

Multilocus phylogeny and Bayesian estimates of species boundaries reveal hidden evolutionary relationships and cryptic diversity in Southeast Asian monitor lizards

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Abstract

Recent conceptual, technological and methodological advances in phylogenetics have enabled increasingly robust statistical species delimitation in studies of biodiversity. As the variety of evidence purporting species diversity has increased, so too have the kinds of tools and inferential power of methods for delimiting species. Here, we showcase an organismal system for a data-rich, comparative molecular approach to evaluating strategies of species delimitation among monitor lizards of the genus *Varanus*. The water monitors (*Varanus salvator* Complex), a widespread group distributed throughout Southeast Asia and southern India, have been the subject of numerous taxonomic treatments, which have drawn recent attention due to the possibility of undocumented species diversity. To date, studies of this group have relied on purportedly diagnostic morphological characters, with no attention given to the genetic underpinnings of species diversity. Using a 5-gene data set, we estimated phylogeny and used multilocus genetic networks, analysis of population structure and a Bayesian coalescent approach to infer species boundaries. Our results contradict previous systematic hypotheses, reveal surprising relationships between island and mainland lineages and uncover novel, cryptic evolutionary lineages (i.e. new putative species). Our study contributes to a growing body of literature suggesting that, used in concert with other sources of data (e.g. morphology, ecology, biogeography), multilocus genetic data can be highly informative to systematists and biodiversity specialists when attempting to estimate species diversity and identify conservation priorities. We recommend holding in abeyance taxonomic decisions until multiple, converging lines of evidence are available to best inform taxonomists, evolutionary biologists and conservationists.

Keywords: conservation genetics, mitochondrial DNA, nuclear DNA, Philippines, Southeast Asia, species delimitation

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Introduction

Delineating and naming species have evolved to be an essential subdiscipline of phylogenetics (Doyle 1995; Wiens & Penkrot 2002; Hey *et al.* 2003; Sites & Marshall 2003, 2004; Leaché & Mulcahy 2007; Wiens 2007; Barrett

& Freudenstein 2011). Although early methods to classify and delimit species utilized small numbers of morphological differences between putative species (Merrell 1981), exclusive reliance on morphology limits our ability to accurately assess species diversity if external characters are conserved (Harris & Sá-Sousa 2002; O'Conner & Moritz 2003; Boumans *et al.* 2007). More recent approaches have embraced the need for consideration of not only diagnostic morphological characters, but

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inferences of evolutionary history as well (Marshall *et al.* 2006a; Leaché & Mulcahy 2007). In fact, the inclusion of genetic data to delimit boundaries between evolutionary lineages has become paramount in biodiversity studies aimed at accurate estimations of species diversity (Wiens & Penkrot 2002; Knowles & Carstens 2007; Rissler & Apodaca 2007; Brown & Diesmos 2009; Welton *et al.* 2010a,b; Barrett & Freudenstein 2011; Setiadi *et al.* 2011; Brown *et al.* 2012).

Incorporating multilocus genetic data into taxonomy and species delimitation has been the focus of many recent studies (Sites & Marshall 2004; Dayrat 2005; Esselstyn 2007; Padial & De la Riva 2009; Barrett & Freudenstein 2011). Of the new approaches developed to investigate species boundaries, the Bayesian method of Yang & Rannala (2010) has ignited both enthusiasm (Leaché & Fujita 2010; Setiadi *et al.* 2011; Brown *et al.* 2012; Fujita *et al.* 2012; Spinks *et al.* 2012) and concern (Bauer *et al.* 2010). The approach provides a mechanism for testing species boundaries in a rigorous and objective Bayesian framework with genetic data. Due to undocumented evolutionary relationships and species diversity, one intriguing system for exploring methods of species delimitation is the water monitors of Southeast Asia (*Varanus salvator* Complex).

Despite being some of the most abundant and conspicuous reptiles of Southeast Asia, the species diversity of water monitors remains highly contested (Pianka *et al.* 2004). In fact, other than body size trends and general colour pattern, past studies have not identified diagnostic, nonoverlapping character differences among recognized species (Gaulke 1991, 1992). Analyses of species boundaries in this group have been varied (Mertens 1942a,b,c; Holmes *et al.* 1975; King & King 1975; Böhme 1988; Becker *et al.* 1989; King *et al.* 1991; Baverstock *et al.* 1993; Card & Kluge 1995; Böhme & Ziegler 1997; Ziegler & Böhme 1997; Böhme & Ziegler 2005), ranging from use of external and reproductive morphology to karyotype and allozyme analyses. Most recently, DNA sequence data have been used to gain insight into phylogenetic relationships (Ast 2001; Welton *et al.* 2010a), historical biogeography (Fuller *et al.* 1998; King *et al.* 1999; Schulte *et al.* 2003; Vidal *et al.* 2012) and body size evolution (Pianka 1995; Collar *et al.* 2011).

Here, we provide the first molecular study of the systematic relationships of this unique assemblage of Southeast Asian lizards. We apply a series of multilocus, phylogeny-based, population genetic, and Bayesian species delimitation approaches to test a variety of taxonomic assessments (Mertens 1959; Gaulke 1991, 1992; Koch *et al.* 2007, 2010a). Our results contradict past approaches based solely on morphological characters and illustrate the utility of multilocus genetic data in estimating species diversity.

Materials and methods

Sample collection

Our data set consists of 81 *Varanus salvator* Complex samples representing natural populations at 56 localities. These include 70 samples from 45 localities in the Philippines, eight samples from three localities in Indonesia and a single sample each from Myanmar, West Malaysia and Singapore. Our sampling includes eight of the 12 currently named taxonomic units within the *V. salvator* Complex (Table 1, Appendix S1, Supporting information). To assess the monophyly of Philippine taxa and the *V. salvator* Complex, we incorporated samples representing 53 of the 94 described taxa (species and subspecies) within the genus *Varanus*, as well as samples from two closely related out-groups, *Heloderma* and *Lanthonotus* (Appendix S1, Supporting information; Caldwell 1999; Lee & Caldwell 2000; Ast 2001; Evans *et al.* 2005; Conrad *et al.* 2011; Welton *et al.* 2013).

Sequencing of DNA

Genomic DNA extraction, PCR amplification and sequencing methods follow those outlined in Welton *et al.* (2013). We screened a suite of candidate nuclear loci from recent studies of higher-level squamate relationships (Townsend *et al.* 2008; Alföldi *et al.* 2011) for intraspecific variability, and among those that amplified easily, selected the four most variable for this study (Table S1, Supporting information). Nuclear loci were combined with previously published mtDNA sequences

Table 1 Taxonomic history of the *Varanus salvator* Complex illustrating the historical uncertainty of species level diversity within the group

One species (Laurenti 1768)	<i>Varanus (Stellio) salvator</i>
Four species (Boulenger 1885)	<i>V. cumingi</i> , <i>V. nuchalis</i> , <i>V. salvator</i> , <i>V. togianus</i>
One species with five subspecies (Mertens 1942a–c)	<i>V. salvator</i> , <i>V. s. cumingi</i> , <i>V. s. marmoratus</i> , <i>V. s. nuchalis</i> , <i>V. s. togianus</i>
One species with eight subspecies (Mertens 1963; Gaulke 1991; Böhme 2003)	<i>V. salvator</i> , <i>V. s. andamanensis</i> , <i>V. s. bivittatus</i> , <i>V. s. cumingi</i> , <i>V. s. komaini</i> , <i>V. s. marmoratus</i> , <i>V. s. nuchalis</i> , <i>V. s. togianus</i>
Seven species with five subspecies (Koch <i>et al.</i> 2007, 2010a; Koch & Böhme 2010)	<i>V. cumingi</i> , <i>V. cumingi samarensis</i> , <i>V. marmoratus</i> , <i>V. nuchalis</i> , <i>V. palawanensis</i> , <i>V. rasmusseni</i> , <i>V. togianus</i> , <i>V. salvator</i> , <i>V. s. andamanensis</i> , <i>V. s. bivittatus</i> , <i>V. s. macromaculatus</i> , <i>V. s. zieglerei</i>

from Welton *et al.* (2013) and Ast (2001; Table S2, Supporting information). We sequenced four nuclear loci [nDNA: two anonymous loci (Alföldi *et al.* 2011; Table S2, Supporting information), and the prolactin receptor (PRLR) and diacylglycerol lipase alpha (DGL- α) genes]: DGL- α (80 in-group, 9 out-group samples), anonymous nuclear locus L52 (63, 15), anonymous nuclear locus L74 (66, 17), and PRLR (59, 9). All ambiguous sites were coded using IUPAC guidelines. Sequencing products were assembled and edited using GENEIOUS (v3.0; Drummond *et al.* 2011). All sequences were deposited in GenBank (accession Nos. KC795009–KC795085 [DGL- α]; KC795086–KC795166 [L52]; KC795167–KC795237 [PRLR]), or Dryad (doi: 10.5061/dryad.m0n61 [L74]).

Sequence alignment and phylogenetic analyses

We produced initial alignments in MUSCLE (v3.7; Edgar 2004), with manual adjustments made in SE-AL (v2.0a9; Rambaut 2002; submitted at Dryad: doi: 10.5061/dryad.m0n61). To assess phylogenetic congruence between mitochondrial and nuclear data, we inferred phylogenies for each locus independently under both maximum likelihood (ML) and Bayesian frameworks. We found weakly supported nDNA topologies, but high support for mtDNA lineages (Figs S1 and S2, Supporting information). Due to the absence of well-supported topological incongruence between mtDNA and nDNA trees, we conducted subsequent analyses using a combined, partitioned, concatenated data set. Following a number of recent studies (Brandley *et al.* 2005; Siler & Brown 2010; Wiens *et al.* 2010), we treated each nuclear locus as a distinct partition and partitioned mitochondrial DNA by codon position and tRNAs, using the Akaike information criterion as implemented in JMODELTEST (v0.1.1; Posada 2008) to select an appropriate model of nucleotide substitution for each of the 11 partitions (Table S3, Supporting information).

Nuclear data were phased for each locus using the program PHASE (v2.1; Stephens *et al.* 2001; Stephens & Scheet 2005). For each population (inferred as islands or biogeographic subregions within islands) from which we had at least four gene copies of a nuclear locus, we conducted a 4-gamete test for recombination in SITES (Hey & Wakeley 1997), to look for patterns of variation consistent with recombination. Furthermore, from each population from which we had at least four gene copies of any locus (i.e. including the mitochondrial locus), we estimated Tajima's *D* (Tajima 1989) using the Population and Evolutionary Genetics Analysis System (pegas) package (v0.4-4; Paradis 2010) in R (R Development Core Team 2008). We estimated the *P*-value of each Tajima's *D* estimate assuming that *D* follows a standard normal distribution under the null hypothesis of neutral

mutation and demographic stability. We used the programs ARLEQUIN (v3.5.1.3; Excoffier & Lischer 2010) and MESQUITE (v2.75; Maddison & Maddison 2011) to estimate standard indices of genetic diversity, including number of heterozygotes and polymorphic sites per locus, as well as haplotype and gene diversity, among phased nuclear loci.

We conducted partitioned ML analyses using the program RAXMLHPC (v7.0; Stamatakis 2006) for the combined data set. We applied the more complex model (GTR + I + Γ) to all subsets, and 1000 replicate ML inferences were performed for the analysis. Each inference was initiated with a random starting tree and used the rapid hill-climbing algorithm of Stamatakis *et al.* (2007, 2008). We then assessed clade support with 1000 bootstrap pseudoreplicates. Partitioned Bayesian analyses in MRBAYES (v3.1.2; Ronquist & Huelsenbeck 2003) were conducted with a rate multiplier to allow substitution rates to vary among subsets. Default priors were used for all model parameters except branch lengths, which were adjusted on subsequent runs to facilitate run convergence (Marshall *et al.* 2006b; Brown *et al.* 2010; Marshall 2010). We ran four independent MCMC analyses, each with four Metropolis-coupled chains set at the default heating scheme. Analyses were run for 40 million generations, sampling every 5000 generations. We assessed stationarity by plotting all sampled parameter values and log-likelihood scores from the cold Markov chains from each independent run against generation time using TRACER (v1.5; Rambaut & Drummond 2007). We also compared split frequencies among independent runs for the 20 most variable nodes using Are We There Yet? (Wilgenbusch *et al.* 2004). We conservatively discarded the first 20% of samples as burn-in.

Population structure

We estimated haplotype diversity and population genetic structure for mitochondrial and nuclear data sets, initially analysing each locus independently. We used the phased nuclear data to estimate statistical parsimony allelic networks using the program TCS (v1.21; Clement *et al.* 2000), which utilizes a 95% connection significance criterion. For comparison, concatenated nuclear and mitochondrial data were analysed with the NeighborNet algorithm in SPLITSTREE (v4.12.8; Huson & Bryant 2006). In addition to analysing the raw, concatenated nuclear and mitochondrial data, we explored the effect of using a standardized distance matrix for nuclear loci [created with the program POFAD (v1.03; Joly & Bruneau 2006)], which facilitates the use of multiple loci and allows for inference of allelic variation which has resulted from population

dynamics (Posada & Crandall 2001; Cassens *et al.* 2005; Zarza *et al.* 2008). Resulting networks can effectively illustrate equally parsimonious inferences and underlying patterns of spatially partitioned genetic variation (Cassens *et al.* 2003).

We applied the Bayesian clustering program *STRUCTURE* (v2.3.4; Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009) to our phased nuclear data to estimate population structure, possible migrants and individuals with admixed population ancestry. We analysed the full data set, as well as one parsed for missing data, estimating frequencies of unique alleles among all samples (due to minimal differences in results, only results from the full data set are presented). Individual samples were coded by shared haplotypes. In the absence of prior knowledge of relationships, and given monitor lizards' inherent capability for dispersal across biogeographic barriers (Hoogerwerf 1954; Gaulke 1991; Rawlinson *et al.* 1992), we used the 'admixture' model for analyses. We varied the a priori constraint of the number of populations from a single, panmictic population throughout all of Southeast Asia ($K = 1$), to a series of populations including all islands (and/or biogeographic subregions within large islands) represented in our sampling ($K = 32$). We ran five independent analyses, each for 5 million iterations, discarding a burn-in of 500 000. We selected the preferred number of populations based on Evanno *et al.* (2005), using the program *STRUCTURE HARVESTER* (Earl & vonHoldt 2012). To distinguish between samples that exhibited mixed vs. pure allelic composition, we used a 90% composition threshold (Pritchard *et al.* 2000) and visualized results with the program *DISTRUCT* (v1.1; Rosenberg 2004).

Bayesian species delimitation

We approached questions of taxonomic diversity on the basis of evolutionary hypotheses inferred from species tree analyses of nine putative lineages. To provide an objective starting point for the program Bayesian Phylogenetics and Phylogeography (BP&P; Yang & Rannala 2010), we estimated topologies using the multi-species coalescent model implemented in *BEAST (v1.7.5; Drummond & Rambaut 2007; Heled & Drummond 2010; see Appendix S2, Supporting information, for detailed *BEAST methodology). We assigned gene copies of each locus to one of nine putative lineages based on recognized species' distributions and the results of our concatenated, multilocus phylogenetic estimate.

We used BP&P to estimate species delimitation under 11 different topologies: the 10 most frequent topologies from the posterior sample of trees from our *BEAST analyses (accounting for nearly 20% of the

posterior sample of trees), and the topology inferred from our concatenated phylogenetic analysis. Under each of these fixed guide topologies, we used BP&P to estimate the probability that each node represents a speciation event (i.e. a split between two distinct species), assuming no admixture following speciation (see Appendix S3, Supporting information, for detailed BP&P methodology).

Results

Sampling, neutrality, recombination, genetic diversity and phylogenetic inference

Our complete, aligned matrices include 146 ND1–ND2 (2531 bp), 89 DGL- α (651 bp), 86 L52 (545 bp), 90 L74 (185 bp) and 74 PRLR sequences (541 bp), respectively. Variable/parsimony-informative characters are: 1610/1460 (mtDNA); 32/16 (DGL- α); 37/18 (L52); 8/4 (L74); and 32/14 (PRLR). We rooted our tree with *Heloderma* based on accepted superfamily Varanoidea relationships (Caldwell 1999; Lee & Caldwell 2000; Townsend *et al.* 2004; Evans *et al.* 2005; Wiens *et al.* 2010; Conrad *et al.* 2011).

There was no evidence of intralocus recombination for any of the nuclear loci from our 4-gamete test. We were unable to reject a model of neutral mutation and demographic stability based on our estimation of Tajima's D ($0.99 > P > 0.06$; Table S4, Supporting information). Our analyses of genetic diversity indicate the presence of heterozygous individuals and polymorphic sites among all nuclear loci, comprised of four individuals for DGL- α (8 polymorphic sites), 2 for L52 (33 polymorphic sites), 4 for L74 (2 polymorphic sites) and two individuals for PRLR (1 polymorphic site). Additionally, average nucleotide diversity for each locus was estimated as 0.0025 (± 0.0017 SD) for DGL- α , 0.1252 (± 0.0682 SD) for L52, 0.2667 (± 0.2471 SD) for L74 and 0.0309 (± 0.0609 SD) for PRLR.

Analyses of the mitochondrial and concatenated data sets resulted in topologies with high bootstrap support (ML) and posterior probabilities (Bayesian; Figs 1 and 2). The inferred topologies were congruent across analyses, and generally, our results support those of Ast (2001) and Collar *et al.* (2011); there is strong support for the monophyly of the *V. salvator* Complex, and within the Complex, the Philippine species are paraphyletic with respect to non-Philippine lineages. In total, eight major, well-supported clades of water monitors are recovered ($BS \geq 70\%$, $PP \geq 0.95$; Fig. 2A–H). Many correspond well to Southeast Asian biogeographic regions (Clade A: Mindanao faunal region; C: Sulawesi; E: Palawan Island; F: Mindoro faunal region; G: Bicol faunal region; H: Visayan faunal region + Romblon Island Group); others

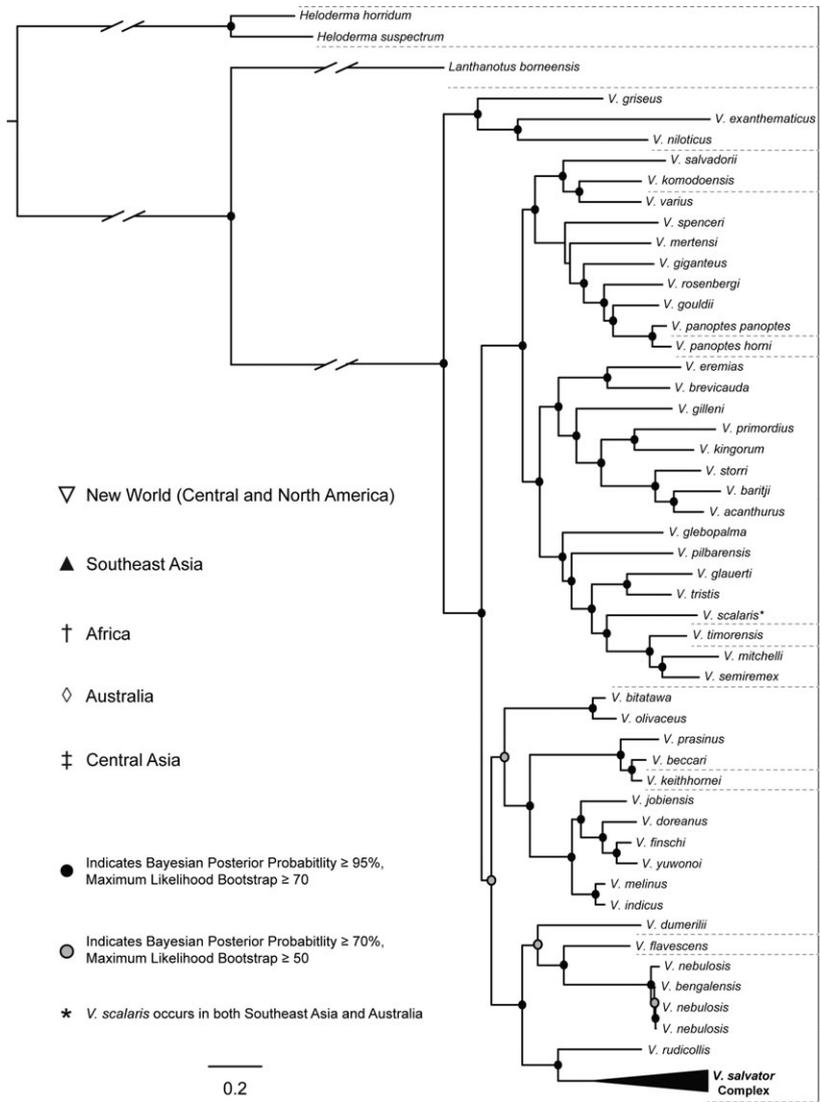


Fig. 1 Maximum likelihood estimate of species-level relationships within Varanidae. Likelihood bootstrap and Bayesian posterior probability nodal support is indicated with shaded circles (see key).

contain samples from multiple regions (D: Sumatra, Java, Myanmar, Singapore). Similarly, well-supported subclades are recovered for specific geological components within major clades (Fig. 2A1–H3). The most surprising general results were our findings of the paraphyletic nature of *V. marmoratus* and the inference of all non-Philippine species nested within a predominantly Philippine clade. Our results indicate a potential relationship between *V. palawanensis* and *V. cf. marmoratus* from the Mindoro faunal region, and a sister relationship between *V. nuchalis* and *V. cf. marmoratus* from the Bicol faunal region (Fig. 2E–H).

Population structure

TCS identified 61 and 44 unique haplotypes from mtDNA and nDNA, respectively (Fig. S3, Supporting information; Table 2). Haplotype diversity is highest

within *V. marmoratus*, with entirely unique variants (distinct networks or individual samples) corresponding to well-supported clades identified in phylogenetic analyses (Fig. 2, Fig. S3, Supporting information). As expected, haplotype diversity was significantly lower in nuclear loci; only in L52 does the partitioning of genetic diversity correspond to major geological components of Southeast Asia’s major landmasses (Fig. S3, Supporting information). *Varanus marmoratus* and *V. nuchalis* exhibit the highest proportions of unique haplotypes (79.4% and 76.5% unique, respectively; Table 2). SplitsTree analyses recovered similar patterns of genetic variation, with greater distinctiveness of sampled taxa apparent in mtDNA (Fig. 3; Table 2), including 13 well-supported clusters (>70 BS; Fig. 3). Analyses of the concatenated nuclear data recovered two poorly supported clusters: one containing *V. marmoratus*, *V. cf. marmoratus* (Bicol and Mindoro faunal regions) and *V. palawanensis*

Table 2 Summary of haplotype diversity within the *Varanus salvator* Complex as inferred by TCS and SplitsTree

Taxon	ND1 + ND2	DGL- α	L52	L74	PRLR	% Unique	mtDNA cluster
<i>V. cumingi</i>	8	2	4	1	2	70.6	+ [†]
<i>V. c. samarensis</i>	2	1	1	1	1	33.3	+
<i>V. marmoratus</i>	19	4	5	2	4	79.4	+
<i>V. marmoratus</i> (Mindoro)	3	3	1	1	1	44.4	+ [†]
<i>V. marmoratus</i> (Bicol)	7	3	3	2	1	56.3	+
<i>V. nuchalis</i>	9	1	4	1	1	76.5	+
<i>V. palawanensis</i>	2	1	1	1 [‡]	2 [‡]	50.0	+
<i>V. s. bivittatus</i>	3	1	1 [§]	1	1	42.9	+
<i>V. s. macromaculatus</i>	6	3	5	1	1	62.5	+ [†]
<i>V. togianus</i>	3	2 [§]	2 [§]	1	1	66.7	+

TCS results are presented by locus (Fig. 3), while those of SplitsTree are indicative of mitochondrial analyses alone (Fig. 3). DGL- α , diacylglycerol lipase- α .

[†]Taxa which are further partitioned by SplitsTree, with clusters corresponding to geographic distributions of lineages. Coding regions of the mtDNA were analyzed including flanking tRNAs.

[‡]Haplotypes which are shared with the typical *V. salvator* form.

[§]Haplotypes which are distinct from the typical *V. salvator* form as inferred by TCS.

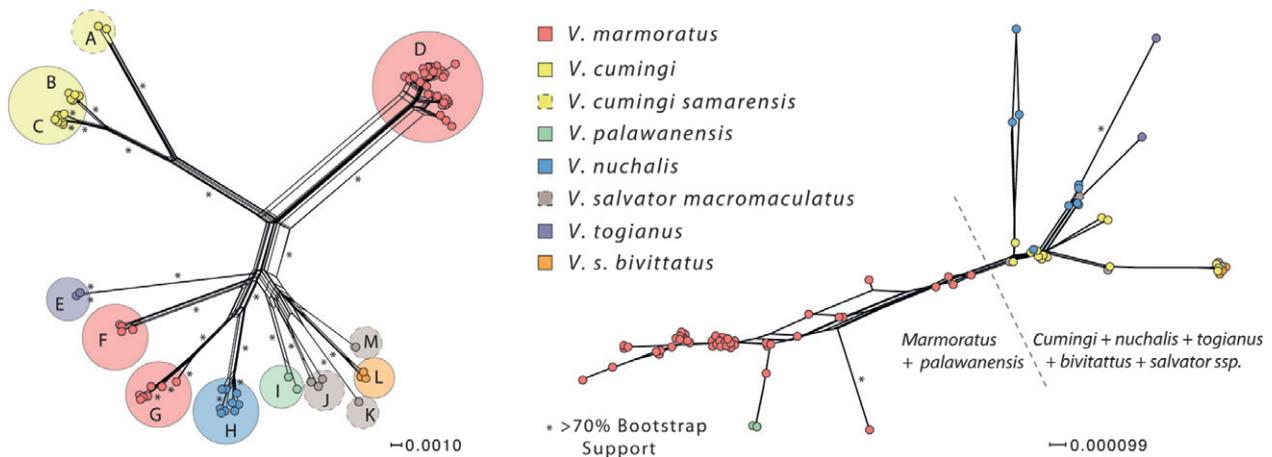


Fig. 3 Mitochondrial and nuclear haplotype networks inferred by SPLITSTREE (Huson & Bryant 2006). Clusters correspond to populations (Philippines, unless noted otherwise) from: (A) Samar and Bohol islands; (B) western Mindanao Island; (C) eastern Mindanao, Camiguin Sur and Talikud islands; (D) northern Luzon, Batan, Calayan and Lubang islands; (E) Sulawesi Island, Indonesia; (F) Mindoro and Semirara islands; (G) the Bicol Peninsula of southeastern Luzon and Catanduanes and Polillo islands; (H) Negros, Panay and Sibuyan islands; (I) Palawan Island; (J) Western Malaysia and Sumatra Island, Indonesia; (K) Myanmar; (L) Java Island, Indonesia; and (M) Sumatra Island, Indonesia.

exhibited increased variance and lower posterior probabilities; Fig. S5, Supporting information). However, subsequent summary of multiple analyses (STRUCTURE HARVESTER; Evanno *et al.* 2005), whereby K is inferred based on the maximal change in log-likelihood values, supported the presence of only two ($K = 2$) 'pure ancestry' populations (Fig. 4). These two populations correspond to: (i) true *Varanus marmoratus* (Babuyan and Batan island groups, and Luzon and Lubang islands) and (ii) *V. cumingi* (Dinagat, Mindanao, Camiguin Sur and Talikud islands), *V. c. samarensis* (Bohol and Samar islands), *V. nuchalis* (Masbate, Negros,

Panay and Sibuyan islands, *V. s. bivittatus* (Java Island), *V. s. macromaculatus* (Western Malaysia, Myanmar and Sumatra Island; Fig. 4) and *V. togianus* (Sulawesi Island). Additionally, the analyses recovered a zone of admixture: *V. cf. marmoratus* (Bicol region of Luzon, Catanduanes, Mindoro, Polillo and Semirara islands) and *V. palawanensis* (Palawan Island; Fig. 4).

Species delimitation

The 10 most frequent topologies from our *BEAST posterior sample differ predominately in the placement of

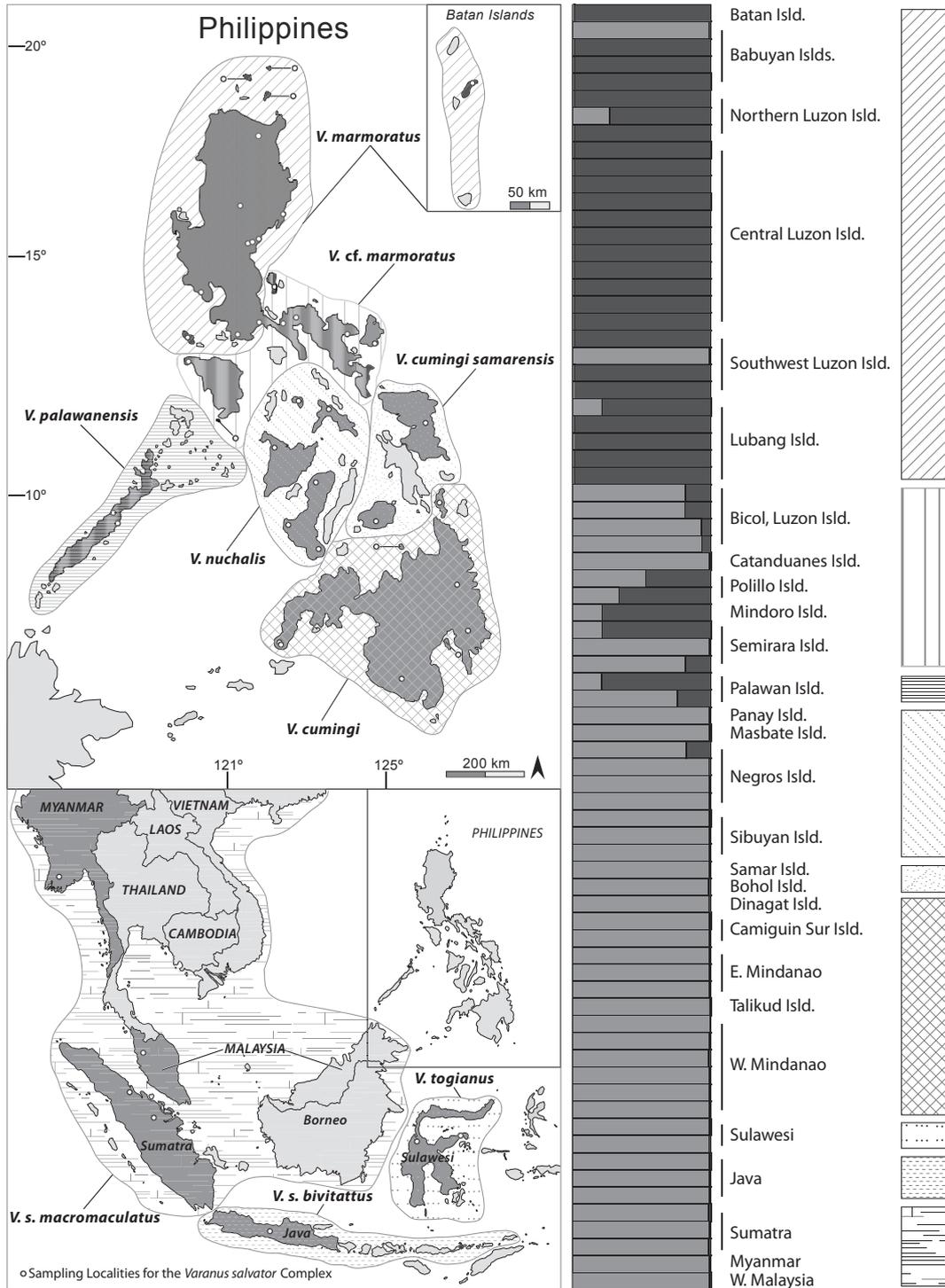


Fig. 4 DISTRUCT (Rosenberg 2004) visualization of STRUCTURE analyses and summarized geographic distribution of major *Varanus* demes (Pritchard *et al.* 2000; Evanno *et al.* 2005) for $K = 2$ populations.

Varanus cf. marmoratus (Mindoro and Semirara islands), *V. palawanensis*, *V. salvator* ssp. and *V. togianus* (Fig. S6, Supporting information). The sister relationship between true *V. marmoratus* and *V. cf. marmoratus* from the Bicol region is recovered in all topologies, with *V. nuchalis*

recovered as sister to both Luzon lineages in nine of 10 trees. The basal status of the lineage containing *V. cumingi* and *V. c. samarensis* is similarly recovered in all topologies. Given these alternative placements of multiple, well-supported phylogenetic lineages, we estimated

Table 3 Summary of results from BPP analyses

Species	Parameter settings (tau, theta)		
	10, 1000 10, 1000	2, 2000 2, 2000	10, 1000 2, 2000
<i>Varanus c. cumingi</i>	0.00–0.45	0.09–0.53	0.00–0.44
<i>Varanus c. samarensis</i>	0.00–0.45	0.09–0.53	0.00–0.44
<i>Varanus marmoratus</i>	0.00–1.00	0.97–1.00	0.67–1.00
<i>Varanus cf. marmoratus</i> (Bicol)	0.21–1.00	0.74–1.00	0.67–1.00
<i>Varanus cf.</i> <i>marmoratus</i> (Mindoro)	0.18–1.00	0.99–1.00	0.16–1.00
<i>Varanus nuchalis</i>	0.77–1.00	0.74–1.00	0.77–1.00
<i>Varanus palawanensis</i>	0.18–1.00	0.99–1.00	0.16–1.00
<i>Varanus togianus</i>	1.00	1.00	0.98–1.00
<i>Varanus salvator ssp.</i>	0.77–1.00	1.00	0.98–1.00

Values indicate ranges of split probabilities among the 11 topologies tested (Fig. S6, Supporting information), where the values are drawn from the node uniting each taxon with its inferred sister taxon. The lineage comprised of both *V. cumingi cumingi* and *V. c. samarensis* received strong support (split probability = 1.0) across all topologies (Fig. S6, Supporting information).

species boundaries in BP&P under all 10 topologies, as well as that recovered from our concatenated gene tree analysis. BP&P analyses were generally consistent across runs for all topologies, with high support recovered for most described species (Table 3; Fig. S6, Supporting information). Given the arbitrary priors applied, it is apparent that the demographic scenario of relatively small ancestral populations and recent divergence times is most likely indicative of the *V. salvator* Species Complex.

Discussion

Since the original description nearly two and a half centuries ago (Laurenti 1768), *Varanus salvator* has undergone numerous taxonomic revisions, defining the complex as a single species (Laurenti 1768), four species (Boulenger 1885), back to a single species (*V. salvator*) with five (Mertens 1942a,b,c) or eight (Mertens 1963; Gaulke 1991, 1992; Böhme 2003) subspecies, and most recently, to six species and six subspecies (Koch & Böhme 2010; Koch *et al.* 2007, 2010a,b; Table 1).

We consider traditional, morphology-based taxonomy as a reasonable basis for hypotheses of species diversity if character-based diagnostic definitions are provided; these are, of course, considered valid under current guidelines of nomenclature (ICZN 1999). However, with respect to water monitors, past studies have been limited by their reliance on a small suite of phenotypic characters and small sample sizes available in museum collections. Although these types of data can provide useful

diagnostic characters (Mertens 1942a,b,c), recent treatments of the *Varanus salvator* Complex have been unable to incorporate statistical analyses of large sample sizes, with little to no incorporation of historical biogeography (but see Gaulke 1991) or underlying genetic variation.

Our sampling of individuals from throughout the range of all but one currently recognized Philippine species allows for robust genetic analyses of populations in the archipelago and includes the major water monitor lineages from the islands of the Sunda Shelf, Sulawesi, and mainland Asia. The absence of a few peripheral populations does not hinder our primary goals of inferring phylogenetic affinities, population structure and species boundaries among Philippine populations.

Phylogenetics and population structure

Although the focal group of this study is the Philippine assemblage of water monitors, our results underscore the necessity of geographically broad sampling to accurately estimate evolutionary relationships and species-level diversity within widespread species complexes. Within the Philippines, the monophyly of all but one of the five described taxonomic units was supported in phylogenetic analyses (*V. c. cumingi*, *V. c. samarensis*, *V. nuchalis*, and *V. palawanensis*). In contrast, the taxon *V. marmoratus*, recovered here as a paraphyletic assemblage, represents three distinct, biogeographically discrete, well-supported clades that are not each other's closest relatives. These newly discovered lineages include a clade from the Mindoro faunal region, and one from the Bicol faunal region and Polillo and Catanduanes islands (Fig. S7, Supporting information). The first of these is sister to *V. palawanensis*, and although this relationship is not strongly supported, the geographic proximity of the Palawan and Mindoro faunal regions provides plausible biogeographic evidence for a close, presumably dispersal-mediated relationship, which has been observed in many other vertebrates (Brown & Guttman 2002; Evans *et al.* 2003; Brown *et al.* 2009; Esselstyn *et al.* 2010; Siler *et al.* 2012). The second lineage is inferred to be sister to *V. nuchalis* from the Visayan faunal region. This relationship is both novel and somewhat surprising, in that this lineage does not share phylogenetic affinities with the rest of Luzon. Biogeographically, however, the Bicol and Visayan faunal regions are geographically proximate, increasing the probability of contemporary gene flow between these regions. The recovery of novel phylogenetic relationships among Philippine water monitors once again highlights the dynamic nature of the Philippine archipelago (Brown & Diesmos 2009)—many vertebrate groups have diversified via apparently complex combi-

nations of vicariance (possibly via sea level oscillation), dispersal, and *in situ* diversification across habitat barriers and, possibly, ecological gradients (Esselstyn & Brown 2009; Esselstyn *et al.* 2009; Linkem *et al.* 2010; Siler *et al.* 2010, 2012; Welton *et al.* 2010a,b, 2013).

The phylogenetic relationships of taxa outside of the Philippines reveal well-supported monophyletic lineages corresponding to major landmasses [i.e. samples from Java (*V. s. bivittatus*) and Sulawesi (*V. togianus*)]. However, mixed affinities are evident on Sumatra, with one sample closely related to *V. s. bivittatus* (Java) and two samples related to *V. s. macromaculatus* (Malaysia and Singapore). The relatively close proximity of Sumatra to the Asian mainland most likely increases the potential for gene flow between these two regions.

Estimates of haplotype diversity depicted in networks, and analyses of population structure, in part mirrored conclusions from our phylogenetic inferences. Of the taxonomic units sampled, all were supported as distinct in mitochondrial haplotype analyses (Fig. 3, Fig. S3, Supporting information; Table 2). The combined results of haplotype network analyses reveal that proportions of unique haplotypes in excess of 50% correspond to recognized species, while values below that are indicative of lower taxonomic units (i.e. subspecies, populations; Table 2). The lineage of *V. cf. marmoratus* from the Mindoro faunal region exhibits a proportion of unique haplotypes below this apparent threshold (44.4% unique), while the lineage from the Bicol faunal region exhibits a proportion greater than the threshold value (56.3% unique; Table 2). Affinities recovered by SplitsTree correspond to expectations based on biogeography (Fig. 3, Fig. S7, Supporting information).

Our STRUCTURE analyses, while not recovering support for all described taxa, did reveal allelic admixture among currently recognized species, subspecies and well-supported lineages, suggesting either contemporary gene flow or the retention of ancestral polymorphisms. With few exceptions, true *Varanus marmoratus* and Sundaland + southern Philippine populations are recovered as two pure ancestry allelic groups (Fig. 4, Fig. S5, Supporting information). Interestingly, the remaining samples exhibit a variably admixed composition of the two pure ancestry allelic patterns (*V. palawanensis* [Palawan Island] and *V. cf. marmoratus* [Bicol region of Luzon Island and Catanduanes, Mindoro, Polillo and Semirara islands]; Fig. 4). The biogeographic distinction between the two pure ancestry groups corresponds to a 'zone of admixture', delineating northern populations (true *V. marmoratus*) from southern (*V. cumingi* ssp., *V. nuchalis* and the remaining non-Philippine taxa; Fig. 4). Despite relatively poor resolution on the taxonomic level, these results corroborate the higher-level systematic relationships inferred by phylogenetic and haplotype analyses.

Although historical and contemporary natural processes of dispersal likely have contributed to these patterns, it is also possible that more recent, human-mediated dispersal has occurred as well and may be responsible for the apparent 'transient' patterns observed within the pure ancestry assemblages (Fig. 4). Water monitors are frequently transported between islands (Welton *et al.* 2013), traded as bush meat, marketed in both legal and illegal pet trade (Gaulke 1998; Welton *et al.* 2013), and likely transported in agricultural shipments (personal observations).

Our analyses indicate the strong possibility of dynamic historical and contemporary gene flow among populations of the *Varanus salvator* Complex. Phylogenetic and population genetic analyses support the distinctiveness, to varying degrees, of all eight taxonomic units sampled and underscore the utility of employing multiple methods to mitochondrial and nuclear data for more robust estimates of species diversity. However, formal taxonomic recognition of all entities detected here is complicated by the possibility of high levels of gene flow among putative taxa and varying levels of genetic divergence between them.

Species delimitation and conservation

With a few exceptions, named water monitor species are phenotypically distinct (Table 4). However, despite past attempts to identify diagnostic characters (body size, coloration, scale counts; Gaulke 1991; Koch *et al.* 2007, 2010a), to date, few nonoverlapping, discrete, (taxonomically diagnostic) character state differences between Philippine species have been identified (Table 4).

Here, we have used multilocus genetic data to evaluate species boundaries (as inferred from morphological data) in Philippine taxa and infer the presence of additional evolutionary units. The majority of our analyses support continued recognition of most named taxa and suggest that novel and distinct lineages from the Mindoro and Bicol faunal regions may warrant recognition upon corroboration of morphological data, and/or other sources of information. However, recognition of lower taxonomic entities (subspecies) was only partially supported by phylogenetic, haplotype and Bayesian delimitation analyses. Specifically, STRUCTURE failed to distinguish between *V. palawanensis* and the Mindoro and Bicol faunal region populations of *V. cf. marmoratus*, and similarly between *V. c. cumingi* and *V. c. samarensis*. Additionally, BP&P provided only variable support for splits between these taxa and no support for the split between *V. c. cumingi* and *V. c. samarensis*. Despite the lack of clear distinction for *V. palawanensis* and the subspecies *V. c. samarensis* in our analyses, their phenotypic distinctiveness (body size and colour pattern, respec-

Table 4 Summary of morphological data used previously (Gaulke 1991; Koch *et al.* 2007, 2010a) to delimit species, and support from phylogenetic (monophyly), unique haplotype diversity, population genetics (TCS haplotypes and NeighborNet clusters), Bayesian species delimitation analyses (BPP) and biogeographic distribution (allopatry) of taxa

	<i>Varanus c. cumingi</i>	<i>Varanus c. samarensis</i>	<i>Varanus marmoratus</i>	<i>Varanus nuchalis</i>	<i>Varanus palawanensis</i>	<i>Varanus salvator macromaculatus</i>	<i>Varanus s. bivittatus</i>	<i>Varanus togianus</i>
Monophyly	+	+	-	+	+	-	+	+
Haplotype	+	+	-	+	+	+	+	+
PN Cluster (>70%)	+	+	-	+	+	+	+	+
BPP	+	-	+	+	+/-	-	-	+
Allopatry	+	+	+	+	+	+	+	+
Body colour (dorsal)	5-6 Transverse bands of yellow ocelli over black background, with occasional yellow paravertebral stripe	5-8 Transverse bands of yellow ocelli over black background	Variably, 4-6 transverse bands of light ocelli over black background	Variably, 4 transverse bands of light ocelli over black background, with occasional light paravertebral stripe	Up to 8 transverse bands of light ocelli, over mostly dark background, mottled with brightly bordered scales	n/a	n/a	n/a
Head colour (dorsal)	Predominantly yellow-gold, with black temporal streak occasionally bordered by lighter streak	Predominantly black, with symmetrical yellow markings	Predominantly black, with 1 or 2 indistinct cross-bands on snout	Predominantly black, but with occasional light markings	Predominantly dark, but occasionally with light markings or light temporal streak	n/a	n/a	n/a
Occipital scales	46-57	47-51	48-59	50-61	46-65	49-65	47-63	44-58
Nuchal scales	26-33	21-30	24-33	19-30	24-32	29-50	28-52	31-42
Scales around midbody	121-150	130-152	115-145	136-169	129-178	135-178	101-175	120-142
Dorsal scales	114-136	114-127	101-123	94-138	116-182	125-172	123-189	117-172
Ventral trunk scales	77-85	81-78	80-92	84-94	78-95	75-95	75-97	77-82
Scales around base of tail	82-108	90-109	85-107	84-110	98-119	88-126	79-128	87-112
Scales around tail, 1/3 from base	42-55	46-55	48-65	43-55	48-70	41-82	51-71	54-63
Narial position	1.9-2.26 Times closer to tip of snout than to eye	2.0-3.29 Times closer to tip of snout than to eye	1.85-2.6 Times closer to tip of snout than to eye	1.78-2.13 Times closer to the tip of the snout than to eye	1.69-2.20 Times closer to tip of snout than to eye	2.0-2.8 Times closer to tip of snout than to eye	1.67-2.88 Times closer to tip of snout than to eye	1.82-2.70 Times closer to tip of snout than to eye

Morphological data summarized from Gaulke (1991, 1992) and Koch *et al.* (2007, 2010a). PN corresponds to phylogenetic networks produced in SplitsTree (considered strongly supported with nonparametric bootstrap values >70%).

tively; Gaulke 1991, 1992; Koch *et al.* 2010a) and clear conservation concern (Brown & Diesmos 2009) support continued taxonomic recognition.

Monitor lizards represent an important group for studies relating to conservation, trade and sustainable harvest given that they represent a commercially important component of local Asian vertebrate faunas (Shine *et al.* 1996; Yuwono 1998; Fa *et al.* 2000; Pernetta 2009; Koch *et al.* 2010a), are a heavily exploited vertebrate group (Shine *et al.* 1996; Shine & Harlow 1998; Mace *et al.* 2007) and are important components of the diet of many indigenous cultures (Mittermeier *et al.* 1992; Nash 1997; Stuart 2004; Welton *et al.* 2010a, 2012). Given the prevalence of water monitors in trade (Schlaepfer *et al.* 2005), we perceive an immediate need for identification of all lineages, some of which may be subject to differential harvesting pressures (Gaulke 1998; Welton *et al.* 2012, 2013).

Our past identification of unrecognized evolutionary lineages of monitor lizards (Welton *et al.* 2010a, 2012) and our new identification of apparently cryptic lineages suggests the existence of additional species diversity or, at least, evolutionary significant units for conservation. This new appreciation of high levels of lineage diversity in Philippine water monitors bolsters the archipelago's designation as a biodiversity hotspot and a global conservation priority (Brown & Diesmos 2009; Welton *et al.* 2010a).

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This study is part of L.J.W. Masters' thesis on phylogeography and conservation trade forensics of water monitors of Southeast Asia. All authors share interests in systematics, biogeography and conservation of vertebrates of the island archipelagos of Southeast Asia.

Data accessibility

DNA sequences: GenBank accessions nos. KC795009–KC795085 [DGL- α]; KC795086–KC795166 [L52]; KC795167–KC795237 [PRLR]; Dryad doi: 10.5061/dryad.m0n61 [L74].

Sequence alignments and control files used in RAxML, *Beast, BP&P, and Structure analyses: Dryad doi: 10.5061/dryad.m0n61.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Specimen voucher numbers, identification and geographic location of samples for this study.

Appendix S2 Description of *BEAST methodology for inferring BP&P starting topologies.

Appendix S3 Description of BP&P species delimitation methodology.

Fig. S1 Mitochondrial (left) and concatenated nuclear (right) maximum likelihood phylogenetic estimates. Only bootstrap support >70% is indicated.

Fig. S2 Individual maximum likelihood gene trees for the four nuclear loci sampled. Only bootstrap support >70% is indicated.

Fig. S3 Mitochondrial and nuclear (ND1–ND2, DGL- α , L52, L74, PRLR) statistical haplotype networks inferred by TCS (Clement *et al.* 2000).

Fig. S4 Distance-based (neighbour-joining) nuclear haplotype network as inferred by the NeighborNet algorithm of SPLITSTREE. Input standardized distance matrix created with the program POFAD.

Fig. S5 DISTRUCT visualization (Rosenberg 2004) visualization of STRUCTURE analyses and summarized geographic distribution of major *Varanus* demes (Pritchard *et al.* 2000) for $K = 6$ populations.

Fig. S6 Bayesian estimates of the *Varanus salvator* Complex species tree as inferred by the program *BEAST.

Fig. S7 Recognized major faunal regions and island groups of the Philippines, including distributions of *Varanus* species.

Table S1 Individual loci, original publications and polymerase chain reaction (PCR) thermal profiles for each. Annealing temperature was varied to improve amplification for problematic samples.

Table S2 Loci and associated primers sequenced for this study. Thermal profiles for PCR and cycle sequencing reactions vary only by annealing temperature (55–58^o) across primers and samples.

Table S3 Estimated models of evolution by data partition, as inferred by JMODELTEST. Final models selected by AIC and applied for partitioned, model-based analyses.

Table S4 Summary of results among loci for tests of neutrality using Tajima's D (Tajima 1989).