THE CHALLENGE OF SPECIES DELIMITATION AT THE EXTREMES: DIVERSIFICATION WITHOUT MORPHOLOGICAL CHANGE IN PHILIPPINE SUN SKINKS

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An accurate understanding of species diversity is essential to studies across a wide range of biological subdisciplines. However, delimiting species remains challenging in evolutionary radiations where morphological diversification is rapid and accompanied by little genetic differentiation or when genetic lineage divergence is not accompanied by morphological change. We investigate the utility of a variety of recently developed approaches to examine genetic and morphological diversity, and delimit species in a morphologically conserved group of Southeast Asian lizards. We find that species diversity is vastly underestimated in this unique evolutionary radiation, and find an extreme case where extensive genetic divergence among lineages has been accompanied by little to no differentiation in external morphology. Although we note that different conclusions can be drawn when species are delimited using molecular phylogenetics, coalescent-based methods, or morphological data, it is clear that the use of a pluralistic approach leads to a more comprehensive appraisal of biodiversity, and greater appreciation for processes of diversification in this biologically important geographic region. Similarly, our approach demonstrates how recently developed methodologies can be used to obtain robust estimates of species limits in “nonadaptive” or “cryptic” evolutionary radiations.

KEY WORDS: BP&P, cryptic species, Eutropis, GMYC, lizard, morphometrics.

Due to its central importance in fields related to the evolutionary study of biodiversity, the practice of species delimitation has a long and contentious history (Wiley 1978; Frost and Hillis 1990; Mayden 1997; de Queiroz 1998; Sites and Marshall 2003). Much of this debate stems from the difficulty of developing a universal species concept (de Queiroz 1999; Esselstyn 2007; Bauer et al. 2010) and involves determining which methodological approaches produce the most accurate results (Marshall et al. 2006; Fujita and Leaché 2011). However, an accurate understanding of species diversity is an essential first step before studies in many other biological fields can be conducted, including studies of species diversification, character evolution, population genetics, ecology, comparative genomics, and conservation (Cracraft 2002; Sites and Marshall 2004; Fujita et al. 2012).

Problems associated with delimiting species are particularly pronounced in some systems and are characterized by challenges that can cause fundamental difficulties for evaluating species rich evolutionary assemblages (Fig. 1). These include many spectacular evolutionary radiations, which biologists are particularly motivated to understand. In several classic examples such as cichlid fishes (Moran and Kornfield 1993; Seehausen 2004; Wagner et al. 2012), passerine birds (Freeland and Boag 1999; Petren et al. 2005; Moyle et al. 2009), Hawaiian silverswords (Baldwin 1997), and ambystomatid salamanders (Shaffer 1984; Shaffer and
Evolutionary biologists have widely embraced the notion that delimiting species ought to be guided by the principle that species are defined as distinct evolutionary lineages (Hennig 1966; Wiley 1978; Frost and Hillis 1990; de Queiroz 2005). Molecular data sets consisting of multiple, unlinked loci have allowed for more rigorous empirical studies delimiting evolutionary lineages that constitute species (Sinclair et al. 2004; Shaffer and Thomson 2007; Carstens and Dewey 2010; Linkem et al. 2010; Kubatko et al. 2011). Although phylogenetic and population genetic methods can be used to construct gene trees and examine population structure, identifying species-level lineages and determining if populations are isolated using genetic data can be challenging, particularly in the face of incomplete lineage sorting or hybridization (Shaffer and Thomson 2007; Frankham et al. 2012; Fujita et al. 2012; Wagger et al. 2012; Welton et al. 2013).

Coalescent-based methods that permit the use of genetic sequence data to identify genetic isolation among—and cessation of gene flow between—lineages (or putative species) have just begun to be developed. Fundamentally, these methods evaluate the likelihood of competing species delimitation hypotheses based on an assumed evolutionary process (Pons et al. 2006; Yang and Rannala 2010; Ence and Carstens 2011; Reid and Carstens 2012). Although these methods incorporate assumptions that may or may not be biologically realistic for a particular species group, they represent important progress toward the development of objective criteria for empirical evaluation of species limits (Leaché and Fujita 2010; Fujita et al. 2012).

Lizards of the genus *Eutropis* (also referred to as "sun skinks") are some of the most common and conspicuous lizards in Southeast Asia, owing to their high abundance, diurnal activity patterns, and generalist habitat preferences. Despite this, the genus has had a long history of taxonomic confusion, due primarily to the fact that sun skinks represent a clade with a generalized external morphology, characterized as evolutionarily highly conserved (Miralles et al. 2005; Miralles and Caranza 2010; Hedges and Conn 2012). Within the Philippines, a region classified as both a Global Biodiversity Conservation Hotspot and a Megadiverse Nation (Conservation International 2008; Brown and Diesmos 2009), few distinct morphological characters clearly differentiate...
currently described species, and the geographic ranges of taxa are not well characterized (Brown and Alcala 1980). Because extreme evolutionary radiations such as these are often characterized by discordance between genetic loci, data type, or other species recognition criteria, we take a pluralistic approach to examine lineage diversification and delimit species in Philippine sun skinks using extensive gene sampling and robust geographical coverage of populations from across this unique archipelago. In addition, our study examines the effectiveness of using recently developed methods and outlines an approach for investigating genetic and morphological diversity, and obtaining accurate estimates of species limits in “nonadaptive” or “cryptic” evolutionary radiations.

Methods
TAXONOMIC AND GENETIC SAMPLING AND IDENTIFICATION OF EVOLUTIONARY LINEAGES

The core Philippine *Eutropis* radiation consists of five described species, one of which is divided into two subspecies (*Eutropis indepensa*, *Eutropis cunningi*, *Eutropis bontocensis*, *Eutropis englei*, *Eutropis multicarinata multicarinata*, and *Eutropis multicarinata borealis*; Brown and Alcala 1980). Two additional species in the genus occur in the Philippines (*Eutropis multifasciata* and *Eutropis rubis*), but these are members of a separate evolutionary radiation (Mausfeld and Schmitz 2003; Datta-Roy et al. 2012) and are not included in this study. Because of the poorly developed taxonomy and difficulty in assigning individuals to species in some cases, we sequenced a large sample of individuals (187) from across the archipelago for the nicotinamide adenine dinucleotide dehydrogenase subunit 2 (ND2) mitochondrial gene (Macey et al. 1997; Linkem et al. 2010) as an initial screen of genetic diversity (see Supplementary Appendix S4 for specific locality information). Our sampling design included two individuals (when possible) of each putative species from each of 79 sampling localities. Previous studies of some species delimitation methods have shown that sampling two individuals dramatically improves both accuracy and precision (Camargo et al. 2012). We also included individuals from the type locality of all described *Eutropis* species in the Philippine radiation with exception of *E. englei*. Because this species is known to occur only in a portion of the country that is logistically challenging for biologists to access, we have been unable to include it in this study. Although the Philippine *Eutropis* radiation (exclusive of *E. multifasciata* and *E. rubis*) represents a monophyletic species group (Mausfeld and Schmitz 2003; Datta-Roy et al. 2012), populations of several species in the group have been reported to occur in eastern Malaysia and the islands of Palau (Crombie and Pregill 1999; Das 2004); accordingly we included several individuals from these populations in our study.

Genomic DNA was extracted from soft tissue using Fujita’s guanidine thiocyanate protocol (Esselstyn et al. 2008). DNA was amplified using standard PCR protocols, and products were purified with a 20% dilution of ExoSAP-IT (Amersham Biosciences, Piscataway, NJ), incubated for 30 min at 37 °C and then at 80 °C for 15 min. Cleaned PCR products were dye-labeled using Big-Dye terminator 3.1 (Applied Biosystems, Foster City, CA), purified using Sephadex (Amersham Biosciences), and sequenced on an ABI 3730 automated capillary sequencer. All PCR products were sequenced in both directions. All DNA sequence data collected for this research was deposited in Genbank (see Supplementary Appendix S3 for accession numbers). Sequences were edited and subsequently aligned using MAFFT in Geneious Pro version 5.3 (Katoh et al. 2005). The alignments were visually examined and translated for coding regions using Mesquite version 2.75 ( Maddison and Maddison 2011). Models of molecular evolution were selected using decision theory implemented in DT-ModSel ( Minin et al. 2003). All alignments and phylogenetic trees generated for this research were deposited in Treebase (accession no. S14377).

We then used a general mixed Yule-coalescent (GMyc) model in combination with the ND2 data to generate a preliminary species delimitation hypothesis. The GMyc model attempts to distinguish between interspecific (modeled by a Yule process) and intraspecific (modeled by the coalescent) branching events on a phylogenetic tree, based on the idea that the rate of coalescence within species should be much greater than between (Pons et al. 2006). There are currently three implementations of the model, all of which we tested on our data set: a maximum likelihood (ML) method with a single threshold (Pons et al. 2006), a multiple threshold ML method that allows the depth of the coalescent–speciation transition to vary along branches of the tree (Monaghan et al. 2009), and a Bayesian method (bGMyc) that accounts for error in phylogeny estimation and uncertainty in model parameters (Reid and Carstens 2012). To generate ultrametric trees for our analyses, we ran both strict clock and uncorrelated lognormal (UCLN) relaxed clock analyses of our 1017 base pair (bp) ND2 data set using BEAST version 1.7.3. Because the inclusion of identical sequences results in many zero length branches at the tip of the tree and can cause the model to overpartition the data set (Reid and Carstens 2012; also noted during preliminary analyses of our data set), we pruned these sequences from our data set for the final BEAST and GMyc analyses (resulting in 128 individuals in the alignment). We performed a likelihood ratio test to see if we could reject a molecular clock for the pruned ND2 data set by comparing the likelihood of the tree after optimizing the branch lengths with and without enforcing a molecular clock using PAUP* version 4.0b10 (Swofford 2002). However, we were unable to reject a global molecular clock for the mtDNA data set (P = 0.08).
We ran our BEAST analyses for 20 million generations, sampling every 2 × 10^3 generations and assessed convergence by ensuring that all parameters had reached stationarity and sufficient (>200) effective sample sizes using Tracer version 1.4 (Rambaut and Drummond 2007), and that the posterior distributions differed from the priors. We checked for topological convergence by ensuring that posterior probabilities were stable and that split frequencies were similar across runs using Are We There Yet? (AWTY: Wilgenbusch et al. 2004; Nylander et al. 2007). For ML GMYC analyses, we used the maximum clade credibility tree generated from the posterior distribution of our BEAST analyses. For bGMYC analyses, we used 100 trees sampled from the posterior distribution of the BEAST analyses and ran the GMYC analyses on each tree for 50,000 generations, discarding the first 40,000 generations as burnin, and using a thinning interval of 100 (as recommended by the authors). Nei’s genetic distance (D_N; Nei 1987) values (with a Jukes and Cantor distance correction) between the groups of populations identified as distinct evolutionary lineages by the GMYC analyses were calculated using DnaSP version 5.10.1 (Librado and Rozas 2009). Based on these analyses of the ND2 data, we then scaled our sampling of individuals down, targeting equal numbers of individuals across all divergent lineages for sequencing of nuclear genes, and further examination of species boundaries.

We chose exon-primed, intron-crossing (EPIC) markers for our study because the conserved exonic portions can anchor primers for use across a phylogenetically diverse species group; whereas the intronic regions typically have higher substitution rates than protein-coding DNA and consequently are more informative for studies of species-level phylogenetics and phylogeography (Thomson et al. 2010). We developed markers by screening primers used in studies of closely related taxonomic groups (birds, turtles, and other squamates) and examining them for appropriate size, function across taxonomic diversity, and variation across species. In some cases, we designed new primers if the original primers failed to work across all taxonomic diversity in Philippine Eutropis, based on the collected sequence data and the Anolis genome (Alföldi et al. 2011). We sequenced each individual for six nuclear genes (see Supplementary Appendix S2 for primer sequences and PCR protocols): the ATP synthetase-B subunit intron (ATPSB; Skinner 2007), the selenoprotein-T intron (SELT; Jackson and Austin 2009), the N-acetyltransferase 15 intron (NAT15; Kimball et al. 2009), the nitric oxide synthase 1 intron (NOS1), the forkheadbox P2 intron (FOXP2), and the t-lactate dehydrogenase M chain (LDHA) gene (Pasachnik et al. 2009). Because sequences from some individuals contained heterozygous insertions/deletions, the program Indelligent version 1.2 (Dmitriev and Rakitov 2008) was used to reconstruct the allelic sequences when necessary.

**FULL DATA SET PHYLOGENETIC ANALYSES**

To further investigate support for species boundaries in our data set, we estimated phylogenetic trees for the full seven-gene data set, the nuclear data only, and each gene individually. Bayesian inference (BI) phylogenetic analyses were performed with BEAST version 1.7.3, using separate strict clock models for each gene, fixing the mean substitution rate to 1.0, and partitioning the data set by gene (with separate substitution models for each partition). We ran our analyses for 100 million generations, sampling every 5 × 10^3 generations, and assessed convergence using Tracer and AWTY as discussed earlier. We examined the effect of analyzing the ND2 data as a single partition, partitioned by codon, or as two partitions (with the first and second codon positions being one partition, and the third codon position being the other) in all analyses of the full data set. However, regardless of partitioning strategy, all analyses consistently produced the same topology, so we chose to analyze it as a single partition in our final analyses. Maximum likelihood gene tree estimates were obtained using RAxML version 7.0.3 (Stamatakis 2006) with nodal support assessed via 100 bootstrap replicates. The evolutionary models for each partition selected using DT-ModSel were used in all BI analyses, whereas all partitions were assigned a GTR + Ψ model in the ML analyses, as this is the only option available in RAxML. Trees were rooted with Eutropis macularia, a closely related species that does not occur in the archipelago (Mausfeld and Schmitz 2003; Datta-Roy et al. 2012).

**COALESCENT-BASED SPECIES DELIMITATION USING NUCLEAR DATA**

We then used the program Bayesian Phylogenetics and Phylogeography (BP&P) version 2.1 in combination with our nuclear DNA (nuDNA) to evaluate potential species boundaries (Yang and Rannala 2010) in several situations where species delimitation was still ambiguous despite an examination of gene trees, morphological data, and geographic range. This method uses a Bayesian framework and an explicit model of lineage sorting based on the coalescent process to estimate posterior probabilities for competing species delimitation models, while integrating over uncertainty in gene trees. It does this using a reversible-jump Markov Chain Monte Carlo (rjMCMC) algorithm in combination with a user-specified guide tree.

BP&P requires a prior on the population size parameter (θ) and the age of the root in the species tree (τ_0), which can affect the posterior probabilities for the models. Both priors are given a γ distribution G(α,β) with mean α/β and variance α/β^2. Larger values for θ and smaller values for τ_0 favor conservative models with fewer species (Yang and Rannala 2010). Estimates of θ exhibit a broad range (~0.0005–0.02) across extant plant and animal species (Zhang et al. 2011). We used a variety of combinations of
priors that assumed large, medium, or small ancestral population sizes and shallow, moderate, or deep divergences. For our priors, we used \( \theta \sim G(1, 10) \), \( \theta \sim G(1, 100) \), \( \theta \sim G(2, 2000) \). The same values were used for \( \tau_0 \), using all the different possible combinations of values for these two parameters to evaluate their effect on the analysis. The program can implement two different rjMCMC algorithms, both of which we tested for consistency across each species delimitation hypothesis, while having the program auto-adjust the fine-tuning parameters. We also ran each analysis multiple times using different starting trees to ensure stability, as well as proper mixing and convergence across runs.

Because BP&P requires that the topology of the phylogeny be known with certainty to accurately delimit species (Yang and Ramnala 2010), we only used BP&P to evaluate species limits in clades with strongly supported topologies in all our phylogenetic analyses of the full data set. Because the ND2 data were used to identify the clades for our species delimitation hypotheses (using the GMYC), we did not include them in the BP&P analyses, and included only the six nuclear genes. This approach allowed us to evaluate the species delimitation hypotheses with independent loci that were not used in the hypothesis formulation.

MORPHOLOGICAL DATA

We also sought to examine the effectiveness of using variation in external meristic (scale counts) and mensural (body measurements) morphological characters to delimit species. In total, we collected morphological data for 145 individuals (see Supplementary Appendix S5 for specimens examined). For each individual, we collected 13 quantitative morphological traits: snout-vent length, axilla–groin distance, head length and head width (measured at the front of the auricular opening), forelimb length, hind limb length, total number of lamellae under toes I–V on the right foot, supra- and infralabial scale counts, ventral scale counts (counted as ventral scales between front and rear limbs), vertebral scale counts (the number of scale rows between the parietal scales and the base of the tail), midbody scale row counts, and number of keels per scale. Morphological data were deposited in the Dryad doi:10.5061/dryad.307g0.

In addition to visually examining the morphological data for characters that could distinguish potential species, we performed three types of multivariate statistical analyses using the morphological data. Principal component analyses (PCA) were performed using a correlation matrix to examine the data for structure that could potentially correspond to species groups. We also performed linear discriminate function analysis (DFA) to determine if individuals could be assigned to the correct species groups identified by molecular data. Finally, we performed a two-step cluster analysis using Schwarz’s Bayesian Criterion and a log-likelihood distance measure as an objective attempt to determine the number of morphological groups present in our data set. All morphological measurements were log-transformed for multivariate analyses to reduce heteroscedasticity and improve normality. Analyses were performed using R version 2.13.0 (The R Foundation for Statistical Computing; http://www.R-project.org), SPSS version 20 (IBM Corp.), and JMP8 (SAS Institute Inc.).

SPECIES TREE ANALYSIS

After assessing species boundaries, species tree analysis was conducted using the program *BEAST (Heled and Drummond 2010) in BEAST version 1.7.3. *BEAST is a Bayesian method that uses a coalescent framework to simultaneously estimate a species-tree topology, divergence times, population sizes, and gene trees from multigene data sets. We ran both strict clock and UCLN relaxed clock analyses, fixing the mean substitution rate to 1.0.

The species population-mean hyperprior and the species Yule process birth prior were both assigned exponential distributions, with means of 0.01 and 1.0, respectively. We used a piecewise linear and constant root population size model. In the strict clock analyses, the relative clock rates for each gene were assigned exponential prior distributions with a mean of 1.0. In the UCLN relaxed clock analyses, the clock means for each gene were also assigned exponential distributions with a mean of 1.0, and the standard deviations were assigned exponential distributions with a mean of 0.05. We ran each analysis for 200 million generations, sampling every 10^4 generations. Convergence was assessed using Tracer and AWTY as described earlier.

Finally, as a cursory examination of diversification rates in Philippine Eutropis, we generated lineage through time plots for each of the post burin-in trees from the posterior distribution of our UCLN relaxed clock *BEAST analyses. We plotted the log number of lineages against relative time using the APE package (Paradis et al. 2004) in R. We tested for a significant departure from the null hypothesis of a constant rate of diversification through time using the constant-rate (CR) test (Pybus and Harvey 2000). We calculated the \( \gamma \)-statistic for each of 10,000 trees drawn from the posterior distribution of two different *BEAST analyses: one employing a more conservative estimate of species diversity (13 species) and one with a more liberal estimate (15 species).

Results

GENERAL MIXED YULE-COALESCENT ANALYSES

Although topologies differed somewhat with respect to the poorly supported, deeper nodes in the tree, both strict and relaxed clock phylogenetic analyses of the ND2 data (when analyzed as a single partition and when partitioned by codon) identified the same strongly supported clades (Fig. 2). These slight changes in topology did not affect our GMYC analyses, which gave consistent
results regardless of the tree used in the analysis. However, each of the different GMYC methods gave a slightly different result. The mean number of species estimated by the single threshold ML model was 19 (with the 95% highest posterior density [HPD] ranging from 4 to 27 species), whereas the mean number of species estimated by the multiple threshold ML model was 27 (with the 95% HPD ranging from 16 to 29). The mean number of species estimated by the bGMYC model was 19 (with the 95% HPD ranging from 9 to 33). After examining the geographic ranges (Fig. 2) and genetic divergences (Table 1) among the lineages identified in the analyses, we adopted the more conservative estimate of species diversity (19) as our working hypothesis. In addition, several of the lineages identified in the GMYC analyses were represented by only 1 or 2 individuals (which could significantly impact the results in coalescent analyses). Because we had poor sampling in the regions where these lineages occur, and the populations together represented well-supported, monophyletic clades in this and subsequent phylogenetic analyses, we conservatively considered them to be a single species in later analyses (Fig. 2; clade numbers four and 15). This resulted in 16 divergent groups of populations we regarded as candidate species, which we targeted for further investigation.

**PHYLOGENETIC ANALYSES**

We collected nuclear sequence data for a subsample of 74 individuals for use in all subsequent analyses. This resulted in 3–6 individuals (mean \( n = 4.6 \)) per divergent clade. Our nuclear data set consisted of a total of 4237 bp, and we were able to sequence all 74 individuals for NAT15 (572 bp), FOXP2 (634 bp), and LDHA (556 bp). ATPSB (1185 bp) was sequenced for 73 individuals, whereas SELT (694 bp) and NOS1 (596 bp) were sequenced for 72 individuals. The combined phylogenetic analyses of all the data identified the same 16 clades as the mtDNA data, and we unambiguously assigned existing species names (based on type localities and examination of type specimens) or clade letters to 13 of them (Fig. 3). Phylogenetic analyses of the combined nuclear gene data only, resulted in support for the monophyly of 15 of the 16 clades (with the exception being the split within clade E in Fig. 3 was not resolved).
Eleven of the 16 clades were found to be monophyletic (although support values were low in some instances) for each of the six nuclear genes (see Supplementary Appendix S6 for individual gene tree estimates). This consisted of the clades that were assigned species names or clade letters in Figure 3, except that clade D, *E. indeprensa*, and *E. cumingi* would be considered a single monophyletic clade in this case. We observed no instances of incongruence between analyses of mtDNA and nuDNA that might indicate hybridization or introgression, as all individuals were consistently assigned to the same 11 monophyletic clades among individual gene tree estimates. Relationships among these clades varied, however, this is not surprising given that most nodes in individual gene trees were not strongly supported, and gene tree heterogeneity is commonly observed in multilocus phylogenetic data sets (Jennings and Edwards 2005; Kubatko and Degnan 2007; Edwards 2008; Liu et al. 2008; Barley et al. 2010). Among clade D, *E. indeprensa* and *E. cumingi*, only three of six nuclear gene trees showed each clade to be monophyletic (ATPSB, SELT, and NOS1). Analysis of mtDNA data revealed significant divergence between the Cordillera Mountain Range populations and all other populations of *E. cumingi*; and actually showed both *E. indeprensa* and clade D populations to be more closely related to other populations of *E. cumingi* than those in the Cordillera (Fig. 2; Table 1). However, none of the nuclear gene trees found the Cordillera Mountain populations to be reciprocally monophyletic with respect to other populations of *E. cumingi*. Within clade E, only two of six nuclear gene trees (ATPSB and LDHA) showed the populations on Mindanao/Bohol and Siargao/Dinagat to form reciprocally monophyletic clades. Among *E. m. borealis* populations, three of six nuclear gene trees (LDHA, SELT, and NOS1) found populations from Luzon/Polillo/Catanduanes/Babuyan Islands and Negros/Panay/Siquijor to form reciprocally monophyletic clades.

**Table 1. Pairwise Nei’s genetic distance (D<sub>np</sub>) values (with a Jukes and Cantor distance correction) between all combinations of each of the 16 clades identified in Figure 2.**

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<td>0.1129</td>
<td>0.1556</td>
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<td>0.1469</td>
<td>0.1471</td>
<td>0.1432</td>
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<td>0.1539</td>
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<td>0.1574</td>
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<td>14</td>
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<td>–</td>
<td>0.1554</td>
<td>0.1442</td>
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<tr>
<td>15</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.1384</td>
</tr>
</tbody>
</table>

**COALESCENT-BASED SPECIES DELIMITATION USING NUCLEAR DATA**

We focused our BP&P analyses on three different groups: the *E. indeprensa* species complex, clade E, and the *E. m. borealis* clade. All analyses gave consistent results regardless of the rjMCMC algorithm and starting tree used (Fig. 3). For both clade E and the *E. m. borealis* clade, our choice of prior distributions for (θ) and (τ₀) did not affect our results, and all analyses supported a split between the Siargao/Dinagat Islands populations and the Mindanao/Bohol populations in clade E, as well as a split between the Luzon/Polillo/Catanduanes/Babuyan Island populations and the Negros/Panay/Siquijor populations of *E. m. borealis*, with a posterior probability for speciation of 1.0 (Fig. 3). For the *E. indeprensa* species complex, all BP&P analyses supported splits between all six clades with speciation posterior probabilities of 1.0 regardless of prior choice, with one exception (Fig. 3). In analyses using our most conservative choice of priors (θ ~ G(1, 10) and τ₀ ~ G(2, 2000), which assume large ancestral population sizes and shallow divergence among species), a split between the Cordillera Mountain populations of *E. cumingi* and the rest of Luzon was not supported.

**MORPHOLOGY**

Trait values for some morphological characters showed little variation across all Philippine *Eutropis* species; and the small variation present was as variable within taxa as between. This included the number of supralabial scales, infralabial scales, and midbody
scale rows. In addition, the number of keels per scale was often as variable within individuals as between individuals. Therefore, we excluded data for these morphological characters and performed all multivariate analyses using data from the remaining nine traits: SVL, axilla–groin distance, head length, head width, forelimb length, hind limb length, total number of lamellae under the toes, vertebral scales rows, and ventral scale rows. We focused our morphological analyses on 10 of the clades from Figure 3: clade C, clade E, clade F, clade G, *E. indeprensa*, *E. cumingi*, *E. bontocensis*, *E. m. multicarinata*, *E. m. borealis*, and the *Eutropis* species from Palau. For the other clades, the sample sizes from which we were able to collect morphological data were
Table 2. Results of principal component analysis.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eigenvalue</td>
<td>6.8024</td>
<td>1.3019</td>
<td>0.3136</td>
<td>0.2255</td>
</tr>
<tr>
<td>Percent</td>
<td>75.5824</td>
<td>14.4658</td>
<td>3.4845</td>
<td>2.5057</td>
</tr>
<tr>
<td>SVL</td>
<td>0.3671</td>
<td>0.1436</td>
<td>0.1861</td>
<td>0.0891</td>
</tr>
<tr>
<td>AG</td>
<td>0.3551</td>
<td>0.1540</td>
<td>0.3832</td>
<td>0.0311</td>
</tr>
<tr>
<td>HDLG</td>
<td>0.3715</td>
<td>0.1073</td>
<td>0.1574</td>
<td>0.0916</td>
</tr>
<tr>
<td>HDWD</td>
<td>0.3699</td>
<td>0.0616</td>
<td>0.3832</td>
<td>0.0364</td>
</tr>
<tr>
<td>ARM</td>
<td>0.3639</td>
<td>0.1370</td>
<td>0.0284</td>
<td>0.1126</td>
</tr>
<tr>
<td>LEG</td>
<td>0.3697</td>
<td>0.0832</td>
<td>0.0466</td>
<td>0.0504</td>
</tr>
<tr>
<td>LAM</td>
<td>0.3309</td>
<td>0.0367</td>
<td>0.8578</td>
<td>0.1968</td>
</tr>
<tr>
<td>VERT</td>
<td>−0.2476</td>
<td>0.5846</td>
<td>0.2155</td>
<td>0.7380</td>
</tr>
<tr>
<td>VEN</td>
<td>−0.1562</td>
<td>0.7560</td>
<td>−0.0888</td>
<td>−0.6187</td>
</tr>
</tbody>
</table>

SVL = snout–vent length, AG = axilla–groin distance, HDLG = head length, HDWD = head width, ARM = forelimb length, LEG = hind limb length, LAM = total lamellae number, VERT = vertebral scale rows, and VEN = ventral scale rows.

Figure 4. Principal component analyses biplots for nine morphological variables shown in Table 2.

Table 3. Results of the discriminate function analysis of 10 clades based on nine morphological variables. Columns represent true identities of individuals based on their genetic clades, whereas rows indicate which group individuals were assigned to by analysis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Clade A</th>
<th>Clade B</th>
<th>Clade C</th>
<th>Clade D</th>
<th>Clade E</th>
<th>Clade F</th>
<th>Clade G</th>
<th>Clade H</th>
<th>Clade I</th>
<th>Clade J</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. m. borealis</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. m. multicarinata</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>E. bontocensis</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eutropis sp. Palau</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clade E</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Clade F</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Clade G</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. indeprensa</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>E. cumingi</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Clade C</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Total N</td>
<td>22</td>
<td>6</td>
<td>20</td>
<td>13</td>
<td>18</td>
<td>13</td>
<td>5</td>
<td>6</td>
<td>14</td>
<td>20</td>
</tr>
</tbody>
</table>

small, so extensive characterization of morphological variation within clades was not possible.

We found snout-vent length, axilla–groin distance, head length, head width, forelimb length, and hind limb length to be strongly correlated ($r = 0.88–0.94$; Supplementary Appendix S1). Total lamellae number was moderately correlated with these variables ($r = 0.69–0.77$; Supplementary Appendix S1). Vertebral and ventral scale rows did not show significant correlation with
the other morphological variables, however, they were moderately correlated with each other \((r = 0.73; \text{Supplementary Appendix S1})\). Principal component analysis found size to be the most important variable in explaining variance in our data set \((explaining 74.8\%)\), as the loadings for PC1 all had similar values for all variables \(\text{with the exception of vertebral and ventral scale rows, which are not strongly associated with body size; Table 2}\). PC2 loaded strongly on the number of ventral and vertebral scale rows, and explained 14.6\% of the total variance. PC3 loaded strongly on the total number of lamellae under the toes, and explained 3.7\% of the total variance. However, most clades did not separate significantly along the axes of variation in morphological size and shape identified in the PCA \((\text{Fig. 4})\).

Discriminate function analyses of the morphological data was only able to assign all individuals correctly for three of 10 genetic clades \(\text{correctly assigning a total of 75.2\% of individuals to their respective genetic clade, highlighting the extremely conserved external morphology of Philippine Eutropis (Table 3)}\). Our cluster analysis found the optimal number of clusters in our data set to be two, with cluster 1 being composed of all individuals from the clades in the \(E.\ multicarinata\) species complex except for \(E.\ bontocensis\), and cluster 2 consisting of all individuals from the clades in the \(E.\ indeprensa\) species complex and \(E.\ bontocensis\) \((\text{Fig. 5})\). However, because our main concern was determining species boundaries, we were more interested in whether or not sister clades showed evidence of morphological differentiation.

Species tree analysis of the molecular data demonstrated strong support for a sister relationship between \(E.\ bontocensis\) and clade F \((\text{Fig. 3})\). These clades were also found to be highly genetically distinct from each other \((\text{Table 1 and Fig. 3})\), despite the fact they occur sympatrically; indicating the two taxa represent distinct species. Discriminate function analysis was able to correctly distinguish between the two clades, and individuals were not misassigned between them. \(Eutropis bontocensis\) was found to exhibit a generally smaller body size and a fewer total number of toe lamellae \((\text{Table 4})\). Similarly, \(E.\ m.\ borealis\) and clade G appear to be highly genetically distinct sister clades \((\text{Table 1, Fig. 3})\). In this case, DFA was again able to distinguish between the two groups based on combinations of the morphological variables \((\text{Tables 3, 4})\), as clade G tended to have slightly larger body size and more vertebral scale rows; however there was significant overlap among all trait values. When comparing clade E and the \(Eutropis\) species from Palau, trait values again overlapped, but in this case DFA was unable to correctly assign all individuals between each group. Discriminate function analysis correctly distinguished between individuals of \(E.\ indeprensa\) and \(E.\ cumingi\), as \(E.\ indeprensa\) had a slightly longer hind limb/SVL ratio and more total toe lamellae.

Discriminate function analysis performed best in distinguishing between the \(E.\ multicarinata\) and \(E.\ indeprensa\) species complexes, with body size generally being much larger in the \(E.\ multicarinata\) species complex. This result was also reflected in our cluster analysis, which identified two morphological clusters.

### Table 4. Ranges of morphological trait values for nine morphological variables for 10 of the clades shown in Figure 3. See Table 2 for character abbreviation interpretations.

<table>
<thead>
<tr>
<th>Clade</th>
<th>(E.\ m.\ borealis)</th>
<th>(E.\ m.\ multicarinata)</th>
<th>(E.\ bontocensis)</th>
<th>(Eutropis) sp. Palau</th>
<th>Clade E</th>
<th>Clade F</th>
<th>Clade G</th>
<th>(E.\ indeprensa)</th>
<th>Clade C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVL</td>
<td>64.1–82.5</td>
<td>61.1–71.3</td>
<td>40.1–61.2</td>
<td>61.0–79.3</td>
<td>64.5–83.9</td>
<td>60.5–78.7</td>
<td>73.7–88.6</td>
<td>54.0–63.8</td>
<td>43.5–60.0</td>
</tr>
<tr>
<td>AG</td>
<td>27.4–41.4</td>
<td>29.6–36.1</td>
<td>22.4–29.5</td>
<td>29.2–40.3</td>
<td>28.6–40.7</td>
<td>27.0–40.3</td>
<td>35.0–41.2</td>
<td>24.9–28.7</td>
<td>21.4–26.9</td>
</tr>
<tr>
<td>HDLG</td>
<td>14.0–17.6</td>
<td>13.5–15.8</td>
<td>9.7–13.8</td>
<td>13.6–18.8</td>
<td>13.6–19.3</td>
<td>12.2–18.0</td>
<td>15.3–19.4</td>
<td>10.3–13.5</td>
<td>8.6–11.6</td>
</tr>
<tr>
<td>HDWD</td>
<td>10.4–13.3</td>
<td>10.0–12.9</td>
<td>6.8–9.8</td>
<td>9.5–12.8</td>
<td>10.0–14.6</td>
<td>10.2–13.0</td>
<td>11.5–17.4</td>
<td>7.9–9.9</td>
<td>6.1–8.6</td>
</tr>
<tr>
<td>ARM</td>
<td>11.0–18.7</td>
<td>14.6–16.9</td>
<td>9.4–13.6</td>
<td>14.8–19.2</td>
<td>13.4–19.8</td>
<td>12.0–19.3</td>
<td>17.9–22.2</td>
<td>11.9–14.9</td>
<td>7.6–11.1</td>
</tr>
<tr>
<td>LEG</td>
<td>15.1–24.8</td>
<td>18.0–23.1</td>
<td>11.4–16.6</td>
<td>18.1–24.3</td>
<td>17.4–24.7</td>
<td>15.6–22.2</td>
<td>21.8–27.3</td>
<td>14.7–18.4</td>
<td>9.4–12.2</td>
</tr>
</tbody>
</table>
largely consisting of these two clades. Although E. bontocensis is an exception to this (having a generally smaller body size than other taxa in the species complex), DFA still assigned 97.8% of individuals correctly to the two groups. Among our comparisons, DFA performed most poorly in distinguishing among E. m. borealis, E. m. multicarinata, clade E, clade F, and the Eutropis species from Palau, only correctly assigning 68.1% of individuals to their actual genetic clade.

SPECIES TREE ANALYSIS

Our strict and relaxed clock species tree analyses using *BEAST resulted in identical topologies (Fig. 6). These results support the existence of two distinct species complexes in the Philippines: the E. indeprensa species complex and the E. multicarinata species complex. Our diversification rate analyses using relative divergence times indicated there has been a significant decrease in speciation rate through time in the Philippine radiation, regardless of the number of species specified in the analysis (Fig. 6).

Discussion

SPECIES DELIMITATION

Despite a recent increase in interest in developing methods for delimiting species, conflicts between data sets, methodologies, and species recognition criteria often makes species delineation difficult in species-rich evolutionary radiations (Frost and Hillis 1990; de Queiroz 1998, 1999; Tobias et al. 2010). Traditionally, this has been examined in cases where species exhibit extensive morphological diversity, but little genetic divergence. This is often the result of rapid or recent divergences among species, or hybridization among lineages, and many well-studied examples exist (Witter and Carr 1988; Seehausen 2004; Petren 2005). In this study, we find an extreme case of an alternative evolutionary phenomenon that poses a challenge to species delimitation: when many evolutionary lineages are highly genetically distinct, but morphologically indistinguishable.

Analysis of our data set revealed that our current systematic knowledge of Philippine Eutropis vastly underestimates the actual lineage (species) diversity present within the archipelago. Concordant gene tree splits from multiple loci provide strong evidence for genetic isolation of populations representing distinct species (Knowles and Carstens 2007; O’Meara 2010), and therefore we find that conservatively, a minimum of eleven distinct species occur in our data set, because each of these clades is supported by seven independent loci. Geographic range data for each of these groups of populations also indicate that these genetic clades are likely isolated from each other: either because they are separated
by large geographic distances, or are highly divergent clades that occur sympatrically (Fig. 6 and Table 5).

However, our coalescent-based analyses indicate strong support for species diversity being as high as 15 candidate species, and provide limited support for as many as 19 distinct evolutionary lineages. A lack of monophyly at all genetic loci does not necessarily preclude the possibility that speciation has occurred, as this could be the result of random variation in the coalescent process, or the failure of lineages to completely sort due to a recent speciation event. We find mixed support in our nuclear data for speciation having occurred among clade D, *E. indeprensa*, and *E. cumingi* (Fig. 6). In addition, these clades occur on adjacent islands and genetic divergences among clades could be the result of phylogeographic structure, with the potential for low rates of gene flow to occur among populations.

Our coalescent-based analyses supported a split between populations on the islands of Mindanao/Bohol and Siargao/Dinagat within clade E. However, because we lacked sampling from NE Mindanao for this clade, we refrain from drawing any strong conclusions. Finally, we found significant genetic divergence between populations of *E. m. borealis* on Luzon/Palilo/Catanduanes/Babuyan Islands and Negros/Panay/Siquijor. However, our data set contains poor sampling in the central Philippine Visayan Islands, where there was evidence of extensive genetic structure among populations (including ~10% uncorrected “P” pairwise mtDNA sequence divergence among individuals from each island). We also lacked sampling on islands separating these two clades (e.g., Masbate), and thus the status of these populations warrants further field sampling and systematic investigation.

**COALESCENT MODELING**

Methods that attempt to model the coalescent process as it relates to species delimitation have only recently begun to be developed (Fujita et al. 2012). In this case, we applied two coalescent modeling methods to our data set, one to generate an initial hypothesis for species delimitation based on mtDNA data (because the chaotic taxonomic history of the group made this problematic), and one to test these species limits using nuclear data. The coalescent models strongly supported the more liberal species delimitation hypotheses in most cases. The few simulation studies that have tested coalescent-based methods have been encouraging, often finding them to perform relatively well even when data sets are small (Yang and Rannala 2010; Zhang et al. 2011; Reid and Carstens 2012), and many recent empirical studies have tested their performance on data sets of various composition (Monaghan et al. 2009; Leaché and Fujita 2010; Barrett and Freudenstein 2011; Burbrik et al. 2011; Setiadi et al. 2011; Brown et al. 2012; Fujita et al. 2012; Spinks et al. 2012).

### Table 5. Table indicating proximity of closest known populations among clades in Figure 6.

<table>
<thead>
<tr>
<th>Clade A</th>
<th>E. bontocensis</th>
<th>Clade E</th>
<th>Clade F</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. m. borealis</td>
<td>AI, 70 km.</td>
<td>NAi, 350 km.</td>
<td>AI, 240 km.</td>
</tr>
<tr>
<td>E. m. multicarinata</td>
<td>SI, 770 km.</td>
<td>SI, 280 km.</td>
<td>SI, 310 km.</td>
</tr>
<tr>
<td>E. m. borealis</td>
<td>Al, 70 km.</td>
<td>SI, 310 km.</td>
<td>Al, 240 km.</td>
</tr>
</tbody>
</table>

Abbreviations indicate closest known populations occur on: AI = adjacent islands, NAI = non-adjacent islands. SI = same island. Distances are approximate distance between closest populations sampled in study.
However, as with any statistical modeling method, it is important that they are used in appropriate systems. For example, the robustness of coalescent approaches to variations in geographic sampling and violations of model assumptions (which are often present in real world data sets such as this one) remain to be examined using simulated and empirical data sets. Of particular concern with respect to our data set are the assumptions of no genetic structuring within lineages and constant population sizes, which are likely unrealistic in island archipelago systems. These assumptions may bias the method toward oversplitting, particularly because in this study we found the method to nearly always support the most liberal groupings.

**MORPHOLOGICAL DATA**

Although sun skinks in general can be characterized as a group in which external morphology has been highly conserved through evolutionary time (Miralles et al. 2005; Miralles and Caranza 2010; Hedges and Conn 2012), our data demonstrate that Philippine *Eutropis* represent an extreme example of this. We found extensive genetic differentiation among species within the Philippine radiation, which has not been accompanied by even moderate levels of differentiation in external morphological traits. In some cases, we were unable to consistently diagnose species based on these morphological characters, and in cases where morphological differentiation appears to have occurred, most of these differences are rather minor, and often involved only slight differences in body size (i.e., species in the *E. indeprensa* species complex generally had a smaller body size than species in the *E. multicarinata* complex).

The fact that speciation in Philippine *Eutropis* is only sometimes associated with relatively small changes in external morphology renders these data problematic for species delimitation. Of course, even when small differences in morphological trait values appear to be diagnostic of certain populations, they could simply be the result of local adaptation. In many cases, we found that morphological trait values varied as much within populations as between them, and were not diagnostic of genetically defined groups that appear to represent distinct species. Interestingly, we also found two examples of sympatric species within the *E. multicarinata* species complex that we were unable to differentiate using morphological data, but which are clearly genetically isolated from each other. This included clade F and *E. m. borealis*, which both occur sympotypically in northern Luzon, as well as *E. m. multicarinata* and clade E, which occur sympotypically in northeastern Mindanao and on Dinagat Island.

In situations where species diverge rapidly or recently, and extensive morphological differentiation has not been accompanied by extensive genetic differentiation, strong directional selection acting on morphological traits, usually through sexual selection or adaptation, is often inferred. When species are well differentiated genetically, but not morphologically (as is the case in this study), it has previously been suggested to be the result of stabilizing selection on important adaptive traits (Schönrogge et al. 2002; Glor et al. 2003). We find it conceivable that a similar phenomenon may be occurring in Philippine *Eutropis*. All species appear to occupy similar microhabitat types and ecological niches (with the exception of *E. bontocensis* and *E. englei*), the two highly morphologically distinct species). Thus, strong selection may be acting to maintain external morphological traits, the values of which may be constrained because they are strongly associated with the similar ecologies of all species (Roughgarden 1972; Stanley 1989; Travis 1989; Johnson and Barton 2005).

**SPECIES DIVERSIFICATION**

Our species tree analysis and our partitioned analysis of the concatenated seven-gene data set resulted in identical topologies, reflecting the strong signal in our data set. The presence of two species complexes is consistent with previous morphological work (Brown and Alcala 1980). Of those, the *E. indeprensa* complex appears to be a more recent radiation, and some of the clades show evidence of incomplete lineage sorting in multiple gene trees if they should indeed be recognized as species. Within the *E. multicarinata* complex, there does not appear to have been a historical split that divided the northern and southern populations (Fig. 6) as was previously hypothesized (Brown and Alcala 1980).

Philippine sun skinks represent an extreme example of a species rich, morphologically conserved evolutionary radiation, although their overlapping geographic distributions make them a particularly intriguing system. Unfortunately, we lack calibration points for our phylogeny, and thus are unable to obtain a reliable estimate for the age of this species complex, although the high sequence divergences among lineages (Table 1) indicate species divergences are likely relatively old. Diversification rate analyses based on relative divergence times appear to indicate that there has been a decrease in speciation rate through time. This could indicate that speciation rates were higher as this clade originally colonized the archipelago, and then slowed as they became more geographically widespread. The two species complexes exhibit overlapping distributions across the archipelago, with the *E. indeprensa* complex being predominantly structured by geographic region (with the exception that two of the clades occur sympatrically on the island of Panay; Fig. 6). The *E. multicarinata* complex exhibits more elaborate biogeographical relationships among candidate species, however, both complexes contain lineages that are restricted to small geographic regions, as well as lineages that appear to be capable of long distance dispersal across biogeographical regions. In addition, unlike most morphologically conserved evolutionary radiations (e.g., “nonadaptive” radiations or “cryptic” species complexes), some morphologically
indistinguishable lineages occur syntopically, raising the question of how these lineages can coexist.

**SPECIES DELIMITATION IN “NONADAPTIVE” OR “CRYPTIC” SPECIES COMPLEXES**

Our study outlines an approach that can be taken to obtain accurate estimates of species boundaries in species complexes where morphology is highly conserved. Our findings suggest that:

1. When lacking, preliminary hypotheses for species limits can be obtained using methods for discovering structure in data without an a priori assumptions of species boundaries (in our case, mtDNA and the GMYC model).
2. EPIC markers can be used to obtain gene tree estimates to provide additional support for lineages that are defined as distinct by mitochondrial data. Introns (Benavides et al. 2007) and anonymous loci (Thomson et al. 2008; Camargo et al. 2012) are also likely to be useful in these situations.
3. Because results based on coalescent modeling can be dependent on model parameters, it is useful to use multiple methods and independent data sets when possible (e.g., using BP&P in combination with presumably unlinked nuclear loci).
4. Although in these systems external morphology can be limited in terms of its utility to delimit species, it can still provide valuable information regarding species boundaries in some cases, such as critically important data for diagnosing sister species.
5. Finally, examining multiple lines of evidence (including geographic range data and ecological data) likely will produce the most biologically meaningful results when delimiting species.

**Conclusions**

We find that species diversity in this unique evolutionary radiation is at least two or three times higher than is currently described, and we identify an extreme case where lineage diversification has not been accompanied by divergence in external morphological traits. The surprising underestimate of species diversity in this group is particularly important because it occurs in a biodiversity conservation hotspot (Brown and Diesmos 2009). Island archipelagos have historically and recently been regarded as models systems for studying evolutionary processes in a diverse range of fields (MacArthur and Wilson 1967; Bock 1970; Losos and Ricklefs 2009; Oaks et al. 2012; Brown et al., in press), however, an accurate understanding of species diversity is critical to these types of studies. Our study represents an empirical example of how recently developed methods can be used to arrive at an enhanced, evolutionary-informed understanding of cryptic biodiversity and reveal previously overlooked, but conceptually intriguing evolutionary radiations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Appendix S1. Correlation coefficients between each of the nine morphological variables.

Appendix S2. Primer information for genes used in study.

Appendix S3. List of genetic samples used in project and associated Genbank numbers for each gene.

Appendix S4. Locality information for all individuals include in study.

Appendix S5. Specimens examined for morphological analyses.