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Phylogeny of Philippine slender skinks (Scincidae: *Brachymeles*) reveals underestimated species diversity, complex biogeographical relationships, and cryptic patterns of lineage diversification

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ABSTRACT

The spectacular, virtually endemic radiation of Philippine semi-fossorial skinks of the genus *Brachymeles* represent one of the few radiations of scincid lizards to possess both fully limbed and limbless species. And yet, nothing is known of the phylogenetic relationships of this exceptional group. Morphologically similar body plans have made it difficult to assess species-level diversity, and the genus has long been recognized as one of the more modest radiations of southeast Asian lizards. However, recent large-scale survey efforts have resulted in the discovery of numerous new species, and taxonomic studies indicate that the diversity within the genus *Brachymeles* is grossly underestimated. In this study we provide the first robust estimate of phylogenetic relationships within the genus *Brachymeles* using a multi-locus dataset and nearly complete taxonomic sampling. We provide statistical tests of monophyly for all polytypic species and two widespread limb-reduced species and our results indicate wholesale deviations from past summaries and taxonomic evaluations of the genus. With few exceptions, we are able to reject the monophyly of all polytypic and widespread species, thereby validating the need for large-scale taxonomic revisions. Our results reveal that the limbless, monotypic, genus *Davewakeum* is nested within *Brachymeles*. Mapping of body form on our preferred phylogenetic tree suggests that limb-reduction and digit loss has occurred on multiple occasions in the history of the genus. A Bayesian reconstruction of ancestral areas indicates strong statistical support for a minimum of five major dispersal events that have given rise to a major component of the observed species diversity on separate Pleistocene aggregate island platforms of the archipelago.

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1. Introduction

Only four genera of scincid lizards are known to possess both fully limbed and limbless species (*Brachymeles*, *Chalcides*, *Lerista*, and *Scelotes*; Lande, 1978; Wiens and Slingluff, 2001; Brandley et al., 2008). Of these four genera, the genus *Brachymeles* is the least well known, with recent studies indicating the recognized diversity of the group is vastly underestimated (Siler et al., 2009, 2010a,b, in press; Siler and Brown, 2010). Within the genus, all but one of the 25 recognized species are endemic to the Philippines (Brown, 1956; Brown and Rabor, 1967; Brown and Alcalá, 1980; Siler et al., 2009, 2010a,b; Siler and Brown, 2010; Siler et al., in press); the exception is *B. apus* from northern Borneo (Hikida, 1982). Thirteen species are pentadactyl (*bicolor*, *boholensis*, *boulengeri*, *gracilis*, sp. A [Masbate Island; Siler and Brown, 2010],

makusog, *mindorensis*, *orientalis*, *schadenbergi*, *talinis*, *taylori*, sp. B [Luzon + Babuyan islands; Siler and Brown, 2010], and sp. C [Jolo Island; Siler and Brown, 2010]), eight are non-pentadactyl, with incompletely developed limbs and reduced numbers of digits (*bonitae*, *cebuensis*, *elerae*, *muntingkamay*, *pathfinderi*, *samarensis*, *tridactylus*, and *wrighti*), and four are entirely limbless (*apus*, *minus*, *lukbani*, and *vermis*). Within the non-pentadactyl species, there exist a wide range of limb- and digit-reduced states. Some species have minute limbs that lack full digits (*bonitae*, *cebuensis*, *muntingkamay*, *samarensis*, *tridactylus*; Duméril and Bibron, 1839; Brown, 1956; Brown and Rabor, 1967; Siler et al., 2009). Other non-pentadactyl species have moderately developed limbs with four digits on the hands and feet (*elerae*, *wrighti*), or four digits on the feet and five digits on the hands (*pathfinderi*; Taylor, 1917, 1925). All species are semi-fossorial and typically found in dry, rotting material inside decaying logs or in loose soil, forest floor detritus, and leaf litter.

The genus *Brachymeles* was first described by Duméril and Bibron (1839) for the small, limb-reduced species *Brachymeles*

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bonitae. As species diversity has accumulated, various authors have noted morphological variation among island populations of many of the polytypic and widespread species (Taylor, 1922; Brown, 1956; Brown and Rabor, 1967; Brown and Alcala, 1980). In *Brachymeles*, several species (*B. Bonitae*, *B. samarensis*, *B. tridactylus*) currently span recognized faunal regions within the Philippines, or Pleistocene Aggregate Island Complexes (PAICs; Brown and Guttman, 2002; Brown and Diesmos, 2002, 2009; Fig. 1, and defy biogeographic boundaries as traditionally conceived (Brown and Diesmos, 2009). Among skinks and other Philippine land vertebrates, multiple lineages have similar widespread distributions, spanning multiple PAICs. These widespread distributions have been the focus of many recent studies, which have revealed that few endemic Philippine reptiles actually possess broad distributions spanning regional faunistic boundaries (Brown et al., 2000, 2009; Brown and Diesmos, 2002, 2009; Siler et al., 2010a,b; Siler and Brown, 2010; Welton et al., 2009, 2010, in press). Although these recent efforts have shed light on cryptic diversity among

Philippine vertebrates, the continued recognition of many widespread species may still compromise our understanding of patterns of regional diversity. Presently, the impressive diversity of endemic vertebrate species in the Philippines is recognized to be distributed among: (1) Pleistocene Aggregate Island Complexes (PAICs; Inger, 1954; Heaney, 1985; Voris, 2000; Brown and Diesmos, 2002), (2) individual islands within PAICs, and (3) upland subcenters of diversity within individual landmasses (review: Brown and Diesmos, 2009).

Historically, the shared body plans and similar external morphological features among populations of *Brachymeles*, and the absence of dense population sampling across the Philippines, proved problematic for diagnosing species (Brown, 1956; Brown and Rabor, 1967; Brown and Alcala, 1980). Although long considered to be a small clade of Southeast Asian lizards (the last revision enumerated only 15 species; Brown and Alcala, 1980), recent studies have significantly increased the known species diversity and expanded the range of variation in body form (Siler et al., 2009,

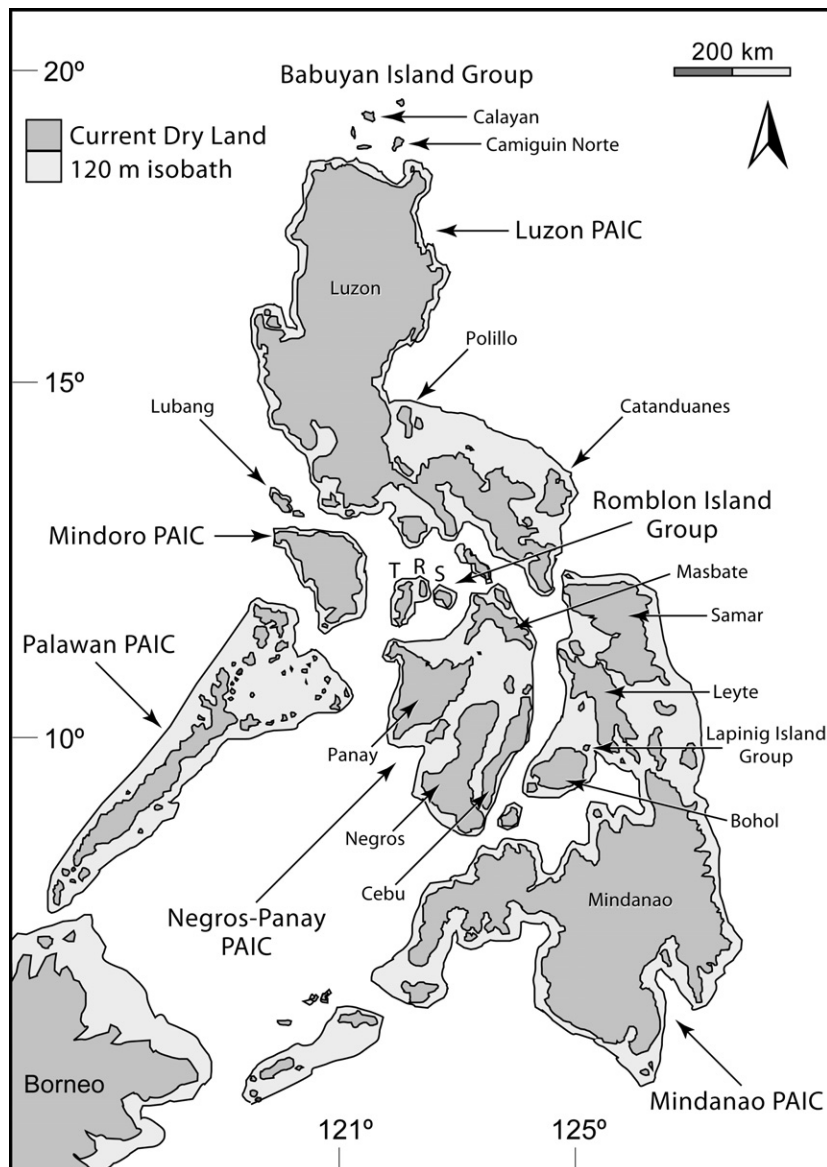


Fig. 1. Map of the Philippines showing the five recognized major Pleistocene Aggregate Island Complexes (PAICs) and additional deep-water islands. Current islands in the Philippines are shown in medium grey; light grey areas enclosed in black 120 m bathymetric contours indicate the hypothesized maximum extent of land during the mid- to late Pleistocene.

2010a,b; Siler and Brown, 2010). Additionally, several rare, mid-to-high elevation species long represented by only a few specimens, in some cases without knowledge of their exact type locality (e.g., *Brachymeles bicolor*, *B. elerae*, *B. wrighti*, *B. pathfinderi*), have recently been rediscovered (Siler, 2010; Siler et al., in press; Siler and Brown, 2010). These studies, coupled with increased sampling throughout the Philippines, have provided a comprehensive dataset with which to begin evaluating the taxonomic stability of polytypic and widespread species across the Philippines. Additionally, the availability of tissue samples for all but two known species of *Brachymeles* now allows for robust estimates of phylogenetic relationships among recognized widespread and polytypic species, and evaluation of species boundaries. For example, Siler and Brown (2010) recently revised two polytypic species (*B. boulengeri* and *B. schadenbergi*) and one widespread species (*B. talinis*); this work resulted in the recognition of ten genetically and morphologically distinct species. Several other species including *B. samarensis* and *B. bonitae* (Brown, 1956; Brown and Rabor, 1967; Brown and Alcala, 1980) are still recognized as having widespread distributions that span multiple historically recognized biogeographic provinces in the Philippines (Dickerson et al., 1925; Kloss, 1929; Inger, 1954; Brown and Diesmos, 2002).

In this study we investigate the biogeography of *Brachymeles* from a phylogenetic perspective, providing the first estimate of phylogenetic relationships for this unique radiation of Southeast Asian lizards. We strove to estimate the phylogenetic position of *Brachymeles* among scincid lizards in order to provide insight into patterns of body form evolution and polarity of character change

and provide the first statistical tests of several hypotheses. We provide the first glimpse into the major body form transitions in *Brachymeles*, particularly with respect to miniaturization, limb-reduction, and digit loss. We also employ a Bayesian ancestral area reconstruction to gain insight into the biogeographical history of the genus. Finally we test the following taxonomic hypotheses: (1) *Brachymeles* is monophyletic; (2) All recognized and formerly recognized polytypic species are monophyletic; and (3) All recognized widespread species are monophyletic. Our data reveal patterns inconsistent with all of the above predictions and at odds with currently recognized taxonomy; we conclude that species diversity within the genus is vastly underestimated and that cryptic patterns of lineage diversification prevail in this poorly known group of Southeast Asian lizards.

2. Materials and methods

2.1. Taxon sampling and data collection

Ingroup sampling included 90 individuals collected from 43 localities, with 23 of the 25 currently recognized species of *Brachymeles* represented (Fig. 2; Appendix 1; Siler and Brown, 2010). The two missing species in our analyses are *Brachymeles vermiformis* and *B. wrighti*. *Brachymeles wrighti* is known from two damaged specimens from northern Luzon Island, and *B. vermiformis* occurs in the Sulu archipelago (where biologists are not permitted to work due to logistical and security obstacles). No tissues have ever been collected for either of these species.

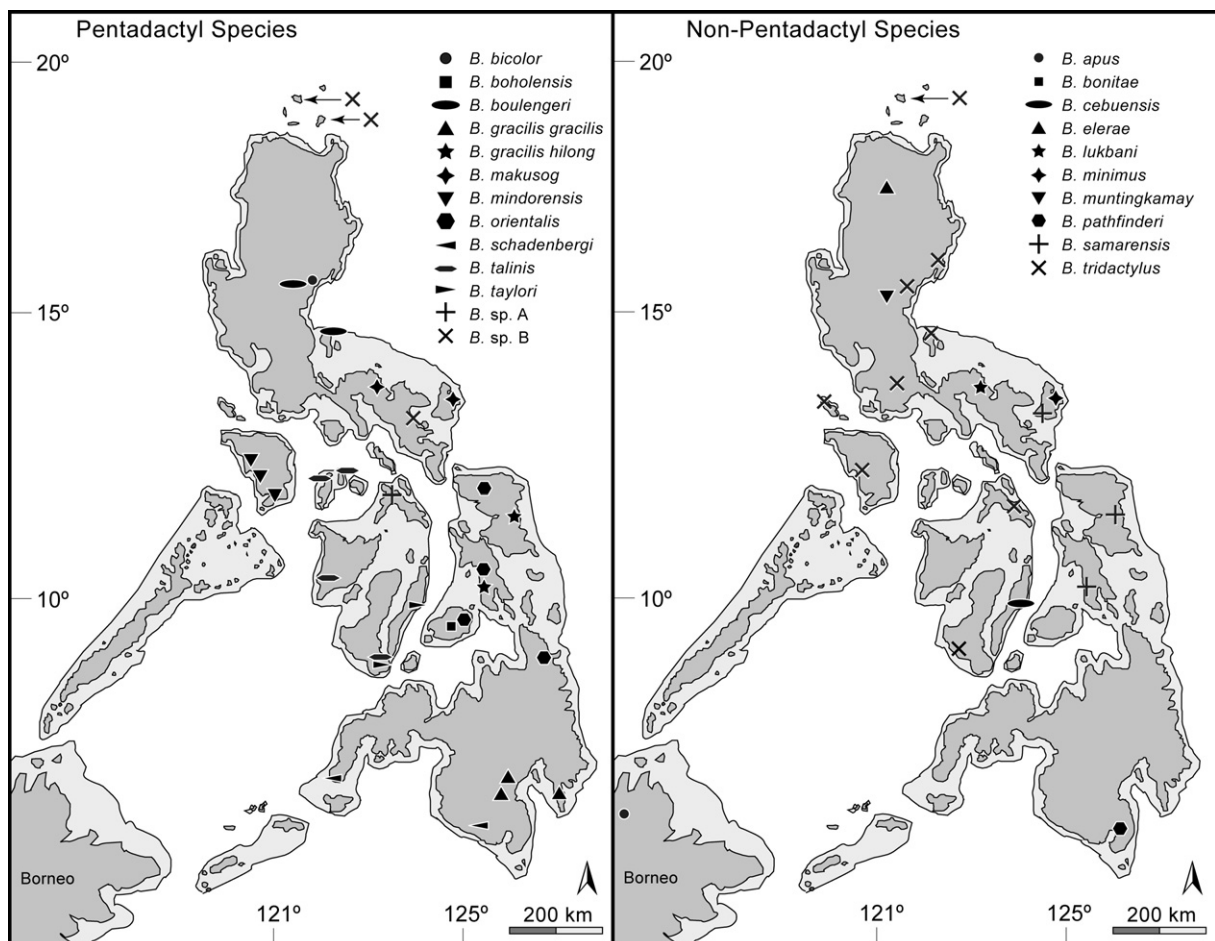


Fig. 2. Distribution of pentadactyl and non-pentadactyl *Brachymeles* samples from the Philippines (see species keys within each map). Current islands in the Philippines are shown in medium grey; light grey areas enclosed in black 120 m bathymetric contours indicate the hypothesized maximum extent of land during the mid- to late Pleistocene.

To assess the monophyly of the genus as well as investigate appropriate outgroup taxa, a broad sampling of scincid species from the subfamilies Lygosominae and Scincinae were included, as well as a single outgroup sample from the family Lacertidae (Appendix 1). For all 108 samples, complete or partial sequences were collected for mitochondrial NADH Dehydrogenase Subunit 1 (ND1), NADH Dehydrogenase Subunit 2 (ND2), ATPase 8 (ATP8), and ATPase 6 (ATP6) genes (Table 1). Additionally, three nuclear loci, Brain-derived Neurotrophic Factor (BDNF), R35, and PTGER4, were completely sequenced for nearly all ingroup samples and many of the outgroup samples (Table 1, Appendix 2). All sequences were deposited in GenBank (Appendix 2).

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, as well as a variety of thermal profiles (Table 1). Amplified products were visualized on 1.5% agarose gels. PCR products were purified with 1 μ L 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 (NC9406038, Amersham Biosciences, Piscataway, NJ) in Centri-Sep 96 spin plates (CS-961, Princeton Separations, Princeton, NJ). Purified products were analyzed with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Continuous gene sequences 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 gene independently using likelihood and Bayesian analyses, and performed pairwise partition homogeneity tests in PAUP 4.0b 10 (Swofford, 2002) with 100 replicates for each pairwise comparison to assess set congruence. Following the observation of no statistically significant incongruence between datasets, we felt justified in using the combined, concatenated, data for subsequent analyses. Exploratory analyses of the combined dataset of 108 individuals (including outgroup taxa with missing data for several genes) and a reduced dataset of individuals with no missing data exhibited identical relationships; we therefore chose to include all available data (108 individuals) for subsequent analyses

of the concatenated dataset. Alignments and resulting topologies were deposited in TreeBase (SN11187).

Parsimony analyses were conducted in PAUP* 4.0b 10 (Swofford, 2002), 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 support, 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). The mitochondrial dataset was partitioned by codon position for the protein-coding region of ND1 and ND2 and by gene region for the short gene regions ATP8 and ATP6. The Akaike Information Criterion (AIC), as implemented in jModeltest v0.1.1 (Guindon and Gascuel, 2003; Posada, 2008), was used to select the best model of nucleotide substitution for each partition (Table 2). The best-fit model for each data partition was implemented in subsequent Bayesian analyses. 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 an incremental heating temperature of 0.05. All analyses were run for 18 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). Finally, 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)]. Although all samples showed patterns consistent with stationarity after 2.5 million generations (i.e., the first 12.5%), we conservatively discarded the first 20% of samples as burn-in.

In preliminary Bayesian analyses of the combined dataset, the independent runs failed to converge. We tried (1) lowering the incremental heating temperature to 0.02, (2) using an unconstrained branch length prior with an exponential distribution of 25 (Siler et al., 2010c; Marshall et al., 2006; Marshall, 2010), and (3) removing outgroup taxa with large amounts of missing data. Although some of the trials of individual permutations of parameters resulted in a failure to converge, the incorporation of the above, plus an unconstrained branch length prior with an exponential distribution and a mean of 25 resulted in convergence. Once complete convergence was achieved, we proceeded with final analyses, presented here.

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

| Locus | Primer name | Sequence | Annealing temperatures | Primer source |
|--------|--------------|------------------------------------|------------------------|-----------------------------|
| NADH 1 | 16dr | 5'-CTACGTGATCTGAGTTCAGACCGGAG-3' | 52–53° | Brandley et al. (2005) |
| | tMet | 5'-TCGGGGTATGGGCCRRARAGCTT-3' | 52–53° | Brandley et al. (2005) |
| NADH 2 | ND2.Brach.F1 | 5'-TTATCGCAACAAAACACCACCC-3' | 52–53° | This study |
| | ND2.Brach.R1 | 5'-AGCYCAGAGGTGATTACAGC-3' | 52–53° | This study |
| | ND2.Brach.R2 | 5'-CCGCTGGATTGGGTGTTTACG-3' | 52–53° | This study |
| ATP8,6 | ATP.F | 5'-CTCAGARATCTGCGGGYCAAATCACA-3' | 58° | Brandley (unpublished data) |
| | ATP.R | 5'-GTGCYTTCTCGRRTAATRTCYCGTCAT-3' | 58° | Brandley (unpublished data) |
| BDNF | BDNF.F | 5'-CCCCAATGAAAGAAGTGASCCTC-3' | 55° | Crottini et al. (2009) |
| | BDNF.R | 5'-TGGGTAGTTCGGCACTGAGAATTCC-3' | 55° | Crottini et al. (2009) |
| PTGER4 | PTGER4.F1 | 5'-GACCATCCCGGCCGTMATGTTTCATCTT-3' | 55° | Townsend et al. (2008) |
| | PTGER4.R5 | 5'-AGGAAGGARCTGAAGCCCGCATACA-3' | 55° | Townsend et al. (2008) |
| R35 | R35.F | 5'-GACTGTGGAYGAYCTGATCAGTGTGG-3' | 55° | Fry et al. (2006) |
| | R35.R | 5'-GCCAAAATGAGSAGAAARCGCTTCTG-3' | 55° | Fry et al. (2006) |

Table 2

Models of evolution selected by AIC and applied for partitioned, phylogenetic analyses.

| Partition | AIC model | Number of characters |
|----------------------------|----------------|----------------------|
| NADH 1, 1st codon position | GTR + Γ | 322 |
| NADH 1, 2nd codon position | GTR + Γ | 322 |
| NADH 1, 3rd codon position | GTR + Γ | 322 |
| NADH 2, 1st codon position | GTR + Γ | 287 |
| NADH 2, 2nd codon position | GTR + Γ | 287 |
| NADH 2, 3rd codon position | GTR + Γ | 287 |
| ATP8 | HKY + Γ | 157 |
| ATP6 | GTR + Γ | 682 |
| BDNF | GTR + Γ | 715 |
| PTGER4 | HKY + Γ | 490 |
| R35 | GTR + Γ | 689 |

Partitioned maximum likelihood (ML) analyses were conducted in RAXMLHPC v7.0 (Stamatakis, 2006) on the concatenated dataset the same partitioning strategy as for Bayesian analysis. The more complex model (GTR + Γ) was used for all subsets (Table 2), 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 100 bootstrap pseudoreplicates employing the rapid hill-climbing algorithm (Stamatakis et al., 2008).

2.3. Topology tests

We tested taxonomy- and phylogeography-based hypotheses to address questions concerning the patterns of *Brachymeles* diversification (Fig. 3): (1) Is the genus *Brachymeles* monophyletic? (2) Are the currently and formerly recognized polytypic species (*B. boulengeri*, *B. gracilis*, *B. schadenebergi*) monophyletic? (3) Are the two “widespread” species (*B. bonita*, *B. samarensis*) monophyletic? (4) Does the PAIC model of diversification explain the patterns of genetic diversity found in widespread species of Philippine *Brachymeles*? And, (5) Are the patterns of genetic diversity in widespread species of Philippine *Brachymeles* similar to those noted in other lineages (Siler et al., 2010c; Jones and Kennedy, 2008; McGuire and Kiew, 2001; Stepan et al., 2003; Alfaro et al., 2004)?

We evaluated each question using Bayesian methods, and the approximately unbiased (AU) test (Shimodaira and Hasegawa, 2001; Shimodaira, 2002) as implemented in Siler et al. (2010c). The topological constraints for these questions are illustrated in Fig. 3, with hypotheses 1–6 derived from taxonomic questions, and hypotheses 7 and 8 derived from the PAIC predictions; the remaining hypotheses (9–11) have been observed in other taxa and are, in part, derived from expectations based on geological history and/or island proximity. Using the full, combined dataset, and the same settings as the RAXML analyses described above, 100 ML searches were performed under each of the 13 constraints. All 1200 trees produced by RAXML (100 from the unconstrained analysis and 100 from each of the 11 constrained analyses), were filtered in PAUP to remove identical topologies. A modified version of RAXML (provided by Alexandros Stamatakis) allowed the per-site likelihoods to be estimated for each of the 116 unique topologies under a partitioned model. An AU test was then performed on the per-site likelihoods from all 116 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. To automate various steps in the process, perl and python scripts were written by J. Oaks and CDS (available by request). For the Bayesian approach, we took the percentage of 11,520 post burn-in trees consistent with each hypothesis to represent the posterior probability that the hypothesis is true.

2.4. Relative time analyses

To test the combined dataset for deviations from a molecular clock, we optimized likelihood scores in PAUP* 4.0b10 with a molecular clock enforced and not enforced on the maximum-likelihood topology. A likelihood ratio test ([LRT] Arbogast et al., 2002; Felsenstein, 2004) significantly rejected a molecular clock ($p \leq 0.00$), and subsequent analyses were conducted within a relaxed clock framework. The chronogram used for ancestral state reconstructions in this study was inferred in a Bayesian framework using BEAST v1.5.3 (Drummond and Rambaut, 2007). A starting tree was designated for each run by manually adjusting the xml BEAUti v1.5.2 (Drummond and Rambaut, 2007) output file. The consensus tree file from Bayesian analyses was imported into R (R Development Core Team, 2008), and using the ape (Paradis et al., 2004), the phylogeny was paired down into individual lineages per species or morphologically distinct, non-monophyletic populations (*B. bonita* and *B. samarensis*). The 47-taxon phylogeny was then converted to a chronogram using the nonparametric rate smoothing method of Sanderson (1997) implemented in the ape (Paradis et al., 2004) package of R, and was exported in Newick format for use as the starting tree for BEAST analyses. Four independent BEAST runs of 50 million generations were completed under the same partitioning strategy as for Bayesian analyses, imposing an uncorrelated lognormal relaxed clock prior on substitution rate (Drummond et al., 2006) and Yule speciation prior. Parameters were sampled every 5000 generations and the initial 50% of each run was discarded as burn-in, leaving a combined 20,000 trees in the posterior distribution. To evaluate convergence among MCMC analyses, trends and distributions of parameters, including the likelihood score, were examined in Tracer (Rambaut and Drummond, 2007).

2.5. Biogeographical reconstructions

To explore whether there is statistical support for historical biogeographic patterns within Philippine species of the genus *Brachymeles*, we compared empirically observed (extant) species distributions to estimates of ancestral distributions using the program BayesTraits version 1.0 (Pagel, 1994; Pagel and Lutzoni, 2002). For all analyses we examined a model of character evolution that assumed equal rates of distributional transitions. For all analyses we seeded the mean and variance of the gamma prior from uniform distributions on the interval 0–20 by enforcing the “Hyperpriorall” command of BayesTraits. The LogCombiner v1.5.4 program of the BEAST v1.5.3 (Drummond and Rambaut, 2007) package was used to combine trees from the posterior distributions of the four independent BEAST runs, producing a file with 2000 trees from the posterior distribution. All 2000 chronograms were then used in analyses of distribution data in BayesTraits in an effort to account for phylogenetic uncertainty. In the program BayesTraits, we ran MCMC chains for 25 million generations, sampling every 5000th generation, and discarded the first 50% of samples as burn-in. The remaining 2500 samples were used to summarize the posterior probabilities of ancestral character states for all nodes of the tree. The “AddNode” command of BayesTraits was used to specify all nodes in the chronograms for visualization of the posterior probabilities of character states at each node.

We ran a series of additional analyses on nodes with ambiguous estimated ancestral character states. The “fossil” command of BayesTraits was used to sequentially enforce the character states making up 95% of the posterior probability at a single node, prioritizing character states with the highest posterior probability. Bayes factors were again applied, and the state supported at each ambiguous node was summarized with the Bayes factors measure of support for that ancestral state (Kass and Raftery, 1995;

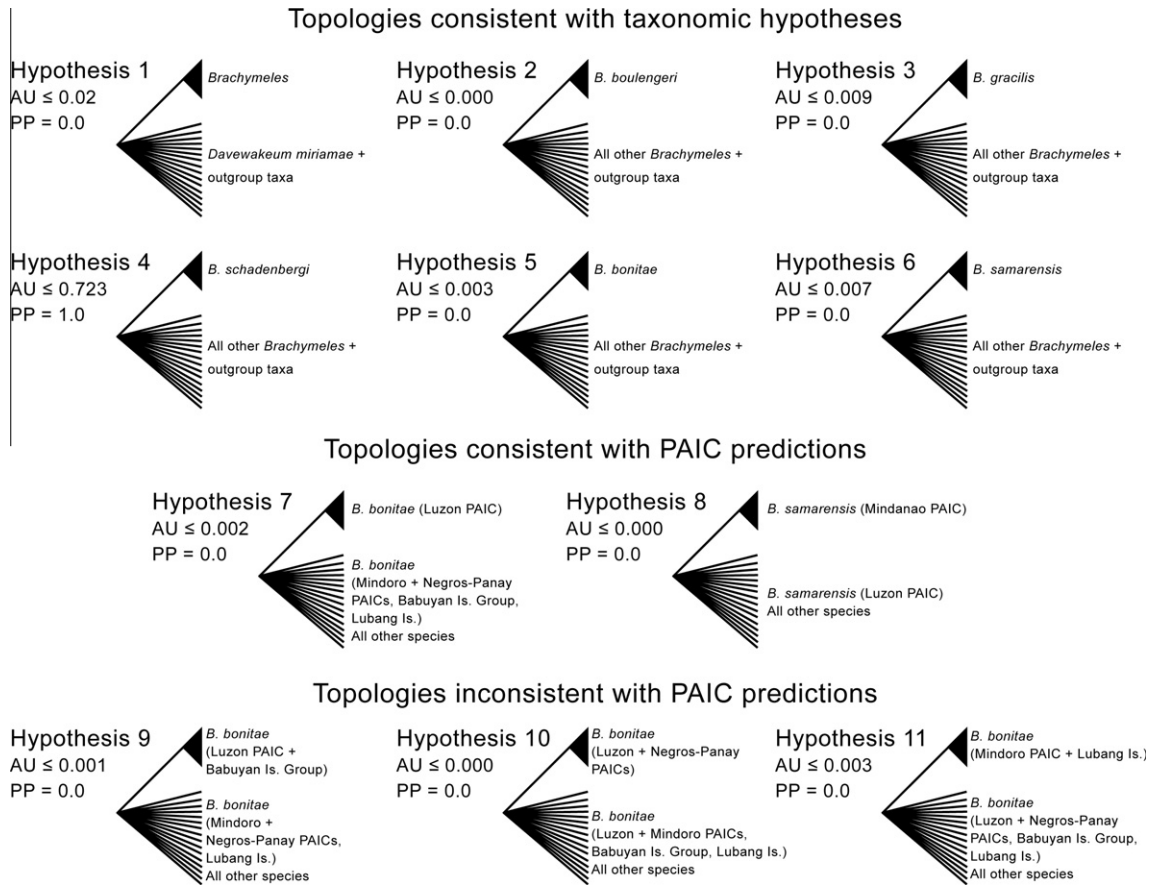


Fig. 3. Six taxonomy-based hypotheses tested in the study. Each hypothesis is illustrated by constraint trees used in AU and Bayesian tests. The highest P -values recovered from each AU test (AU), and the posterior probabilities (PP) of the constraint topology, are shown.

Nylander et al., 2004). We enforced the ancestral states for all nodes sister to Philippine *Brachymeles* to be considered non-Philippine in distribution.

3. Results

3.1. Taxon sampling, data collection, and sequence alignment

The complete, aligned matrix contains 82 samples of *Brachymeles*, representing 23 of the 25 recognized taxa, for the mitochondrial genes and nuclear loci. Seventeen additional samples are included as outgroups, consisting of representatives from the subfamilies Lygosominae and Scincinae within the family Scincidae as well as a single representative from the lizard family Lacertidae. Following initial unrooted analyses, and the results of recent squamate evolution studies (Whiting et al., 2003; Townsend et al., 2004; Wiens et al., 2006; Brandley et al., 2005, 2008), we rooted the tree using samples of *Takydromus sexilineatus* from China. Within each gene, variable and parsimony-informative characters are observed as follows: 118 and 107 out of 158 (ATP8); 357 and 339 out of 683 (ATP6); 504 and 464 out of 966 (ND1); 575 and 531 out of 861 (ND2); 115 and 69 out of 715 (BDNF); 127 and 95 out of 490 (PTGER); 304 and 220 out of 689 (R35). The number of most parsimonious trees and consistency index (CI) resulting from MP analyses of the combined dataset is 78 trees (CI = 0.318).

3.2. Phylogenetic analyses

Analyses of the combined data (ND1, ND2, ATP8, ATP6, BDNF, PTGER4, R35) result in topologies with high Maximum Likelihood

bootstrap support and posterior probabilities (Fig. 4). Topologies are congruent across these analyses (Fig. 4). All analyses support three clades of outgroup scincid taxa (Fig. 4). Outgroup samples from the subfamily Lygosominae are never recovered as part of a single clade (Fig. 4); however, given the possibility that the chosen root for analyses and the outgroup sampling strategy likely influence outgroup relationships, additional taxa should be obtained for a more exhaustive analysis of scincid relationships before definitive conclusions may be drawn.

The genera *Eumeces* and *Plestiodon* are recovered as a single, strongly supported clade in all analyses (Fig. 4, Clade 1). Although this clade is often supported as the closest group of outgroup species to *Brachymeles* + *Davewakeum* (ML; Fig. 4), support for its placement was always low, and in Bayesian analyses, the clade is regularly recovered as part of a three clade polytomy of outgroup samples (results not shown). The species *Davewakeum miriamae* is always recovered nested within the genus *Brachymeles* between the clade *B. apus* + *B. cf. apus* from Borneo and all Philippine *Brachymeles* (Fig. 4).

Although the genus *Brachymeles* (as currently recognized) is never recovered as a monophyletic group, all analyses strongly support the monophyly of *Brachymeles* + *Davewakeum* (Fig. 4, Clade 2). The two limbless species *Brachymeles apus* (Borneo) and *Davewakeum miriamae* (Thailand) are always recovered as the two lineages most closely related to all Philippine *Brachymeles* (Fig. 4). Within the Philippines, all limbless species and the majority of limb-reduced species are recovered as part of two reciprocally monophyletic sub-clades, and together are sister to all pentadactyl species and the remaining non-pentadactyl taxa (Fig. 4, Clade 3). Sampled populations of the currently recognized widespread,

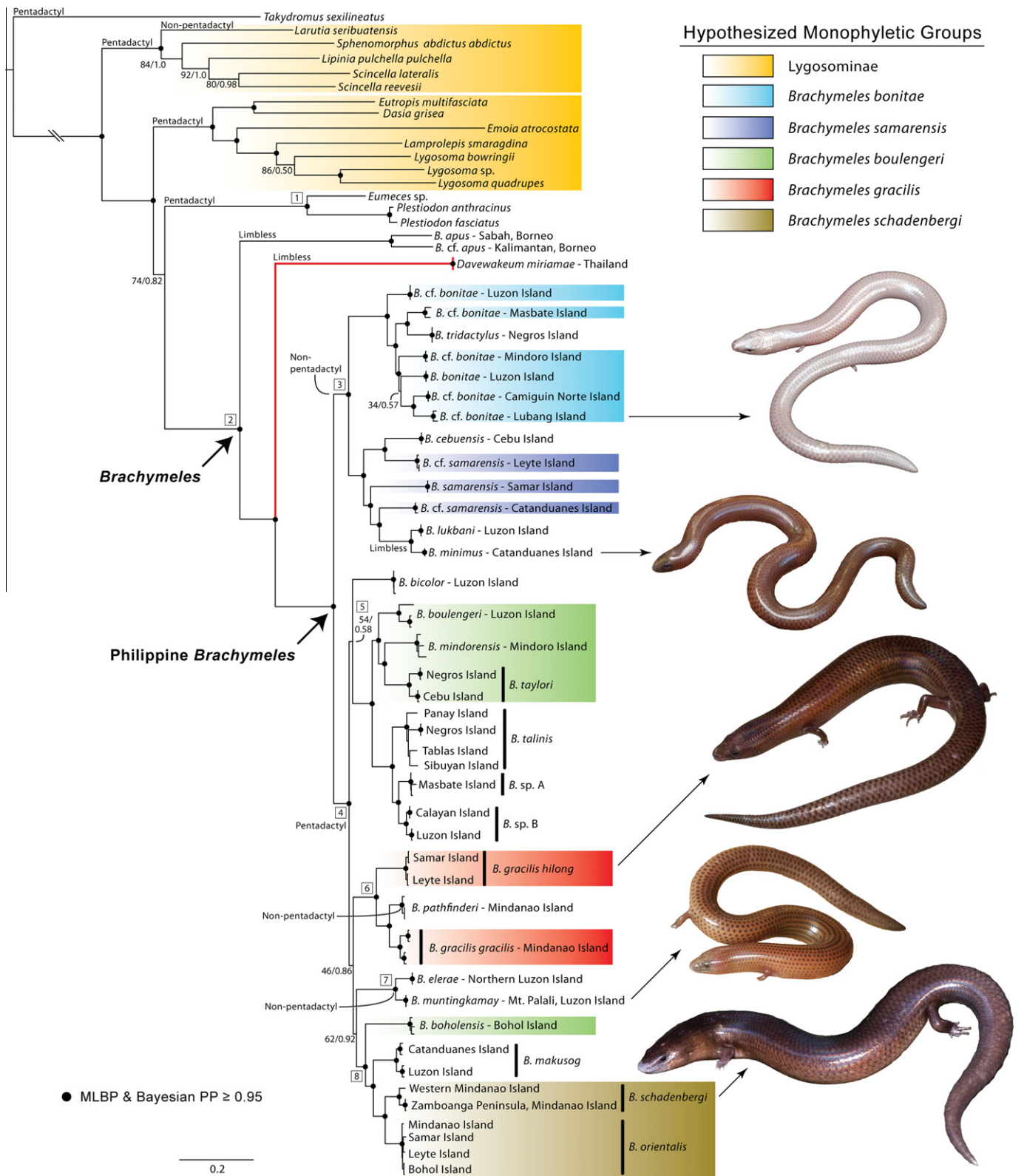


Fig. 4. Hypothesized relationships of *Brachymeles*, illustrated by ML estimates (–ln L 54137.110363). Nodes supported by $\geq 95\%$ Bayesian PP and ML bootstrap support were considered significantly supported and are indicated by black circles. Terminals are labeled with abbreviated taxonomic names, followed by general geographic distribution (Appendix 1). The placement of the genus *Davewakeum* is indicated by red branches. Colored boxes highlight hypothesized monophyletic groups. Labeled clades denote general body plans observed for species included in this study. Alpha and numerical labels correspond to clades referred to in the Results and Discussion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

limb-reduced species *Brachymeles bonitae* and *B. samarensis* are never recovered as monophyletic (Fig. 4, Clade 3). *Brachymeles cebuensis*, one of only two species of *Brachymeles* with unequal

numbers of fingers and toes (three fingers/two toes) is recovered as sister to *B. samarensis* from Leyte Island (Fig. 4, Clade 3). Within Clade 1, all analyses recover a population of *Brachymeles bonitae*

with one fore-limb digit and one hind limb digit as sister to the tridactyl species *B. tridactylus*, and the five other populations of *B. bonitae* (Fig. 4). Additionally, a morphologically distinct population of *B. cf. bonitae* with limbs but no digits is supported to be the sister species to *B. tridactylus* (Fig. 4, Clade 3).

All pentadactyl species and three non-pentadactyl species of *Brachymeles* are always recovered as part of a clade, sister to Clade 3 (Fig. 4, Clade 4). Although the two species formerly part of the *Brachymeles schadenbergi* species complex (*B. orientalis* and *B. schadenbergi*) are always recovered as reciprocally monophyletic lineages (Fig. 4, Clade 8), the polytypic species *B. gracilis*, and the four species formerly part of the *B. boulengeri* species complex, are strongly supported to be paraphyletic (Fig. 4, Clades 5, 6, 8). All analyses recover four well-supported clades within Clade 4 (Fig. 4). Although Bayesian analyses provided high support for the relationships between these clades, likelihood analyses provide less support (Fig. 4, Clade 4).

Three non-pentadactyl species are recovered as part of Clade 4 in all analyses (Fig. 4). The tetradactyl species *Brachymeles elerae* and the tridactyl species *B. muntingkamay* are always recovered as sister taxa (Fig. 4, Clade 7); however, the relationship of Clade 7 to the other major clades in Clade 4 is less well supported. *Brachymeles pathfinderi*, the only other species with unequal digit numbers (five fingers/four toes), is recovered with strong support to be nested within the polytypic species *B. gracilis* (Fig. 4, Clade 6).

Brachymeles bicolor, the longest species, is recovered as sister to a subclade of three medium-sized species (*B. boulengeri* + *B. mindorensis* + *B. taylori*) and three large species (*B. talinis* + two undescribed species [Siler and Brown, 2010]). *Brachymeles boholensis*, formerly recognized as a subspecies of the *B. boulengeri* species complex, is consistently supported as part of Clade 8, sister to *B. orientalis*, *B. schadenbergi*, and *B. makusog* (Fig. 4).

3.3. Topology tests

Results from the Bayesian methods and the approximately unbiased (AU) test were highly consistent. Among the taxonomy-based hypotheses, both methods rejected hypotheses of monophyly for the genus *Brachymeles*, the widespread species *B. bonitae*, *B. gracilis*, and *B. samarensis*, and the former polytypic species *B. boulengeri* (Fig. 3). Additionally, both methods failed to reject the monophyly of the former polytypic species *B. schadenbergi*, now recognized as two distinct sister species (Siler and Brown, 2010; Fig. 3). All biogeography-based hypotheses were rejected by both methods (Fig. 3).

3.4. Body form evolution

Our simplified mapping of major body plan variation onto the preferred phylogenetic tree (Fig. 4) makes it clear that the evolutionary history of *Brachymeles* involves multiple instances of evolutionary shifts in body size, limb-reduction, and digit loss. Although a full understanding of these trends will require a comprehensive analysis of both external (body size, limb proportions, digital states) and internal (vertebral numbers and elongation) morphology, preliminary trends can be ascertained on the basis of results presented here. If it is assumed, for example, that the ancestors of *Brachymeles* possessed similar body plans as the genus' putative closest relatives (*Eumeces*, *Plestiodon*, *Lygosoma*, *Emoia* *Dasia* and *Eutropis*), then limb-reduction and loss may have occurred along the lineages leading to *B. apus* and *B. cf. apus*, in the lineage leading to *Davewakeium miriamae*, in the lineage leading to the *B. bonitae* and *B. samarensis* complexes, and independently in the lineages leading to *B. pathfinderi* and *B. muntingkamay* + *B. elerae*. It is interesting to note that all of the known diversity of pentadactyl species in the genus is endemic to the Philippine.

3.5. Historical biogeography

Although the placement of *Brachymeles* within the family Scincidae remains somewhat ambiguous, the impact of ancestral range for all *Brachymeles* does not appear to heavily impact ancestral reconstructions within the genus (not shown). The results of analyses of ancestral areas are never significantly impacted by placing restrictions on the ancestral character states among outgroup taxa and the node giving rise to all *Brachymeles* (not shown). Without *a priori* knowledge of the true patterns of diversification within the Philippines, we conservatively chose a model allowing for equal rates of transition among major faunal regions of the Philippines. Ancestral state reconstruction analyses resulted in many nodes where the reconstructed ancestral range is ambiguous (Fig. 5). The Luzon PAIC is the preferred ancestral range in most cases with varying degrees of support (Fig. 5). Importantly, all analyses resulted in unambiguous support for ancestral ranges at eleven nodes in the phylogeny, supporting the Luzon, Negros-Panay, and Mindanao PAICs as the ancestral ranges for several clades of Philippine *Brachymeles* (Fig. 5A–C). Given strong statistical support for ancestral areas at eleven nodes, we are confident in hypothesizing five dispersal events (Fig. 5), including clear dispersal from Luzon to Mindoro, Luzon to the central Visayan islands, dispersal from the Visayas to Mindoro and Luzon, and a clear instance of dispersal from the Mindanao island group to southern Luzon. Considering the moderate to strong statistical support for a specific reconstruction at seven ambiguous nodes (indicated by Bayes factors of 4.13–9.10; Kass and Raftery, 1995; Nylander et al., 2004), we infer the possibility of five additional dispersal events (arrows not illustrated in Fig. 5).

4. Discussion

4.1. Taxon sampling

Our widespread sampling of species across the Philippines allows for fine-scale resolution of phylogenetic relationships and an unprecedented and comprehensive taxonomic review (Siler et al., 2009, 2010a,b; Siler and Brown, 2010; Siler et al., in press). The two species missing in our analyses are *Brachymeles wrighti* from northern Luzon Island and *B. vermis* from the Sulu Archipelago in the southern Philippines. Both species are represented by only a few museum specimens worldwide, and no genetic samples have ever been collected. Previous studies that have included samples of *Brachymeles* have not been able to confidently place the genus *Brachymeles* within the family Scincidae (Brandley et al., 2005, 2008). Although our attempt to sample widely from outgroup taxa results in some well-supported relationships, the most closely related species to the genus *Brachymeles* remains unclear (see also Brandley et al., 2005). We are unable to collect full sequence data for all included outgroup taxa (Appendix 2), and missing data may have contributed to weaker support for outgroup relationships. It is anticipated that additional outgroup and gene sampling will aid resolution of these relationships.

4.2. Phylogeny and cryptic genetic diversity

Cryptic diversity has been documented as a global phenomenon (Pfenninger and Schwenk, 2007; Bickford et al., 2007), and we now suspect the phenomenon to also characterize Philippine slender skinks (Siler et al., 2009, 2010a,b; Siler and Brown, 2010; Siler et al., in press). Although we focus on diversity of skinks of the genus *Brachymeles*, our results support the taxonomic issues identified in numerous studies for the family Scincidae (e.g., the non-monophyly of Lygosominae), and phylogenetic studies across the

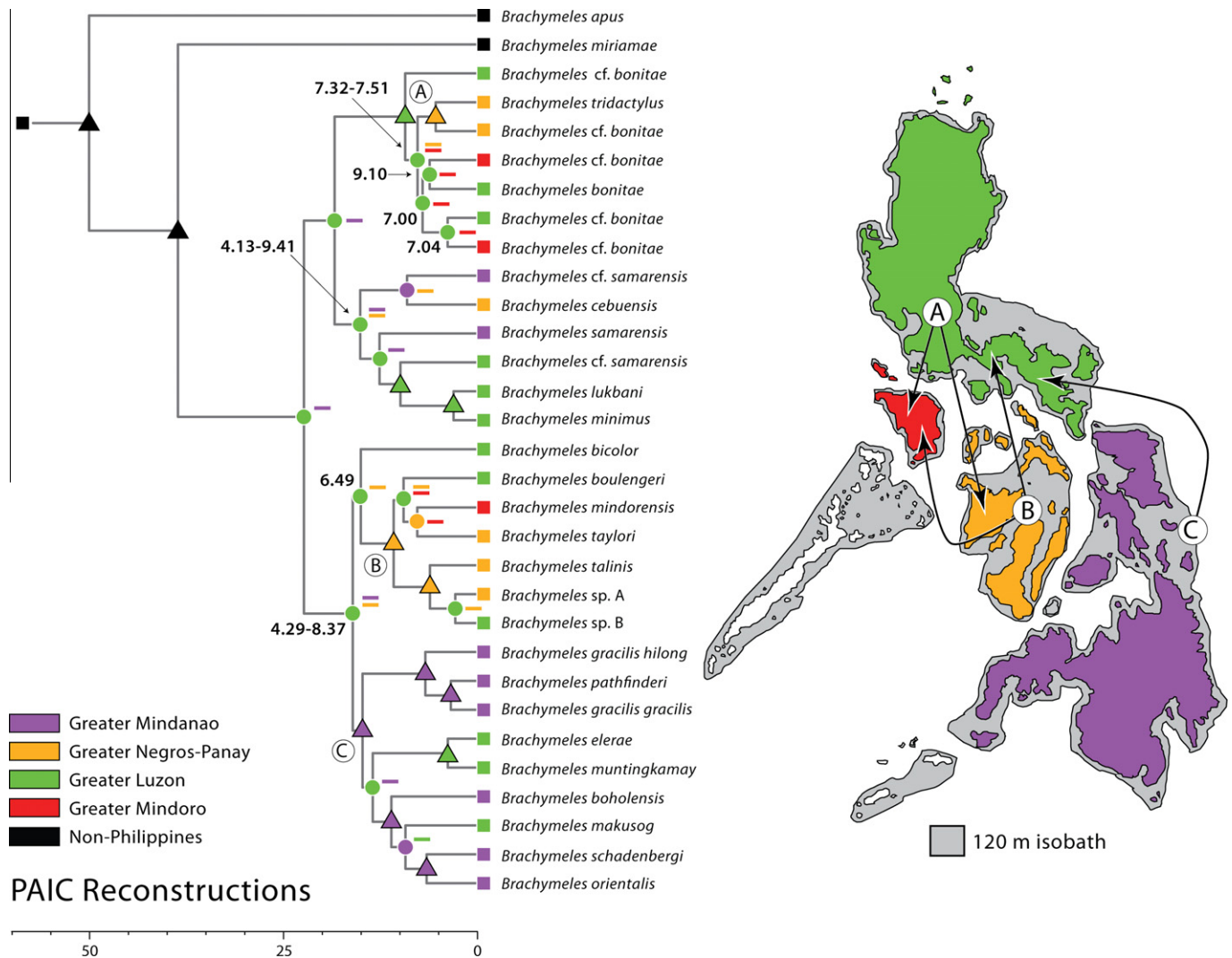


Fig. 5. Maximum clade credibility chronogram and estimated ancestral states of geographic range in *Brachymeles* skinks. Ancestral area reconstructions are indicated at each node. Triangles indicate unambiguous reconstructions of an ancestral area (posterior probability ≥ 0.95), colored according to the hypothesized state (see key). Circles represent ambiguous character reconstructions, with colors representing the preferred area. Colored blocks at each ambiguous node represent alternate states recovered as possibilities in Bayes Traits analyses. Bayes factors are provided as an indication of the moderate to strong statistical support for preferred states at nodes with ambiguously reconstructed ancestral ranges. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

family are needed to provide future taxonomic revisions. Although we did not expect to find that *Davewakeum miriamae* is nested within the genus *Brachymeles*, this result, in retrospect, is not surprising when comparing morphology of this genus to species of *Brachymeles*. *Davewakeum* shares several unique morphological features with *B. apus*, *B. bonitae*, and *B. samarensis*, including the fusion of the mental and first infralabial scales, the presence of a moderate-sized interparietal scale, nasal scales, two loreal scales, a frontal scale, frontoparietal scales, the presence of a single scale row on the lower eyelid (Siler et al., 2010b; Siler, pers. obs.), and a general elongated, limb-reduced body plan.

With the results of this study, and the fact that the genus *Brachymeles* (Duméril and Bibron, 1839) was described well before *Davewakeum* (Heyer, 1972), we consider *Davewakeum* to be a junior synonym of *Brachymeles* and, consequently, *Brachymeles miriamae*, new combination, to be the fifth limbless species of *Brachymeles*. We note that at least one additional known limbless species (*B. cf. apus*, from Kalimantan, south Borneo) awaits description (Iskandar and Bickford, unpublished data).

The conservative body plans within the genus *Brachymeles* have led to confusion over species boundaries, the long accepted

recognition of polytypic species, and the recognition of “widespread” species with distributions across accepted faunal boundaries (Brown, 1956; Brown and Rabor, 1967; Brown and Alcala, 1980). With the exception of *Brachymeles schadenbergi*, the results of this study do not support the monophyly of the currently and previously-recognized polytypic species in the genus (Figs. 3 and 4). Additionally, all currently recognized subspecies are both strongly supported divergent lineages in the phylogeny and represent unique morphologies, most likely worthy of specific rank (Fig. 4; Siler and Brown, 2010; CDS, unpublished data).

The degree to which convergent morphology has led to the underestimation of diversity within the genus can further be exemplified by examining the “widespread” species densely sampled in this study. Populations of the previously-recognized widespread pentadactyl species, *B. talinis*, have recently been revised to represent five unique pentadactyl species, each with non-overlapping geographic distributions (Fig. 4): *B. makusog* (Siler et al., 2010a), *B. talinis*, *B. sp. A* (Siler and Brown, 2010), *B. sp. B* (Siler and Brown, 2010), and *B. sp. C* (Siler and Brown, 2010). Additionally, populations of the two former polytypic species, *B. boulengeri* and *B. schadenbergi*, have recently been revised, and all subspecies

elevated to full, readily diagnosed species (Siler and Brown, 2010; Fig. 4, Clade 5, 8).

Currently, *Brachymeles bonitae* and *B. samarensis* are recognized to have atypical distributions that span multiple PAICs (Fig. 4, Clade 3). *Brachymeles samarensis* is known to occur on two islands in the Greater Luzon PAIC (Catanduanes, Luzon), as well as two islands and one small island group in the Mindanao PAIC (Leyte, Samar, Lapinig Island Group; Fig. 1 and 2). In comparison, *B. bonitae* has the widest recognized distribution of any species in the genus. Populations referable to this species occur in three distinct PAICs (Luzon, Mindoro, Negros-Panay) and two small deep-water island groups (Babuyan, Romblon). However, our results indicate that both *B. bonitae* and *B. samarensis* are complexes of numerous, morphologically similar species (Fig. 4, Clade 3). Although populations within both species complexes share similar body plans, unique sets of characters do exist, including differences in the numbers of fore- and hind limb digits (Siler and Brown, unpublished data). These inter-population differences have historically been recognized as morphological variation within widespread species (Brown, 1956; Brown and Rabor, 1967; Brown and Alcalá, 1980). However, upon our observation that character differences coincide with deep genetic divergences and biogeographic breaks in lineage distributions, we have begun to suspect that many inter-population variants in *B. samarensis* and *B. bonitae* will prove to be full species in accordance with any modern lineage-based species concept. Recently, taxonomic revisions such as this have been made for the *B. boulengeri* and *B. shadenbergi* complexes (Siler and Brown, 2010).

Recent studies have revealed numerous other “widespread” Philippine endemic reptiles to actually represent complexes of cryptic species, with few species actually possessing distributions that span recognized faunistic boundaries (Brown et al., 2000; Brown and Diesmos, 2002, 2009; Siler et al., 2010a,b; Siler and Brown, 2010; Welton et al., 2009, 2010, in press). Exceptions do exist (Brown and Alcalá, 1970), but many of these have turned out to represent invasive species with suspected histories of human mediated introductions (Diesmos et al., 2006; Brown et al., 2010). It is clear that the diversity of *Brachymeles* is vastly underestimated, and detailed morphological comparisons are needed to revise the taxonomy within the genus.

Finally, some level of uncertainty remains in the species diversity of *Brachymeles* outside of the Philippines (Fig. 4). The apparent disjunct distribution of *Brachymeles* in Southeast Asia may be an artifact of high levels of extinction outside of the Philippines or an absence of discovery. The phylogeny suggests that at least one genetically distinct, undescribed limbless species exists in southern Borneo (Kalimantan, Indonesia; Fig. 4), and it is highly probable that other undescribed species will eventually be discovered in other parts of Asia. It is noteworthy that the entire diversity of limbed, pentadactyl, reduced-limbed, and limbless body forms are found in the Philippines (Fig. 4); we take this pattern as a testament to the rich processes of diversification found within this small but remarkable island archipelago (Brown and Diesmos, 2009).

4.3. Diversification and body form evolution within a semi-fossorial genus

Previous surveys of body plan diversity within *Brachymeles* have focused solely on morphological variation (Brown and Rabor, 1967; Brown and Alcalá, 1980, 1995), and have mentioned the potential for undocumented cryptic diversity within the genus as an ancillary possibility, not an expectation, and certainly not a prevailing phenomenon. However, a number of studies have shown that the evolution of a burrowing lifestyle is correlated with decreasing dispersal abilities (Selander et al., 1974; Patton and

Yang, 1977; Patton and Feder, 1978) as well as changes in body form (see Crottini et al., 2009 for review). Several lineages of *Brachymeles* have undergone a reduction in limb size and digit numbers (Fig. 4), which may further reduce their vagility (Daniels et al., 2005; Mulvaney et al., 2005). Over time, reduced dispersal abilities may lead to an increasingly patchy distribution, reduction in gene flow among populations, and the accumulation of inter-population genetic differences (Nevo, 1979). This process also could be amplified within an island archipelago or a geographically complex island such as Luzon or Mindanao. We expect that this process has contributed to cryptic lineage diversification in this unique south-east Asian radiation.

The results of phylogenetic analyses provide evidence for three losses of external limb elements, and three distinct instances of changes in digit states. Although five species of *Brachymeles* are externally limbless (*B. apus*, *B. minimus*, *B. miriamae*, *B. lukbani*, *B. vermis*), it is currently unknown whether internal girdle elements are present in any of the five species, or if any of them retain some vestigial limb elements. Additionally, there is evidence for up to four independent losses of auricular openings, with the openings being completely covered by scales in *B. apus*, *B. cf. apus*, *B. miriamae*, all species of *Brachymeles* in Clade 3 of Figure 4, and *B. muntingkamay*. It is also interesting to note that all species and populations with unequal digit numbers in the fore- and hind limbs have fewer toes than fingers in *Brachymeles* (*B. bonitae*, *B. cebuensis*, *B. pathfinderi*, *B. samarensis*), in contrast to many previous studies which have shown that reductions in digit number are more common in the fore-limbs of scincid lizards (Brandley et al., 2008; Skinner and Lee, 2010).

4.4. Biogeographic patterns

Although numerous previous studies have observed some phylogenetic patterns at least partially consistent with PAIC-based models of diversification (e.g., Heaney et al., 1998; Kennedy et al., 2000; Brown and Diesmos, 2002; Brown and Guttman, 2002; Evans et al., 2003; Esselstyn et al., 2004), we rejected all topologies predicted from a PAIC-based model (Fig. 3). Additionally, we rejected all hypotheses derived from patterns observed in other taxa (Alfaro et al., 2004; McGuire and Kiew, 2001; Siler et al., 2010c).

Phylogenetic analyses and ancestral state reconstructions provide support for multiple dispersal events in *Brachymeles* leading to complex and biogeographically convoluted distribution patterns observed today. Ancestral range reconstructions unambiguously estimate the ancestral range for 11 nodes in the chronogram, supporting a minimum of five geographic range shifts between major faunal regions in the Philippines (Fig. 3). Of these hypothesized inter-PAIC transitions, with the exception of a dispersal out of the Mindanao PAIC, all geographic transitions are hypothesized to have originated in the Luzon or central Visayan PAICs (Fig. 3A–C). Additionally we note that although only five inter-PAIC dispersal events are unambiguously reconstructed (Fig. 5A–C), the ancestral range for 5 additional nodes can be inferred with strong statistical support (Bayes factors 6–10; Kass and Raftery, 1995; Nylander et al., 2004). Therefore, the possibility remains that additional instances of between-PAIC dispersal took place. Given that the major PAIC platforms of the archipelago have never been connected by dry land (Kloss, 1929; Inger, 1954; Heaney, 1985; Voris, 2000; Yumul et al., 2003, 2008), suggesting that faunal exchange among PAICs necessitates dispersal across ocean channels (review: Brown and Diesmos, 2009), we find it reasonable to conclude that much of the historical dispersion of *Brachymeles* throughout the archipelago has been through the process of waif dispersal over-water. Clearly the evolutionary and biogeographic history of semi-fossorial slender lizards has been heavily impacted by faunal exchange

throughout the archipelago. This may appear at odds with the general assumption of low vagility assumed for reduced-limbed lizards with a burrowing lifestyle, but we find it conceivable, and even plausible, that dispersal between islands is mediated by frequent rafting of mats of vegetation, topsoil, and logs; these possible vectors are frequently observed washing out of the mouths of rivers following heavy storms (CDS and RMB, *personal observations*).

5. Conclusion

Our study provides a comprehensive, phylogenetic analysis for a closely related group of lizards with a remarkable range of body form diversity. We have included samples from nearly all recognized species within the genus *Brachymeles*, and our intraspecific sampling has uncovered cryptic genetic diversity within many species (e.g., *Brachymeles bonita* and *B. samarensis*). This study provides the foundation for a robust model system with which to address patterns of body form evolution, processes of diversification, and species delineation. With the exception of the recently published *Lerista* dataset (Skinner et al., 2008; Skinner, 2010; Skinner and Lee, 2010), our estimates of phylogeny represent one of the most comprehensive datasets for fine-scale studies of limb-reduction and loss in squamate reptiles.

It is clear that currently recognized diversity of skinks of the genus *Brachymeles* is vastly underestimated, and that numerous taxonomic revisions will be necessary to fully appreciate the processes of diversification within this nearly endemic Philippine radiation. New species await description (e.g., the new limbless species in southern Borneo; Fig. 4), and likely await discovery, and future survey work should focus on regions outside of the Philippines (e.g., Borneo, Malay Peninsula, Indochina). This study has revealed another case of extensive cryptic diversity in a once recognized assemblage of “widespread” Philippine species (Fig. 4). Together with numerous recent studies (Brown et al., 2009; Esselstyn et al., 2009; Esselstyn and Brown, 2009; Oliveros and Moyle, 2010; Siler et al., 2010c; Linkem et al., 2010) this effort has resulted in wholesale discovery of numerous new species and cryptic evolutionary lineages of endemic Philippine vertebrates. Once considered a small radiation of Asian skinks, the recognized species diversity of the genus *Brachymeles* will likely increase by more than 300% over the next five years (Siler et al., unpublished data).

We rejected all PAIC-based models of diversification (e.g., Heaney et al., 1998; Kennedy et al., 2000; Brown and Diesmos, 2002; Brown and Guttman, 2002; Evans et al., 2003; Esselstyn et al., 2004), as well as all patterns observed in other studies (Alfaro et al., 2004; McGuire and Kiew, 2001; Siler et al., 2010c; Fig. 3). However, the results of this study provide evidence for five major dispersal events across faunal zone boundaries that have given rise to the major clades of *Brachymeles* species diversity in the Philippines (Fig. 5). Surprisingly, all but one of these dispersal events are hypothesized to have originated in the Luzon or central Visayan PAICs (Fig. 3A–C). The results of this study, coupled with our knowledge of the geologic history of the region (Kloss, 1929; Inger, 1954; Heaney, 1985; Voris, 2000; Yumul et al., 2003, 2008), suggests that much of the historical faunal exchange of *Brachymeles* throughout the archipelago has been through the process of over-water (waif) dispersal. Without time-calibrated phylogenies, and the absence of closely related fossil calibrations, it is difficult to say when the hypothesized dispersal events occurred, leading to the complex distribution patterns observed today.

The transition from quadrupedal to limbless body plans has occurred repeatedly in independent lineages of squamate reptiles (i.e., snakes, lizards, amphisbaenians; Wiens and Slingluff, 2001; Greer, 1991; Pough et al., 2004). It is clear that these transitions also occur repeatedly within single radiations of closely related

species (Fig. 4). The results of this study provide the first evidence of repeated limb, digit, and auricular opening loss in the genus *Brachymeles*. Given our results, and those of other studies that explicitly test morphological patterns of body form change within squamate reptiles (for review, see Wiens and Slingluff, 2001; Brandley et al., 2008), we are left with many unanswered questions. Given the apparent evidence for repeated body form change in *Brachymeles*, can we estimate the number of times characters have been lost (or potentially gained) in *Brachymeles*? Do the patterns of morphological changes observed within this unique radiation of Southeast Asian lizards support previous hypotheses of correlated morphological evolution associated with limb-reduction and loss in squamate reptiles (for review, see Wiens and Slingluff, 2001; Brandley et al., 2008)? Is there evidence for a gradual, evolutionary sequence involved in the process towards limb loss? What inferences can we make from statistical reconstructions (estimates) of ancestral morphology and character state change on the phylogeny? These and other broad-scale evolutionary questions that address the processes of body form evolution must be assessed within a comparative framework, and require the addition of robust morphological datasets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ymp.2010.12.019](https://doi.org/10.1016/j.ymp.2010.12.019).

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