environment and Natural Resources (DENR) for facilitating collecting and export permits necessary for this and related studies, wherein we are particularly grateful to M. Lim, C. Custodio, and A. Tagtag. Financial support for fieldwork for CDS was provided by a Panorama Fund grant from The University of Kansas Natural History Museum and Biodiversity Center, a Madison and Lila Self Fellowship from the University of Kansas, Fulbright and Fulbright-Hayes Fellowships, as well as an NSF DEB 0804115 to CDS and NSF EF-0334952 and DEB 0743491 to RMB. For the loans of specimens (museum abbreviations follow Leviton et al., 1985), we thank J. Vindum and A. Leviton (CAS), R. Sison (PNM), J. Ferner (CMNH), A. Resetar and H. Voris (FMNH), R. Crombie (USNM), T. LaDuc (TNHC), D. Bickford (RMBR), and M. Lakim of Sabah Parks (SP). Critical reviews of the manuscript were provided by C. Linkem, J. Sukumaran, and L. Trueb. We thank the CAS's Stearns Fellowship for funding multiple visits to examine comparative material and CDS and RMB thank A.C. Alcala, M. Diesmos and their families for their support and hospitality during numerous visits to the Philippines.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.01.027.

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