BIODIVERSITY RESEARCH

Critically endangered island endemic or peripheral population of a widespread species? Conservation genetics of Kikuchi's gecko and the global challenge of protecting peripheral oceanic island endemic vertebrates

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ABSTRACT

Aim To highlight the significant conservation challenge of evaluating peripheral endemic vertebrates in island archipelago systems and to assess empirically the complexities of approaches to conservation genetic studies across political and biogeographic boundaries. To demonstrate the poignant need for international collaboration and coordination when species delimitation problems with high conservation concern involve island endemics with biogeographically peripheral ranges.

Location Southeast Asia, Lanyu Island, Taiwan, and the Philippines.

Methods Genetic samples were collected and sequenced for one mitochondrial gene and five nuclear loci for species of the *Gekko mindorensis-G. kikuchii* species complex in Southeast Asia. We used maximum likelihood and Bayesian phylogenetic methods and coalescent-based species delimitation analyses to estimate phylogeographic relationships, construct multilocus haplotype networks and test putative species boundaries.

Results Phylogenetic and population genetic analyses suggest that Kikuchi's Gecko may represent a peripheral population of a widespread species distributed from the northern Philippines to Taiwan. However, we identify a discrepancy between inferences of species boundaries resulting from methods based on allele frequencies versus coalescent-based methods that incorporate evolutionary history. Coalescent-based analyses suggest that *G. kikuchii* may be a distinct evolutionary lineage. Our study underscores the need for coalescent-based methods in conjunction with population genetic approaches for conservation genetic assessments of widespread species.

Main conclusions This study joins a few recent works suggesting that Philippine-derived anomalies in the fauna of Lanyu (and possibly greater Taiwan) are worthy of careful reconsideration. Determining whether each is the result of recent human-mediated introduction or (possibly more ancient) natural dispersal should be the goal of future studies on this seldom-conceived biogeographic relationship. Isolated species endemic to islands on the outer periphery of biogeographic and political regions represent particular conservation challenges. This is especially true if a species occurs on an isolated island that is allied biogeographically with one nation, but politically administered by another.

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Diversity and Distributions

INTRODUCTION

Oceanic islands often possess unique and highly endemic faunas, with insular communities characterized by combinations of species that may occur nowhere else on earth (Darlington, 1957; Carlquist, 1965; Gillespie *et al.*, 2008; Losos & Ricklefs, 2009). Individual species may be endemic to these small isolated land masses, limited in geographic distributions and characterized by smaller population sizes and restricted demographic scale (Grant, 1998; Pruett *et al.*, 2010). Isolation, founder effects and genetic bottlenecks may all lead to rapid loss of genetic variation as a result of drift or selection (Mayr, 1942; Clegg *et al.*, 2002; Fleischer & McIntosh, 2001; Kisel & Barraclough, 2010). These phenomena may lead to the genetically unique, celebrated, endemic island lineages recognized today (Van Dyke *et al.*, 2008; Losos & Ricklefs, 2009; Wilson *et al.*, 2009; Pruett *et al.*, 2010).

Unfortunately, many of these island endemic species can be more vulnerable to extinctions than continental counterparts (Blackburn *et al.*, 2004; Duncan & Blackburn, 2004; Didham *et al.*, 2005; Frankham, 1997, 1998; Frankham *et al.*, 2001; Reed *et al.*, 2003; Spielman *et al.*, 2004; for review, see Jemieson, 2007), a situation that is exacerbated by habitat destruction and anthropogenic changes that heavily affect island ecosystems (Frankham, 1998; Whittaker & Fernández-Palacios, 2007; Kingsford *et al.*, 2009; Caujapé-Castells *et al.*, 2010).

As vital as it is for conservation biologists to effectively develop and implement appropriate management strategies for island populations, it is also critical to first identify, diagnose and define island endemic lineages so that the global conservation community will appreciate their distinctiveness, unique evolutionary histories, and systematic affinities (Kier *et al.*, 2009; Costello *et al.*, 2013). Moreover, from an initial practical perspective, the conservation community can do nothing to leverage legal mechanisms, financial resources and conservation information networks until unique evolutionary lineages from islands are correctly identified and formally recognized under our current system of taxonomic recognition and conservation status assessment (IUCN, 2012). Put simply, the first step in protection of a unique island endemic is its correct identification as a unique evolutionary lineage, that is, a species (Simpson, 1961; Wiley, 1978; Frost & Hillis, 1990; de Queiroz, 2007).

Island ecosystems and the species that they support may be threatened by a myriad of anthropogenic factors, including overpopulation, unchecked development, systematic and near complete resource extraction, and political factors. Furthermore, peripheral islands may fall under political jurisdiction of one government, while being biogeographically related to another land mass that falls under another country's governance. The protection of this class of peripheral island unique evolutionary lineages represents a special conservation challenge. Celebrated examples include the many islands of eastern Indonesia (many of which are biogeographically more closely related to Australopapuan faunas than to the remaining Sundaic islands of central Indonesia; Taylor, 2003), Bougainville (politically Papua New Guinea, but faunistically allied with the Solomon Islands), Hawaii (politically United States, but with faunas derived from throughout the Pacific Ring of Fire land masses; Gillespie *et al.*, 2012), Palawan (politically Philippine, but with a mammal fauna derived from Malaysian Borneo (Esselstyn *et al.*, 2004, 2010) and many others (Gillespie & Claque, 2009).

One small island with a compellingly curious vertebrate fauna is Lanyu Island ('Orchid' Island, or 'Botel Tobago'), southeast of Taiwan (Fig. 1). Under the jurisdiction of Taiwan (Republic of China), Lanyu Island is well known for its unique combinations of animals and plants, of varying phylogenetic and biogeographic affinities (Biogeographical Society of Japan, 1932; Lin & Yen, 1982; Su & Ho, 1982; Shen & Tsai, 2002). Lanyu supports several morphologically distinctive endemics, and many species shared with Taiwan and the Asian mainland (Okada, 1936; Ota *et al.*, 1988; Ota, 1991a; Oliveros *et al.*, 2011), and ostensibly a few species shared with the northern Philippines to the south (Fig. 1; Oliveros *et al.*, 2011). An interesting Lanyu biogeographic dichotomy has been documented, with the majority of its species, and more dispersal-prone, volant species predominantly shared with Taiwan and the Asian mainland (Esselstyn & Oliveros, 2010; Oliveros & Moyle, 2010), but some

Figure 1 Hypothesized relationships of Philippine species of *Gekko*, focusing on sampled populations of the *G. mindorensis-G. kikuchii* species complex, illustrated by the maximum clade credibility tree resulting from Bayesian analyses. Nodes supported by ≥ 95% Bayesian posterior probabilities (PP) and ≥ 70% maximum likelihood bootstrap proportion (MLBP) are indicated with black dots. Terminals are labelled with taxonomic names, or in the case of the focal complex, sampling localities (Appendix S1). Numerical labels correspond to unique sampling localities, with surrounding coloured boxes highlighting the eight well-supported clades of *G. mindorensis* (Siler *et al.*, 2012) and samples of *G. kikuchii*. Each sampled locality shown on the map of the Philippines, Lanyu Island, and Taiwan, with the type locality for *G. mindorensis* represented by a black star. Putative, taxonomically stable, species boundaries based on *a priori* expectations shown in the upper right for reference with numerical labels corresponding to regional localities.
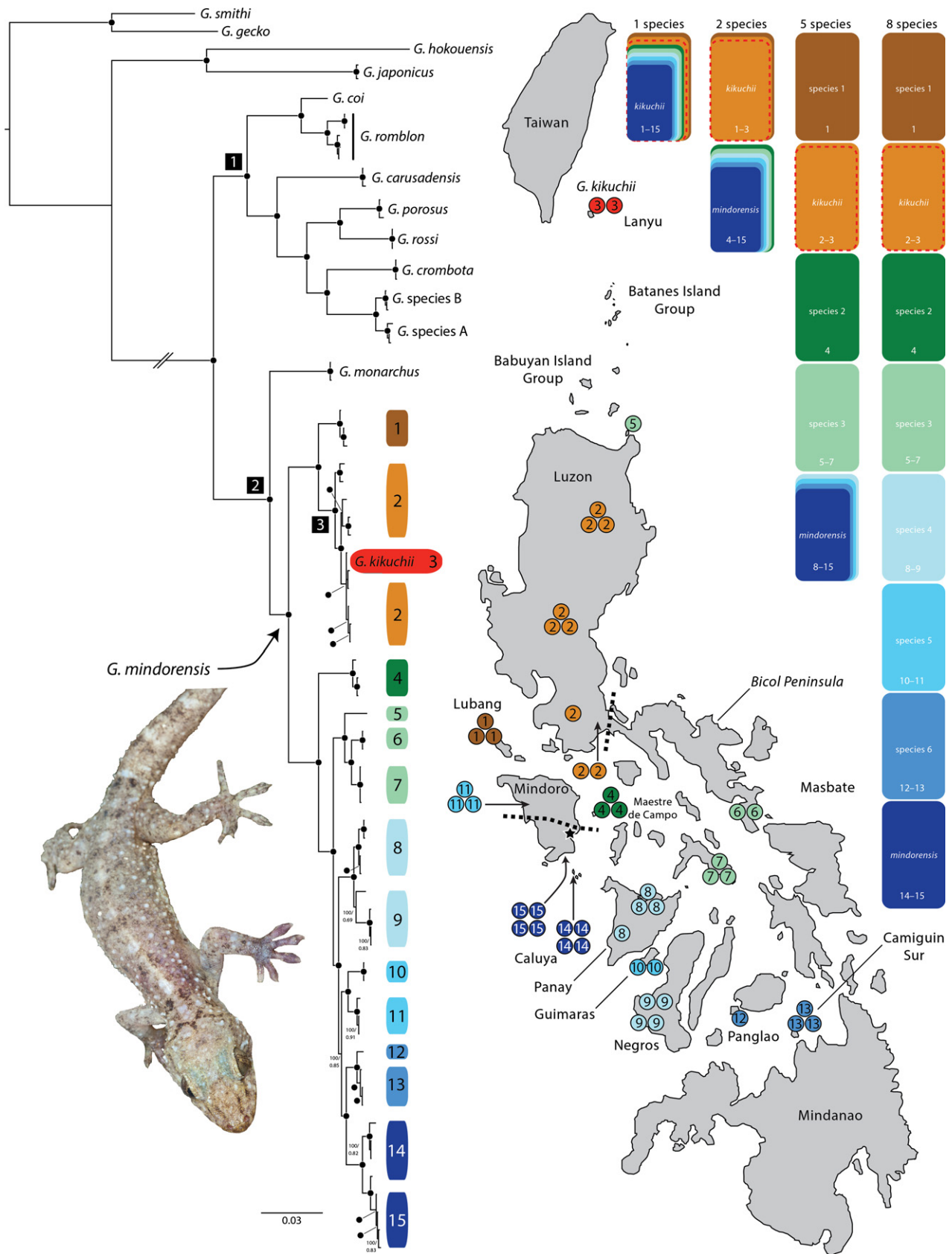
non-volant land vertebrates aligned with the Philippines (Okada, 1936; Ota, 1987, 1991b; Oliveros *et al.*, 2011).

Lanyu Island is home to the rare endemic Orchid Island Gecko, *Gekko kikuchii*. Discovered and formally named just a century ago (Oshima, 1912), this species has been compared to *Gekko mindorensis*, a widespread Philippine species (Taylor, 1919; Brown & Alcala, 1978; Siler *et al.*, 2012), and *Gekko monarchus*, a more widespread species known from the Malaysian Peninsula and several adjacent islands of Indonesia and the southwestern Philippines (Oshima, 1912; Taylor, 1919; Ota *et al.*, 1990; Siler *et al.*, 2012). For more than two decades, *G. kikuchii* has been classified by the Forestry Bureau, Government of Taiwan (FBGT), as endangered or rare, and strictly protected under the Wildlife Conservation Act originally enforced in 1990 and revised in 2009; *G. kikuchii* is currently assigned to category II, with an area of occurrence $< 3 \text{ km}^2$ and limited only to the southeastern portion of Lanyu Island (also, see IUCN, 2012).

More recently, the taxonomic status of *G. kikuchii* has been called into question. Now considered 'Data Deficient' by the IUCN (2012), this species broadly overlaps phenotypically and karyologically with *G. mindorensis* [Wang, 1962; Brown & Alcala, 1978; Bauer, 1994; H. Ota, (unpublished data)] In fact, several authors have treated Philippine *G. mindorensis* as a junior synonym of *G. kikuchii* (Bauer, 1994; Ferner *et al.*, 2001; Kluge, 2001; Brown *et al.*, 2013a,b), suggesting that the species may not be endemic to Lanyu Island at all. However, this proposition has never been critically evaluated.

In a recent phylogenetic study, Siler *et al.* (2012) demonstrated that Philippine *G. mindorensis* is composed of at least eight highly divergent genetic lineages or clades, highlighting the need for a comprehensive taxonomic reconsideration of this taxon, particularly with respect to the taxonomically confused and morphologically similar Lanyu Island endemic, *G. kikuchii.*

In this study, we set out to answer three questions concerning the status of the endangered Lanyu Island gecko: (1) How do comparisons of different methods and data types impact interpretation of species boundaries for *G. kikuchii* and *G. mindorensis* and the recognition of *G. kikuchii* as a rare, microendemic Taiwanese species? (2) Do genetic and morphological data suggest the presence of cryptic diversity in Philippine *G. mindorensis*? Finally, in difficult or ambiguous cases of species delimitation, (3) do coalescent-based methods that take into account the history of allelic variation arrive at different conclusions than genetic analyses limited to allele frequencies?

Here, we apply topology-based tests of phylogenetic relationships, population-structure analyses, coalescent-based species delimitation procedures, and explicitly geographical analyses of external morphology to the *G. kikuchii* problem. We provide a comprehensive analysis of species boundaries across a putatively widespread Philippine vertebrate (*G. mindorensis*) and a reconsideration of the conservation status of a small island endemic vertebrate (*G. kikuchii*). This study underscores important lessons for multilocus, integrative species delimitation involving small, peripheral, isolated island endemics and has major implications for conservation assessments of peripheral island species globally.

METHODS

Sampling and data collection

Ingroup sampling included data for 72 individuals used in a recent phylogenetic study of Philippine *Gekko* (Siler *et al.*, 2012), representing all 12 currently recognized species of *Gekko* from the northern Philippines, *G. monarchus*, and all divergent clades of *G. mindorensis* identified by Siler *et al.* (2012); Fig. 1 and Appendix S1 in Supporting Information). Outgroup samples were chosen based on Siler *et al.* (2012), and included five individuals representing four species: *Gekko gecko*, *G. hokouensis*, *G. japonicas*, and *G. smithi* (Appendix S1). Sequence data were available for the mitochondrial gene NADH dehydrogenase subunit 2 (*ND2*), as well as the nuclear gene phosducin (*PDC*), on GenBank for 77 and 61 individuals, respectively (Siler *et al.*, 2012). We sequenced four additional nuclear loci for the majority of samples, with one exception: *R35* (13 individuals), *L52* (75 individuals) *L78* (76 individuals), *L145* (72 individuals). The latter four of these loci were developed for use in the recent *Anolis* genome study (Alföldi *et al.*, 2011). All novel sequences were deposited in GenBank (Appendix S1). Polymerase chain reaction (PCR) and sequencing protocols are summarized in Appendix S2.

Sequencing, alignment and phylogenetic analyses

Initial alignments were produced in Muscle 3.8.31 (Edgar, 2004), and manual adjustments were made in MacClade 4.08 (Maddison & Maddison, 2005). To assess phylogenetic congruence between the mitochondrial and nuclear data, we inferred the phylogeny for each subset independently using likelihood and Bayesian analyses. Following the observation of no strongly supported incongruence between datasets, we conducted subsequent phylogenetic analyses using a combined dataset. Exploratory analyses of the combined dataset of 77 individuals, including individuals lacking sequence data for one of the six loci, and a reduced dataset of 61 individuals (no missing data for all loci except *R35*) supported identical relationships, and we therefore chose to include all available data (77 individuals) for subsequent analyses of the concatenated *ND2 + PDC + L52 + L78 + L145 + R35* dataset. Alignments and resulting topologies are deposited in Dryad (doi: 10.5061/dryad.mj3v6).

Partitioned Bayesian analyses were conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Each of the three datasets, *ND2*, *PDC* and *R35*, were partitioned by codon position (Gamble *et al.*, 2011; Siler *et al.*, 2012), whereas each of the three remaining loci, *L52*, *L78* and *L145*, were each analysed as a single character set, respectively. The

Akaike (1973) information criterion (AIC), as implemented in jModeltest (2.1.4; Guindon & Gascuel, 2003; Darriba *et al.*, 2012), was used to select the best model of nucleotide substitution for each partition (Table 1). A rate-multiplier model was used to allow substitution rates to vary among subsets, and default priors were used for all substitution parameters. We ran eight independent Markov chain Monte Carlo (MCMC) analyses, each with four Metropolis-coupled chains, an incremental heating temperature of 0.02, and an exponential distribution with a rate parameter of 25 as the prior on branch lengths (Marshall *et al.*, 2006; Marshall, 2010; Siler *et al.*, 2010, 2011). All analyses were run for 50 million generations, with parameters and topologies sampled every 5000 generations. To assess chain 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 1.4 (Rambaut & 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 5 million generations (i.e. the first 10%), we conservatively discarded the first 20% of samples as burn-in.

Partitioned maximum likelihood (ML) analyses were conducted in GARLI 2.0.1019 (Zwickl, 2006) for all datasets under the same partitioning strategy used in Bayesian analyses and the AIC-selected substitution models for each subset (Table 1). We performed 10 independent search replicates with starting trees generated using random-order stepwise additions with 100 attachments per taxon. Each replicate was terminated when there were no topological improvements in log-likelihood greater than 0.01 for more than 10,000 generations, and no overall improvement in log-likelihood > 0.001 for more than 500 generations. To assess clade support, we

ran 1000 bootstrap replicates under the same settings, except with one tree search per bootstrap replicate.

Population assignment and biogeographic boundaries

As currently recognized, *G. kikuchii* and *G. mindorensis* are valid, unique taxa. Our possible *a priori* alternate expectations of population structure were as follows: (1) individuals of *G. kikuchii* may represent a unique population within the data; (2) little to no admixture between *G. kikuchii* and *G. mindorensis*; (3) individuals of *G. mindorensis* might represent a number of identifiable populations with a higher degree of admixed individuals; (4) individuals of *G. kikuchii* and *G. mindorensis* may be assigned to the appropriate species-specific population with high probability; and (5) individuals of *G. kikuchii* may be recovered as part of a genetically structured *G. mindorensis* species complex. To distinguish between these alternate predictions, we used the Bayesian clustering method of the program Structure (v2.3.3; Pritchard *et al.*, 2000; Falush *et al.*, 2003), with allelic data from our five nuclear loci, to estimate the number of populations among *G. kikuchii* and *G. mindorensis* samples, as well as infer the probabilities of individuals belonging to each of the estimated populations. We phased each nuclear locus, using the program PHASE v. 2.1.1 (Stephens & Donnelly, 2003) and retained haplotypes with the highest probabilities for subsequent analyses. Analyses were run under the correlated allele frequency model and the admixture ancestry model (Falush *et al.*, 2003). All other options were left with default settings. To consider the entire space of biologically plausible species boundaries for this study, we considered the number of possible demes (putative species), represented by (K), to range from one (*G. kikuchii* and *G. mindorensis* are the same species) to 15 (*G. kikuchii* and each sampled island population of *G. mindorensis*, with two exceptions, represent unique species). The two exceptions for these analyses were as follows: (1) we treated populations from the Bicol Peninsula (Fig. 1, Population Code 6) as unique from populations on Luzon Island proper (Fig. 1, Population Code 2), and (2) we treated north and south Mindoro Island as unique biogeographic entities following recent geologic studies (Zamoros *et al.*, 2008; Fig. 1, Population Codes 11, 14, 15). We conducted 20 independent runs in Structure for each of the 15 values of K (1–15). We ran each analysis for 5 million iterations, with a burn-in of 500,000. The rate of change in likelihood scores across values of K (ΔK) was evaluated in Structure Harvester (WEB v.0.6.93; Dent & vonHoldt, 2012) following the method of Evanno *et al.* (2005) to determine the number of preferred genetic clusters. The results of structure analyses inferring three demes were visualized using the program DISTRUCT (v1.1; Rosenberg, 2004).

To investigate whether there is evidence of spatial boundaries among morphological features of populations in the *G. mindorensis* Complex, we performed two statistical analyses of morphological data, using a dataset of 16 meristic and

Table 1 Models of evolution selected by AIC and applied for partitioned, model-based phylogenetic analyses.

Partition	AIC model	Number of characters
ND2, 1st codon position	HKY + I + Γ	346
ND2, 2nd codon position	HKY + Γ	346
ND2, 3rd codon position	GTR + Γ	346
PDC, 1st codon position	HKY + I	137
PDC, 2nd codon position	HKY + I	136
PDC, 3rd codon position	GTR + I	136
R35, 1st codon position	HKY	214
R35, 2nd codon position	JC	213
R35, 3rd codon position	HKY	213
L17	HKY + Γ	258
L52	GTR	327
L78	GTR + Γ	348
L145	HKY + Γ	347

AIC, Akaike information criterion.

mensural morphological characters measured from 166 adult specimens representing all eight divergent clades within the *G. mindorensis* Complex (including *G. kikuchii*; Appendix S3) identified by Siler *et al.* (2012) and 13 of the 15 island populations identified in this study. Adult specimens were not available for populations from Panglao or Palaui islands; these islands were not included. Selected characters follow Linkem *et al.* (2010) and Brown *et al.* (2011), and include: Snout–vent length, head length, tibia length, supralabial and infralabial counts, preanofemoral pore-bearing scale count, midbody tubercle row number, paravertebral tubercle row number, midbody transverse ventral and dorsal scale numbers, longitudinal ventral and paravertebral scale numbers in the axilla–groin region, toe and finger scansor numbers, dorsal banding number in the axilla–groin region, and the presence or absence of continuous preanofemoral pore series (Appendix S3). First, we conducted geographically explicit analyses in the program GENELAND (v.4.0; Guillot *et al.*, 2005). Twenty independent analyses were performed with spatial modelling. Geographic coordinates were in decimal degrees with an uncertainty of 0.0001. The number of populations was allowed to vary from 1–15, matching the approach we followed for Bayesian species delimitation and population assignment analyses. Each analysis was run for 1,000,000 MCMC iterations, sampling every 1000th iteration. Post-processing included a spatial realm of 362 × 300 pixels and a burn-in of 500 samples. Additionally, to explore our morphological dataset for natural clusters of populations, we performed a Two-Step Cluster Analysis in SPSS v21.0.0.0 (IBM Corp., Armonk, NY, USA) using Schwarz's (1978) Bayesian Criterion and a log-likelihood distance. All morphological measurements were log-transformed prior to analysis (Appendix S4), and the morphological dataset was deposited in DRYAD (doi: 10.5061/dryad.mj3v6).

To visualize population genetic structure, we generated phylogenetic networks for the mitochondrial and nuclear datasets, respectively, by employing the Neighbour-Net algorithm (Bryant & Moulton, 2004) in the program SPLITSTREE v4.10 (Huson & Bryant, 2006; Fig. S1). Prior to constructing networks for nuclear datasets, we reduced each alignment of phased alleles to the set of unique alleles. To assess the support for inferred splits in each network, a bootstrap analysis was conducted with 1000 pseudoreplicates.

Bayesian species delimitation

We explored support for hypothesized species boundaries using the program Bayesian Phylogenetics and Phylogeography (BPP v.2.0; Yang & Rannala, 2010). To provide an objective starting topology for BPP, we used the multispecies coalescent model implemented in the program *BEAST (v1.6.2; Drummond & Rambaut, 2007; Heled & Drummond, 2010) to estimate relationships among the 15 geographically defined populations. For detailed description of these analyses, see Appendix S4. Phased gene sequences were assigned to lineages on the basis of sampling locality (Fig. 1), and the

two most common topologies observed in the *BEAST posterior distribution of species trees were used as guide trees. Under both guide trees, we ran four independent analyses for an initial burn-in period of 1 million generations, followed by sampling every 1000 generations for an additional 5 million generations. We confirmed the burn-in period and sampling was sufficient by examining the log-likelihood scores of each of the four independent chains over generations, and ensuring the effective sample sizes of all parameters were > 500. We used reversible-jump MCMC algorithm '0' (equations 3 and 4; Yang & Rannala, 2010), with an ε setting of 10. We used a uniform prior over rooted trees, and exponential priors on population size parameters (Exp(400)) and the root age of the species tree (Exp(40)). In addition to analysing all six loci under these settings, we also analysed each locus independently. Furthermore, to explore the robustness of our results, we explored analyses run under algorithm '0' with ε set to 20, under algorithm '1' with α set to 1 and 2, and with and without sites containing gaps included.

Assessing support for topological hypotheses

To explore the phylogenetic position of *G. kikuchii* in relation to *G. mindorensis*, and biogeographic affinities of Philippine populations of *G. mindorensis*, we estimated the probability of the following taxonomic- and biogeographic-based hypotheses (Table 2): (1) Is *G. kikuchii* monophyletic? (2) Is *G. mindorensis* monophyletic? And (3) Do the recognized Philippine faunal regions [Pleistocene Aggregate Island Complexes (PAICs)] form monophyletic clades? Support for these topological hypotheses are outlined in Table 2. We estimated the posterior probability of each hypothesis as the proportion of 16,004 post burn-in trees from partitioned Bayesian analyses (summarized above) consistent with each hypothesis.

RESULTS

Taxon sampling, data collection, and sequence alignment

The complete, aligned matrices contain < 3% missing data for mitochondrial data, and an average of < 5% missing data for each nuclear locus (excluding *R35* with only 11 sampled). We rooted the tree using the sample of *Gekko gecko* (Siler *et al.*, 2012). Within each dataset, variable characters were observed as follows: (*ND2*) 722 of 1259, (*PDC*) 24 of 419, (*L17*) 28 of 258, (*L52*) 37 of 327, (*L78*) 21 of 350, (*L145*) 24 of 358 and (*R35*) 3 of 640.

Phylogenetic analyses and support for topological hypotheses

Analyses of the combined data resulted in topologies with high bootstrap support (ML) and posterior probabilities

Table 2 Summary of Bayesian posterior support for *a priori* topological hypotheses.

Hypothesis		Description of constraint	Posterior probability
H$_1$	Monophyly of *Gekko kikuchii*	Both samples of *G. kikuchii*	**0.05**
H$_2$	Monophyly of *Gekko mindorensis* populations	All *G. mindorensis* samples	**0.0**
H$_3$	Monophyly of *G. mindorensis* (Luzon faunal region)	All *G. mindorensis* samples from Luzon	**0.0**
H$_4$	Monophyly of *G. mindorensis* (Mindanao faunal region)	All *G. mindorensis* samples from Mindanao	1.0
H$_5$	Monophyly of *G. mindorensis* (Mindoro faunal region)	All *G. mindorensis* samples from Mindoro	**0.0**
H$_6$	Monophyly of *G. mindorensis* (Visayan faunal region)	All *G. mindorensis* samples from Guimaras, Masbate, Negros and Panay	**0.0**

Significant *P*-values at α ≤ 0.05 are shown in bold.

(Fig. 1), with relationships congruent across all analyses. The results mirrored those observed in a recent phylogenetic study of species of the genus *Gekko* in the Philippines (Siler *et al.*, 2012). Among the diversity of species from Philippines included in this study, all micro-endemic species from the central and northern Philippines form a single clade (Fig. 1, Clade 1). As observed in the study described by Siler *et al.* (2012), the widespread species, *G. mindorensis* and *G. monarchus* are supported to be sister taxa, both of which are sister to the radiation of northern micro-endemic species (Fig. 1, Clade 2). Generally, lineages within the Philippines are highly structured, genetically divergent and well supported (Fig. 1). One exception is the minimal genetic divergence between Lanyu Island and portions of Luzon; in fact, *G. kikuchii* is recovered with high support to be nested within a clade of populations of *G. mindorensis* from Luzon proper, most closely related to the northernmost sampled populations on Luzon Island (Fig. 1, Clade 3). Analyses of individual loci consistently recover *G. kikuchii* nested within a clade of *G. mindorensis* (Fig. S1).

Analyses provided no support (posterior probability approaching zero) for the two taxonomic (H1 and H2) and three of the four biogeographic hypotheses (H3, H5 and H6) investigated (Table 2). The results provided strong support for the monophyly of populations of *G. mindorensis* from the Mindanao faunal region in the southern Philippines (H$_4$; Table 2).

Population assignment of individuals

Structure analyses support three genetic clusters (K = 3; Fig. 2), with all but four of the 15 populations assigned to one of the clusters with high estimated membership fractions (q > 0.90; Fig. 2). As observed in phylogenetic analyses, *G. kikuchii* and populations of *G. mindorensis* from Luzon Proper were recovered as members of the same genetic cluster (q > 0.96; Fig. 2). Populations from Caluya, Masbate and Southern Mindoro islands were identified as composed of admixed individuals, with membership fractions shared by two genetic clusters ($q_{1,2}$ = 0.23–0.77; Fig. 2).

Analyses of external morphological phenotypes recovered five (GENELAND) and two (TwoStep Cluster Analysis) discrete clusters, respectively (clusters based strictly on meristic and mensural data; Figs 3 & S2; Appendix S3). With one excep-

tion, the geographically explicit analyses of morphological spatial boundaries recovered groups that do not follow *a priori* expectations of species boundaries (Brown & Diesmos, 2009; Brown *et al.*, 2013a,b).

Strongly supported genetic structure observed among locus-specific phylogenetic networks were consistent with the results of phylogenetic analyses (Fig. S1). Although the mtDNA dataset contained 35 unique haplotypes, each nuclear locus contained fewer unique haplotypes (six unique haplotypes for *L52*; 11 for *L78*; 12 for *L145*; four for *PDC*; three for *R35*), and phylogenetic networks of the nuDNA datasets provide less structural resolution among the major clades of *G. mindorensis* (Fig. S1).

Bayesian species delimitation

Results for the Bayesian species delimitation analyses are summarized in Fig. 3. Following our objective approach to test support for species splits across a geographically split starting topology (assuming 15 geographically based populations within the *G. mindorensis* Complex), the results supported nearly all splits with speciation probabilities near or equal to 1.0 (Fig. 2), regardless of topology or parameters used (Appendix S4).

DISCUSSION

Gekko kikuchii: distinct Lanyu endemic species or recent Philippine transplant?

Is *G. kikuchii*, a distinct Lanyu Island endemic species worthy of formal taxonomic recognition and conservation prioritization (IUCN, 2012)? Our results present a compelling mixture of support, further highlighting the systematic and conservation challenge posed by peripheral, isolated, island species (Lesica & Allendorf, 1995; Abbitt *et al.*, 2000; Yakimowski & Eckert, 2007; Gibson *et al.*, 2009). First, multilocus phylogenetic estimates depict surprising relationships between members of the *G. kikuchii-G. mindorensis* Group (Figs 1 & S1). The fact that the Lanyu population is nested inside of the Luzon Island clade suggests that *G. kikuchii* might be a recent colonist (possibly human-mediated, e.g., Ota *et al.*, 2004; Kuraishi *et al.*, 2009; Brown *et al.*, 2010) of Lanyu from northern Luzon. Second, our cluster analyses of
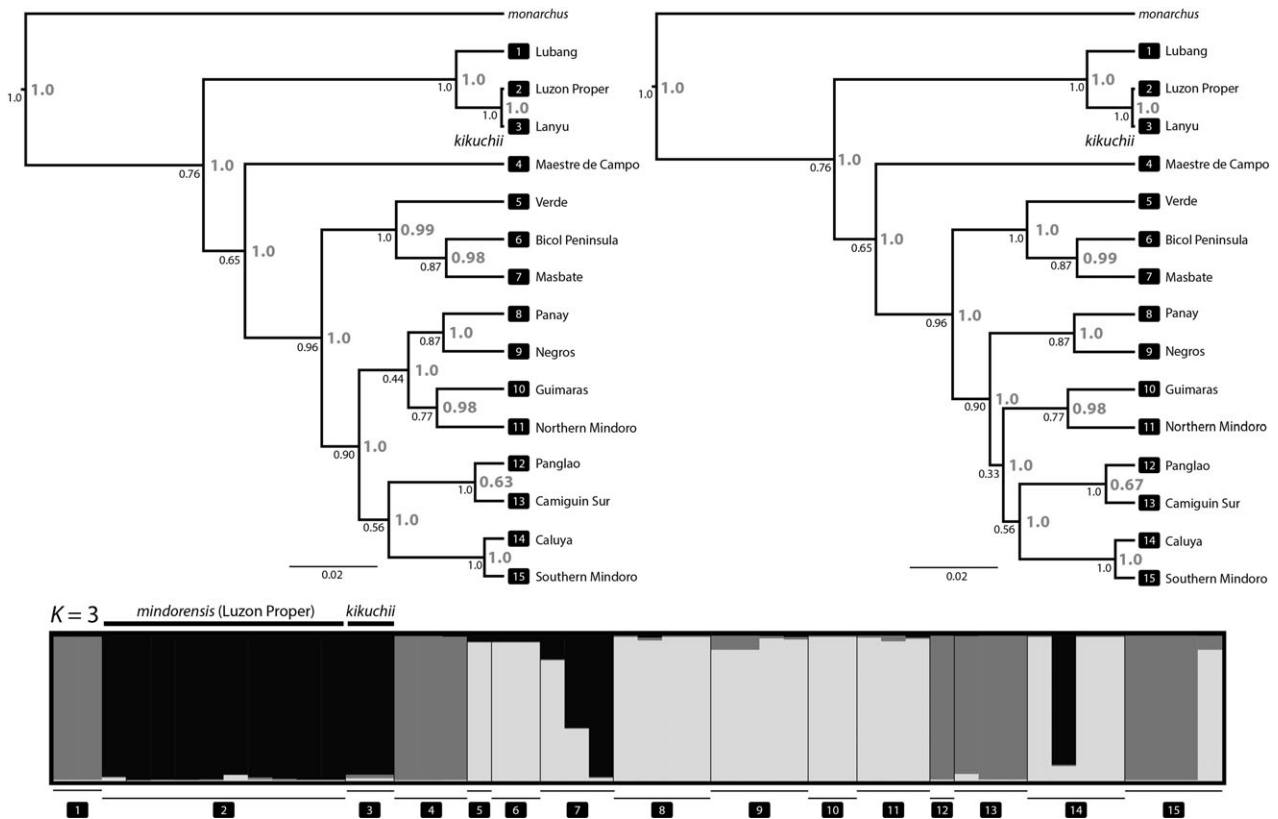
Figure 2 (Top) BPP guide trees representing the two most common topologies observed in *BEAST posterior distribution of species trees with results of Bayesian lineage delimitation analyses inferred by BPP. Posterior probabilities of phylogenetic relationships and inferred lineage splits are provided at each node in small, black and large, grey text, respectively. (Bottom) Graphical representation of STRUCTURE results for $K = 3$ genetic groups. Shades correspond to one of the three genetic groups, and vertical bars display probability of each individual belonging to one of these inferred groups.

external morphological phenotypes without *a priori* assignment to groups (based on phylogenetic analyses) resulted in clusters of morphological variation that defy both phylogenetic relationships (Fig. 1) and biogeographic expectations (Brown & Diesmos, 2009; Brown *et al.*, 2013a,b) in four of five of the cases of identified geographical phenotypes (Figs 3 & S2), and both clusters recovered in TwoStep Cluster analyses (Fig. 3). When we consider the possibility of true cryptic species diversity, these results should not be surprising, as standard morphological characters traditionally used to diagnose species of geckos may not be as useful for genetically distinct, but morphologically cryptic, island endemic species. Third, Bayesian assessments of topological relationships based on biogeographic expectations were nearly all improbable (Table 2). Finally, our Bayesian species delimitation procedure, like many other recent empirical applications of this method (Leaché & Fujita, 2010; Brown *et al.*, 2012) inferred species splits between nearly all geographically defined populations ($n = 15$; Fig. 2).

Given the apparent conflict between results of different methods, how should systematists and conservation specialists apply the goals of integrative taxonomy (Sites & Marshall, 2004; Padial & De la Riva, 2009) and objectively arrive at partitioning strategies for vertebrates in an island archipel-

ago, especially when each major island population arguably represents its own, cohesive, evolutionary lineage (*sensu* Frost & Hillis, 1990; de Queiroz, 1999)? As species delimitation procedures become more objective, statistical, automated and analytically driven (Fujita *et al.*, 2012), one possible pitfall is that the methods may be applied formulaically or without the reasonable oversight of an investigator who takes into account multiple sources of data and consciously applies a reasonable, conservative systematic philosophy in accordance with the Code of Zoological Nomenclature (Esselstyn, 2007; Bauer *et al.*, 2010). However, even pluralistic approaches, with multiple data types and simultaneous considerations of phenotypic, genetic and geographical sources of information can result in conflicting support for species boundaries, as is observed in this study. With the myriad of possibilities represented in our results, additional data likely are needed before comprehensive taxonomic revisions can be applied.

Conservation challenges associated with recognition of peripheral island endemic species

The taxonomic recognition of *G. kikuchii* as a valid species may be an artefact of biogeographic and political peripheralization. It is clear that the political boundaries and regional
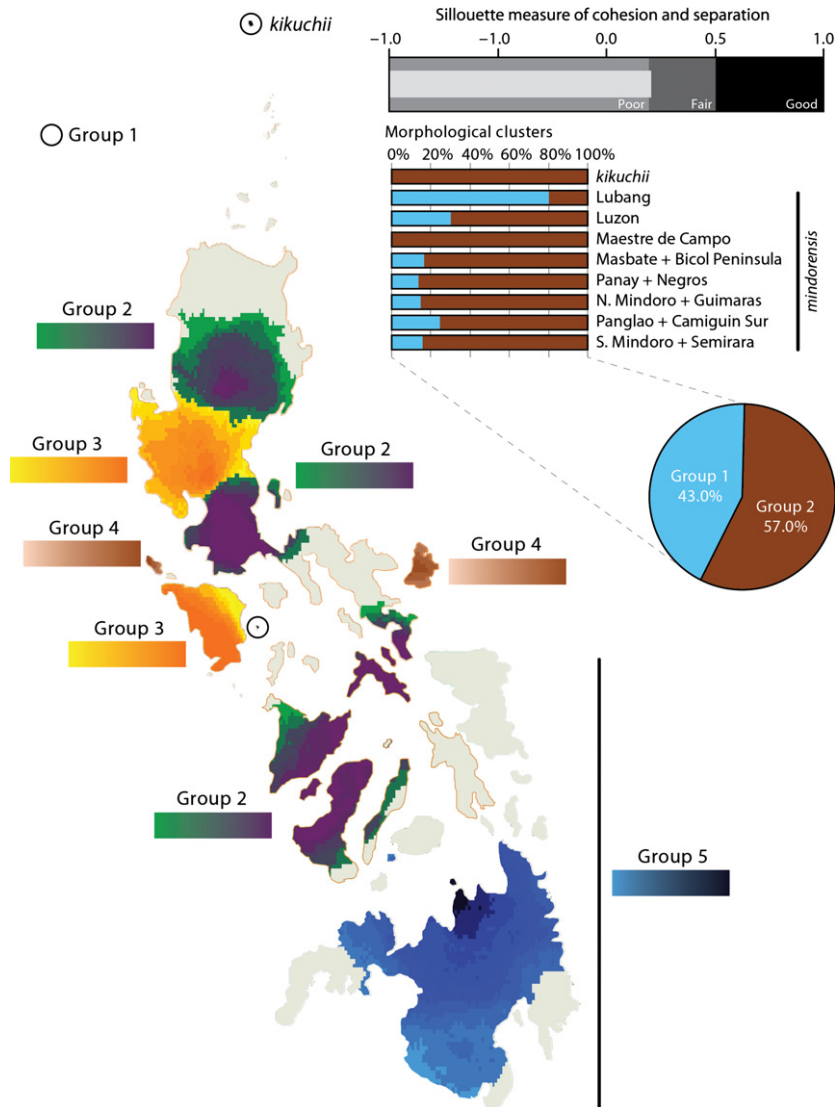
Figure 3 Summarized results of GENELAND analyses showing the distribution of the five inferred morphological clusters (for full results, see Fig. S2), with gradients representing the posterior probabilities of membership in each morphological cluster, trending from light (low) to dark (high). Results of Two-Step Cluster Analysis showing two inferred phenotype groups and the percentage membership of each sampled population in each group.

focus of taxonomists (Joppa *et al.*, 2011), combined with biogeographic peripheralism can lead to oversights that may result in both Type I (Frost & Hillis, 1990: recognizing more species than are actually present) or Type II (failing to recognize distinct species) errors in biodiversity characterization. The consequence, of course, from conservation perspectives is that resources may be inappropriately spent on insignificant or peripheral population units (Type I scenario) or that unrecognized but distinct evolutionary lineages (species) may be insufficiently protected (Type II scenario). We suspect that both possibilities may represent conservation challenges for species isolated along the boundaries of biogeographic and/or political boundaries. Of course, the latter (Type II) is a more egregious threat to continued survival of isolated island species (Daugherty *et al.*, 1990; Russello *et al.*, 2005; Spinks *et al.*, 2012). Aside from the circumstances of discov-

ery and description, the consequence for continued taxonomic confusion and misdirection of conservation resources is clearly illustrated by the case of *G. kikuchii* and *G. mindorensis* (Roesler *et al.*, 2011; Reptile Database, 2012; Brown *et al.*, 2013a,b), with the conservation community standing at an impasse with regard to the status of the Lanyu Island population (IUCN, 2012).

A distinct Lanyu Island species obscured by recent mitochondrial introgression?

One of our more intriguing results is the discrepancy between inferences of species boundaries resulting from coalescent-based methods for species delimitation versus methods based on allele frequencies that do not explicitly use historical information in allelic variation. Structure analyses

found no support for the uniqueness of *G. kikuchii*, whereas coalescent-based approaches implemented in this study (BPP) find strong support for its unique identity (Figs 2 & 4). This strong support is largely driven by the pattern of one and four segregating sites in the *L52* and *L145* loci, respectively, which are very unlikely to arise under ancestral processes within a single population. Although our mtDNA and nuDNA datasets possess no unique haplotypes for *G. kikuchii* (Fig. S1), the unique haplotypes among populations of *G. mindorensis* from Luzon Proper, particularly those recovered for loci *L52* and *L145*, appear to be driving

these analytical inferences (Fig. S1). By explicitly modelling the relationships among the gene copies, the BPP model is able to detect the signal in locus *L52* and *L145* that is difficult to explain under a model lumping *G. kikuchii* and *G. mindorensis* populations on Luzon Island into a panmictic population (Fig. 4), especially considering the relatively slow mutation rates of these two loci. This clearly shows the potential benefits of coalescent-based methods that utilize the full information in the sequence data rather than distil the data into haplotype frequencies. It should be noted that we currently lack sampling from large regions of northern
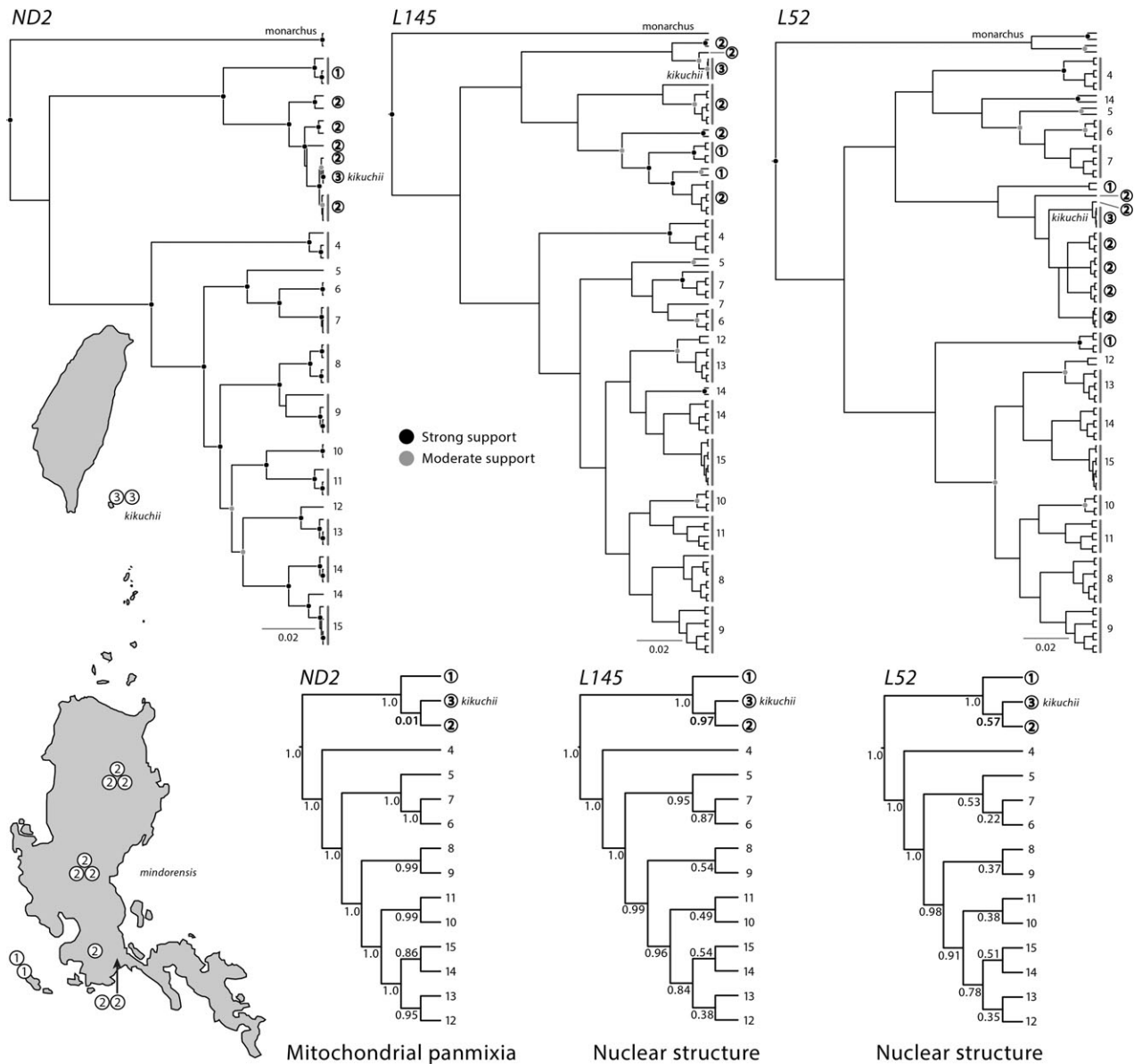

Figure 4 Summary of conflicting results between mitochondrial and nuclear datasets. (Top) Consensus gene trees for three of the loci from the posterior samples of the *BEAST analysis. Node support (strong, ≥ 95% PP; moderate, 75–94% PP) shown for reference. (Bottom) Summary of results of locus-specific BPP analyses where numbers at nodes indicate the estimated posterior probabilities of lineage splits. Numbered labels correspond to sampled populations outlined in Fig. 1, with circled numbers highlighting the clade of interest.

(and western) Luzon Island, and the inclusion of additional populations from Luzon Proper might further impact the results of such analyses.

The support for recognition of a distinct (from Luzon) Lanyu Island endemic inferred from slowly evolving nuclear loci (rather than the mitochondrial data) under a coalescent-based method suggests the interesting possibility that the Lanyu Island population may have been a historically distinct, albeit recently diverged, evolutionary lineage. Furthermore, it is plausible that recent mitochondrial introgression of the Luzon Island genome into the Lanyu population may have obscured the signature of a distinct evolutionary species in the other analyses. Further analyses with many more unlinked nuclear loci will be necessary to explore this possibility further.

Biogeographic and conservation implications: the Luzon–Taiwan (Lanyu) link

This study represents a compelling demonstration of a possible Luzon-to-Taiwan (Lanyu Island) faunal transplant (or dispersal event) from the Philippines. Other cases of suspected northern dispersal events originating in the Philippines include the presence of the skink *Eutropis cumingi* on Lanyu (Ota & Huang, 2000), and the frog *Polypedates leucomystax* in Japan (Ota *et al.*, 2004; Kuraishi *et al.*, 2009). Past studies, in contrast, documented a few cases of bird, insect, mammal or plant groups that appear to have peripherally invaded the Philippines from the north (Dickerson, 1928; Oliveros & Moyle, 2010; Esselstyn & Oliveros, 2010; Oliveros *et al.*, 2011), only extending to the Batanes or Babuyan islands (or the highlands of Luzon in the case of some plants; Dickerson, 1928). This study joins a few recent works suggesting that Philippine-derived anomalies in the fauna of Lanyu (and possibly greater Taiwan) are worthy of careful reconsideration. Determining whether each is the result of recent human-mediated introduction or (possibly more ancient) natural dispersal should be the goal of future studies on this seldom-conceived biogeographic relationship and significant resulting conservation concern. Ascertaining the temporal framework for naturally occurring faunal exchanges between the northern Philippines and Taiwan (Lanyu) provides opportunities for future research (Oaks *et al.*, 2013).

Taxonomic conclusions and conservation action

Three possible alternate classifications are immediately evident; we could conclude that (1) two species are evident as the result of our analyses (*G. kikuchii* applied to Lanyu and Luzon populations, *G. mindorensis* applied to remaining populations; Fig. 1); (2) three species (additional recognition of a new species from the small islands of the central Philippines; Fig. 1); or (3) conceivably five or even eight species (Fig. 1) may be present. Preliminary surveys of meristic and mensural morphological variation across the 160+ specimens examined here have allowed for the identification of some

traditionally diagnostic differences between several of these populations (Appendix S3; CDS *et al.*, unpublished data). Regardless of strong evidence for unique, albeit cryptic, evolutionary lineages likely represented by allopatric populations of *G. mindorensis* (Fig. 5), the observed discrepancy between disparate datasets used in this study prevents us from suggesting concrete taxonomic changes at this time, with one exception. No matter which of the possible classifications listed above is supported in future studies, we are confidant that populations of *G. mindorensis* from central and northern Luzon Island and *G. kikuchii* represent the same unique evolutionary lineage.

Therefore, we recognize the species *G. kikuchii* as the unique evolutionary lineage distributed throughout central and northern Luzon Island in the Philippines and Lanyu Island based on the following (Fig. 5): (1) The results of concatenated and coalescent-based analyses are conclusive in the placement of *G. kikuchii* nested with strong support within the divergent lineage of *G. mindorensis* composed of populations from central and northern Luzon Island. Not only do statistical tests of topology reject the reciprocal monophyly of the two species (Table 2), but it is clear that the addition of unsampled populations from western Luzon would not result in their reciprocal monophyly; (2) the lineage is geographically isolated and circumscribed by biogeographic barriers recognized as boundaries to species distributions (i.e., Bicol Peninsula vs. Luzon Proper); (3) percentage divergences for the mitochondrial data (5.8–14.0% uncorrected pairwise divergence) show that the monophyletic lineage is distinguished from congeners by levels of genetic divergence equal to, or greater than, those between previously defined species of Philippine gekkonid lizards – viz., species of the genus *Gekko* and *Cyrtodactylus* (Welton *et al.*, 2010a,b; Brown *et al.*, 2011); (4) the lineage shares the same specialized karyotype ($2N = 44$: see Appendices S3, S4); (5) populations from throughout the geographic range of the lineage (Lanyu–Central Luzon Island Proper) broadly overlap in traditional diagnostic morphological characters (Appendix S3); (6) although morphologically similar to the remaining divergent evolutionary lineages of *G. mindorensis*, the Luzon-Lanyu clade trends towards a unique morphological identity, with observed tendencies among individuals to have smaller heads, shorter tails, fewer precloacofemoral pore-bearing scales, fewer midbody dorsal scales, fewer paravertebral scales and fewer toe scansors (Appendix S3); (7) in this study, we have included populations from near the type localities for both species, and the populations from the type island of *G. mindorensis* (southeast Mindoro Island; Taylor, 1919) are recovered in a distinct clade deeply divergent to the Luzon-Lanyu clade of *G. kikuchii* (Figs 1 & 5); and (8) the species name *G. kikuchii* (Oshima, 1912) is the oldest available name for the Luzon-Lanyu lineage (vs. *G. mindorensis* Taylor, 1919).

Although the new recognition of *G. kikuchii* renders *G. mindorensis* paraphyletic (Fig. 5), the revised understanding of *G. kikuchii* provides a necessary first step towards
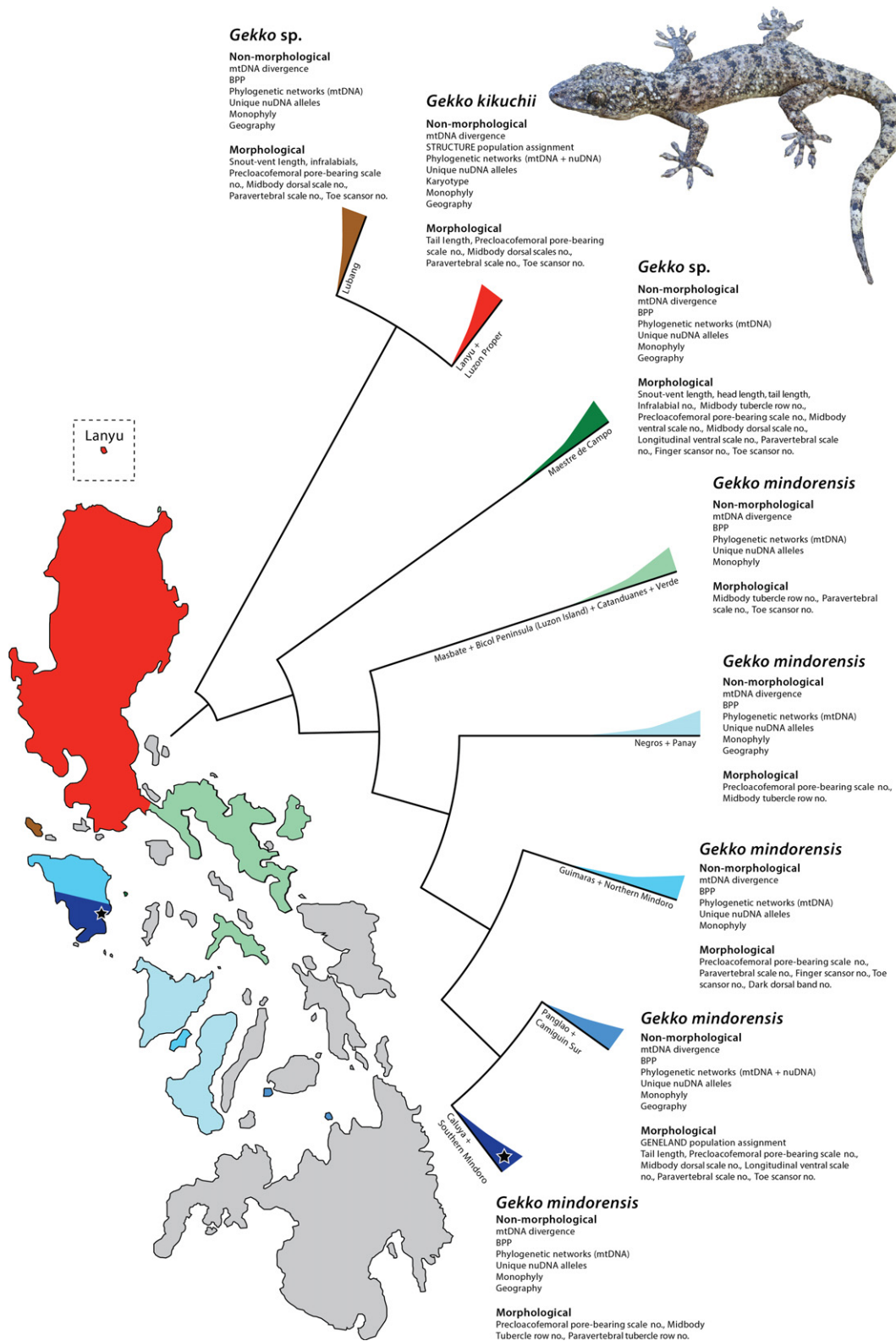
Figure 5 Taxonomic decision key summarizing data in support of taxonomic conclusions drawn in this study and proposed future directions for taxonomic revisions. The tree represents relationships supported in phylogenetic analyses and shows the revised recognition of *Gekko kikuchii* with the seven remaining divergent lineages of *Gekko mindorensis* recovered in this study. Terminal labels highlight regional distributions and corresponding lines of supporting evidence for each clade. In many cases, morphological lines of evidence represent observed trends and are not all non-overlapping in states (Appendix S3). The clade containing populations from near the type locality for *G. mindorensis* represented by a black star.

protecting this unique, cryptic lineage. We expect future studies will support conservatively at least two additional unique lineages within *G. mindorensis* worthy of recognition as species: (1) Lubang Island populations and (2) Maestre de Campo Island populations (Fig. 5). The recognition of these lineages in future studies would, at a minimum, resolve and stabilize the taxonomy of the *G. mindorensis* Complex and definitively identify targets for future conservation action and policy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Summary of specimens of Philippine species of *Gekko* corresponding to genetic samples included in the study, with GenBank acession numbers provided for reference.

Appendix S2 Polymerase-chain reaction (PCR) and sequencing protocols.

Appendix S3 Distribution of selected diagnostic characters in *Gekko kikuchii* and *G. mindorensis*.

Appendix S4 Additional details: methods, history, and taxonomy.

Figure S1 Gene networks of all unique alleles sampled from one mitochondrial locus and five nuclear loci of *Gekko kikuchii* and *G. mindorensis*.

Figure S2 Maps of posterior probabilities of membership in each of five inferred morphological clusters identified by GENELAND trending from light (low) to dark (high).

BIOSKETCH

All authors are generally interested in the fields of historical biogeography, evolution, and biodiversity conservation. Cameron D. Siler and Jamie R. Oaks are involved in research programmes investigating the evolutionary history and conservation biogeography of Southeast Asian amphibian and reptile lineages. Jamie R. Oaks is interested in developing, implementing, and applying phylogeographic methods to test hypotheses about processes of diversification. Hidetoshi Ota and Kerry Cobb are interested in the systematics, biogeography, and conservation of East Asian herpetofauna. By combining active field programmes with modern molecular and bioinformatic techniques, we hope to contribute to a synthetic understanding of the mechanisms driving diversification in Southeast Asia, and aid in the development of new approaches to conservation genetics.

Author contributions: C.D.S. and R.M.B. conceived the ideas and, together with colleagues in the Philippines, conducted fieldwork; C.D.S. collected all data at the University of Kansas; C.D.S. analysed the data; J.R.O. assisted in data analysis; K.C. assisted in data collection; C.D.S. led the writing; R.M.B., J.R.O., and H.O. assisted in writing.

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