Molecular phylogenetic evidence for ancient divergence of lizard taxa on either side of Wallace's Line

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Wallace's Line, separating the terrestrial faunas of South East Asia from the Australia–New Guinea region, is the most prominent and well-studied biogeographical division in the world. Phylogenetically distinct subgroups of major animal and plant groups have been documented on either side of Wallace's Line since it was first proposed in 1859. Despite its importance, the temporal history of fragmentation across this line is virtually unknown and the geological foundation has rarely been discussed. Using molecular phylogenetics and dating techniques, we show that the split between taxa in the South East Asian and the Australian–New Guinean geological regions occurred during the Late Jurassic to Early Cretaceous in two independent lizard clades. This estimate is compatible with the hypothesis of rifting Gondwanan continental fragments during the Mesozoic and strongly rejects the hypothetical origin of various members of the Australian–New Guinean herpetofauna as relatively recent invasions from South East Asia. Our finding suggests an ancient fragmentation of lizard taxa on either side of Wallace's Line and provides further evidence that the composition of modern global communities has been significantly affected by rifting and accretion of Gondwanan continental plates during the Middle to Late Mesozoic.

Keywords: Wallace's Line; biogeography; Gondwana; Agamidae; Varanidae; molecular clock

1. INTRODUCTION

Wallace's Line, one of the best-studied biogeographical phenomena in the world, separates continental Asia, The Philippines, Borneo and Western Indonesia from Sulawesi, Eastern Indonesia and the Australian–New Guinean region (figure 1; Wallace 1859; Whitmore 1981; Briggs 1987; Van Oosterzee 1997). It marks a geographical separation between divergent assemblages of birds, mammals, reptiles, insects and fishes, whose phylogenetic distinctness is confirmed by recent molecular phylogenetic studies (DeBoer & Duffels 1996; Keogh 1998; Barker et al. 2002). Although numerous authors have noted a distinct difference in faunal compositions east and west of Wallace's Line, considerable disagreement exists regarding the origin and position of this break (Whitmore 1981). These disagreements result from the complicated geological history of the region, as well as the widely disparate hypotheses for the timing and origin of the separation. For over a century, the origin of this faunal separation has been the subject of significant scientific debate. Only in the past two decades has integration of phylogenetics, biogeography and plate tectonics begun to provide insight into the origin and development of this faunal division (Macey et al. 2000; Metcalfe et al. 2001; Barker et al. 2002).

The two prevailing hypotheses for the origin of a major faunal change across Wallace's Line invoke either Late Tertiary (less than 25 Myr ago) dispersal events (Tyler 1979; Baverstock & Donnellan 1990; Honda et al. 2000) or ancient fragmentation of Gondwanan plates (Moody 1980; Estes 1983; Macey et al. 2000). The first hypothesis claims that climatic changes and the close proximity of the Australia–New Guinea plate to Laurasia during the Miocene would have allowed Laurasian groups to disperse to Australia or New Guinea and to occupy open niches not available in South East Asia (Cogger & Heatwole 1981). Alternatively, a Middle to Late Mesozoic (75–160 Myr ago) separation of these faunas by the breakup of Gondwanaland indicates that the primary diversification of major groups occurred among continental fragments moving in isolation across the Tethys Sea.

Understanding the complex geological history of South East Asia is essential for identifying the factors shaping these faunas. Three major geological events are especially noteworthy. First, much of continental south Asia contains fragments that broke from the northern Gondwanan margin during the Late Palaeozoic and accreted to Laurasia by the Triassic (Metcalfe 2001). However, some disagreement exists regarding the timing of later accretionary events, such as that of Lhasa and West Burma (Richter & Fuller 1996; Metcalfe 2001). Second, the Indian subcontinent began separating from eastern Africa, Madagascar and western Australia between 160 and 128 Myr ago, became completely separated ca. 100 Myr ago and accreted to Asia ca. 50 Myr ago (Johnson & Veevers 1984; Windley 1988). Third, Australia separated from Antarctica and rapidly moved north to its current position after India had accreted to southern Asia. Only in the last 10–20 Myr has the northern margin of the Australian plate had close contact with the island regions of Borneo, Java

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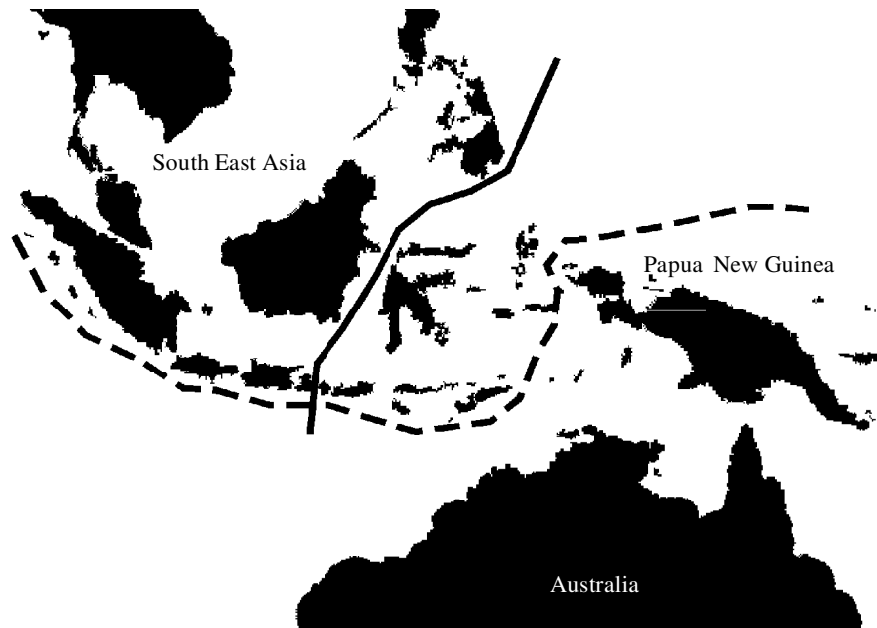
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Figure 1. Map of South East Asia and northern Australia and Papua New Guinea indicating the location of Wallace's Line (solid line). The dotted line indicates the approximate boundary of the Indian continental plate.

and Western Indonesia (Hall 2001). The roles of these events in fragmenting taxa across Wallace's Line are testable using molecular dating techniques.

Estimation of a divergence date for faunas on either side of Wallace's Line requires comparison of related taxa separated by this barrier for organisms with poor aquatic dispersal. Lizards are poor over-water dispersers, and two lizard groups are ideal for estimating the age of Wallace's Line: (i) the agamid lizard subfamily Amphibolurinae and (ii) the clade containing the anguimorph sister groups Varanidae and Lanthanotidae. Each group is composed of subgroups containing taxa distributed throughout Australia and much of Papua New Guinea, or Thailand, Indochina, Burma and South China (Macey *et al.* 2000; Ast 2001). As noted above, these latter areas are Gondwanan in origin, arising as blocks that split from either the northern margin of Australia or the Indian subcontinent.

To provide a date with error estimates for the divergence of lizard taxa on either side of Wallace's Line, phylogenetic analyses were conducted on mitochondrial DNA sequences from 71 amphibolurine taxa (*ca.* 90% of currently recognized species) and three outgroup taxa representing a broad level of divergence among agamid subfamilies (Hydrosaurinae, Leiolepidinae and Draconinae). Thirty-four of these sequences are newly reported, with the remainder coming from Macey *et al.* (1997*b*, 2000) and Melville *et al.* (2001). In addition, we performed phylogenetic analyses of sequence data reported by Ast (2001) for 49 taxa from *Lanthanotus, Varanus* and five anguimorph outgroups (Anguidae, Helodermatidae), with two additional outgroup sequences from Macey *et al.* (1999) (Shinisauridae, Xenosauridae). Sequences analysed contain *ca.* 1700 bases from the mitochondrial genes encoding ND1 (subunit one of nicotinamide adenine dinucleotide (NADH) dehydrogenase), through the genes encoding tRNA^{Ile}, tRNA^{Gln}, tRNA^{Met}, ND2 (NADH dehydrogenase subunit two), tRNA^{Trp}, tRNA^{Ala}, tRNA^{Asn}, tRNA^{Cys}, tRNA^{Tyr} and COI (subunit I of cyto-

chrome *c* oxidase). The evolutionary rate of this mitochondrial genomic region has been calibrated for agamid lizards (Macey *et al.* 1998*b*), with similar rates found in diverse vertebrate groups (evidence reviewed by Weisrock *et al.* (2001)). These data were used to test the two prevailing hypotheses that address the age and origin of faunal divergence across Wallace's Line: that it is the result of Late Tertiary (less than 25 Myr ago) dispersal events or that it is an effect of Middle to Late Mesozoic (75–160 Myr ago) fragmentation of Gondwanan plates.

2. MATERIAL AND METHODS

(a) *Specimen information, laboratory protocols and alignment of DNA sequences*

Specimen voucher, locality and GenBank accession numbers for newly reported amphibolurine agamid and outgroup taxa are presented in electronic Appendix A available on The Royal Society's Publications Web site. Information for previously published varanoid, amphibolurine and outgroup taxa is available from Ast (2001), Macey *et al.* (1999, 2000) and Melville *et al.* (2001).

Genomic DNA was extracted from liver, muscle or blood using Qiagen QIAamp tissue kits. Different primer combinations were used to amplify from genomic DNA. Amplifications of genomic DNA were conducted using a denaturation at 94 °C for 35 s, annealing at 53 °C for 35 s and extension at 70 °C for 150 s with 4 s added to the extension per cycle, for 30 cycles. Negative controls were run for all amplifications. Amplified products were purified on 2.5% NuSieve GTG agarose gels and reamplified under similar conditions. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis *et al.* 1982). Template DNA was eluted from acrylamide passively over 3 days with Maniatis elution buffer (Maniatis *et al.* 1982). Cycle-sequencing reactions were run using a Promega fmol DNA sequencing system with denaturation at 95 °C for 35 s, annealing at 45–60 °C for 35 s and extension at 70 °C

for 1 min for 30 cycles. Sequencing reactions were run on Long Ranger sequencing gels for 5–12 h at 38–40 °C.

Amplifications of the mitochondrial genomic segment extending from the ND1 gene to the COI gene from genomic DNA were done with different primer combinations. Most samples were amplified with L3914, or L4178b in combination with H4980. In addition, all samples were amplified with L4437b in combination with H5934, H6159 or H6564. Both strands were sequenced using L3914, L4178b, H4419a, H4419b, L4437b, H4584, H4629, L4645, L4831, L4882b, H4980, L5002, L5239, L5549a, L5556, H5934, H5934b and H6159. All primers are from Macey *et al.* (1997*a*) except L3914 (erroneously reported in Macey *et al.* (1998*a*) as L3878), H4419b, L4882b, H4629 (Macey *et al.* 2000), H6159 (Weisrock *et al.* 2001), H4584, L5239 (Melville *et al.* 2001) and L5549a (Townsend & Larson 2002). One new primer was used in this study: H5937c, 5′-GTTCCAATATCCTTATGRTT-3′. Primer numbers refer to the 3′ end on the human mitochondrial genome (Anderson *et al.* 1981), and L and H denote extension of light and heavy strands, respectively.

Alignment of tRNA genes was based on secondary structural models (Kumazawa & Nishida 1993; Macey & Verma 1997). Secondary structures of tRNAs were inferred from primary structures of the corresponding tRNA genes using these models. Gaps were treated as missing data. Unalignable regions in three length-variable loops (D, T and variable loops) of some tRNA genes and some intergenic sequences were excluded from phylogenetic analyses. The alignments used in phylogenetic analyses are available in TREEBASE (Study accession number S826, Matrix accession numbers M1325, M1326).

(b) *Phylogenetic analyses*

Phylogenetic trees were estimated using PAUP* beta v. 4.0b10 (Swofford 2002) with 100 heuristic searches using random addition of sequences under the maximum parsimony criterion (MP). Bootstrap resampling was applied to assess support for individual nodes using 1000 bootstrap replicates with 10 heuristic searches featuring random taxon addition. Decay indices were calculated for all internal branches of the tree using TREEROT v. 2b (Sorenson 1999). Maximum-likelihood (ML) analyses were performed using the best-fitting model of sequence evolution for the overall shortest tree from unweighted parsimony analysis obtained from MODELTEST v. 3.06 (Posada & Crandall 1998). Posada & Crandall (2001) found that the starting tree did not significantly influence the estimated parameters found by MODELTEST. Simultaneous optimization of ML parameters and phylogenetic hypotheses for these datasets was computationally impractical. The best-fitting model parameters were fixed and the overall most parsimonious trees were used as starting trees for branch swapping in 10 heuristic searches with random taxon addition to find the highest-likelihood topology.

Divergence times were estimated using the non-parametric rate-smoothing algorithm (NPRS) (Sanderson 1997) in TREE EDIT (Rambaut & Charleston 2001). This method is appropriate when evolutionary rates vary between lineages. To determine whether evolutionary rates were variable among lineages, the likelihood values of the overall most parsimonious topology were calculated with and without a molecular clock enforced in PAUP* and subsequently used to perform a likelihood ratio test (LRT). The test statistic is chi-squared distributed with $n = 2$ degrees of freedom where n is the number of sequences (Muse & Weir 1992). Outgroups were removed prior to rate-smoothing

calculations to prevent overestimation of the mean evolutionary rate across the phylogeny. Branch lengths on the overall most parsimonious topology were calculated using ML distances to obtain the probability of change along each branch in the phylogeny. These parameters correct for multiple substitutions, which may produce saturation of nucleotide changes in parsimony analyses and underestimation of divergence times. Branch lengths were scaled by multiplying each by 1611 or 2042 (number of included base positions for each dataset) to obtain the estimated number of substitutions on each branch, and dividing by 10.5 or 13.3 (estimated numbers of substitutions per million years using a calibration of 0.65% change (Macey *et al.* 1998*b*; Weisrock *et al.* 2001) per lineage per million years times 1611 or 2042.) These scaled branch lengths were then corrected using the non-parametric rate-smoothing algorithm to obtain times for each divergence event. Error estimates of divergence times were obtained by simulating 1000 datasets in SEQGEN (Rambaut & Grassly 1997) using the topology from parsimony analyses and parameters from the likelihood analysis. Each dataset was then used to estimate branch lengths and to calculate divergence times as above.

3. RESULTS

In the phylogenetic analysis of 1611 unambiguous sites in 74 aligned sequences of amphibolurine agamids and outgroups, 1171 (1075 ingroup only) are variable and 971 (893 ingroup only) are phylogenetically informative (parsimony criterion). Analysis of the mitochondrial DNA data provides a single most parsimonious phylogenetic tree with a length of 9231 steps (figure 2). Hierarchical LRTs using MODELTEST find that the most complex model (GTR + I + Γ; Tavaré 1986; Yang 1994) best explains the DNA sequence data and topology of the overall most parsimonious tree. Model parameters for the amphibolurine dataset are as follows: alpha = 0.632; proportion of invariant sites = 0.206; substitution rates R(a) = 0.393, R(b) = 5.521, R(c) = 0.512, R(d) = 0.192 and R(e) = 5.530; and estimated base frequencies A = 0.423, C = 0.325, G = 0.077, T = 0.176. A single optimal ML tree is found with a negative log likelihood of 38 954.5. The hypothesis of ultrametric evolution was strongly rejected on the parsimony tree ($-2 \ln \Lambda = 517.2$; d.f. = 72; $p < 0.0001$); thus, the use of NPRS is appropriate for this topology. Results of an ML analysis are congruent with this topology, and support a sister-taxon relationship between the South East Asian species, *Physignathus cocincinus*, and the Australian and New Guinean species. It is also noteworthy that species in the genus *Hypsilurus*, distributed both in Australia and New Guinea, are closely related, as expected from the close geographical proximity of these regions throughout the Earth's history.

The mitochondrial DNA dataset for Varanoidea (Ast 2001) was analysed in combination with sequences of *Shinisaurus* and *Xenosaurus* (Macey *et al.* 1999) for an overlapping region sequenced above with additional data from the ND1 gene. This dataset contains 2042 unambiguous sites in 56 aligned sequences with 1315 (1180 ingroup only) variable sites and 1149 (960 ingroup only) phylogenetically informative sites (parsimony criterion). Two overall most parsimonious trees are obtained, each with a length of 9253 steps (figure 3). ML model parameters are as follows: alpha = 0.613; proportion of invariant
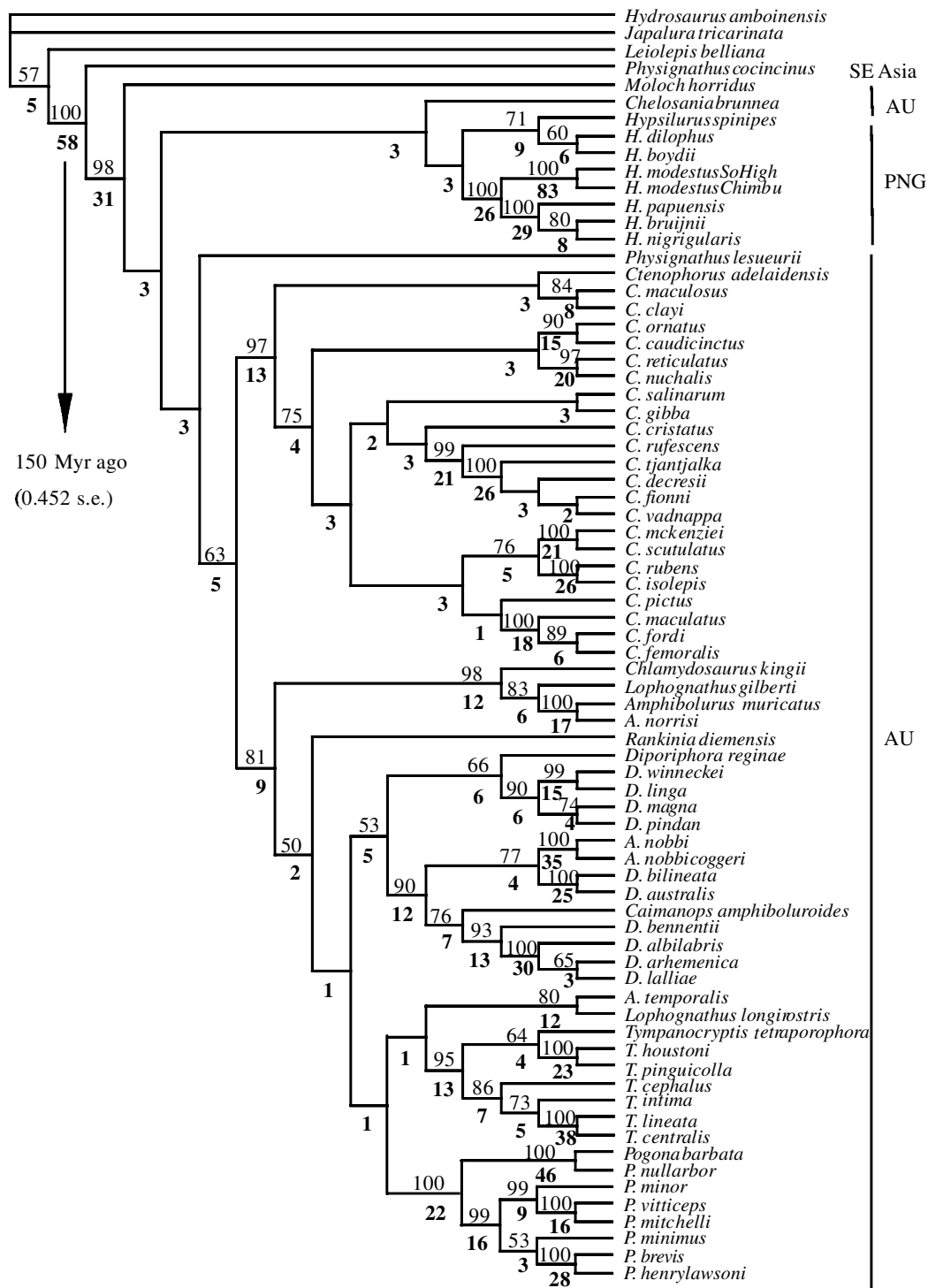
Figure 2. Phylogenetic relationships among amphibolurine agamid lizards. A single most parsimonious tree based on analysis of 1611 aligned base positions (971 parsimony informative) of mitochondrial DNA sequence data (length = 9231 steps). Bootstrap values are indicated above the branches and decay indices are denoted in bold below the branches. AU, taxa distributed in Australia; PNG, taxa found in Papua New Guinea.

sites = 0.279; substitution rates R(a) = 0.261, R(b) = 7.553, R(c) = 0.559, R(d) = 0.364 and R(e) = 5.383; and estimated base frequencies A = 0.382, C = 0.354, G = 0.062, T = 0.202. A single optimal ML tree is found with a negative log likelihood of 38 530.9. The hypothesis of ultrametric evolution was strongly rejected for the two parsimony trees ($-2 \ln \Lambda = 206.8$, d.f. = 54, $p < 0.0001$; $-2 \ln \Lambda = 210.3$, d.f. = 54, $p < 0.0001$), and the use of NPRS is also appropriate for these topologies.

Results of parsimony and maximum-likelihood analyses for *Varanus*, *Lanthanotus* and outgroups are congruent with previous studies (Ast 2001; figure 3). The Bornean genus *Lanthanotus* is strongly supported as the sister taxon to *Varanus*. This latter genus is composed of four major groups. A weakly supported clade containing African species is the sister group to all remaining *Varanus* species. The next group is composed of species found entirely to the west of Wallace's Line. In the third major clade, the
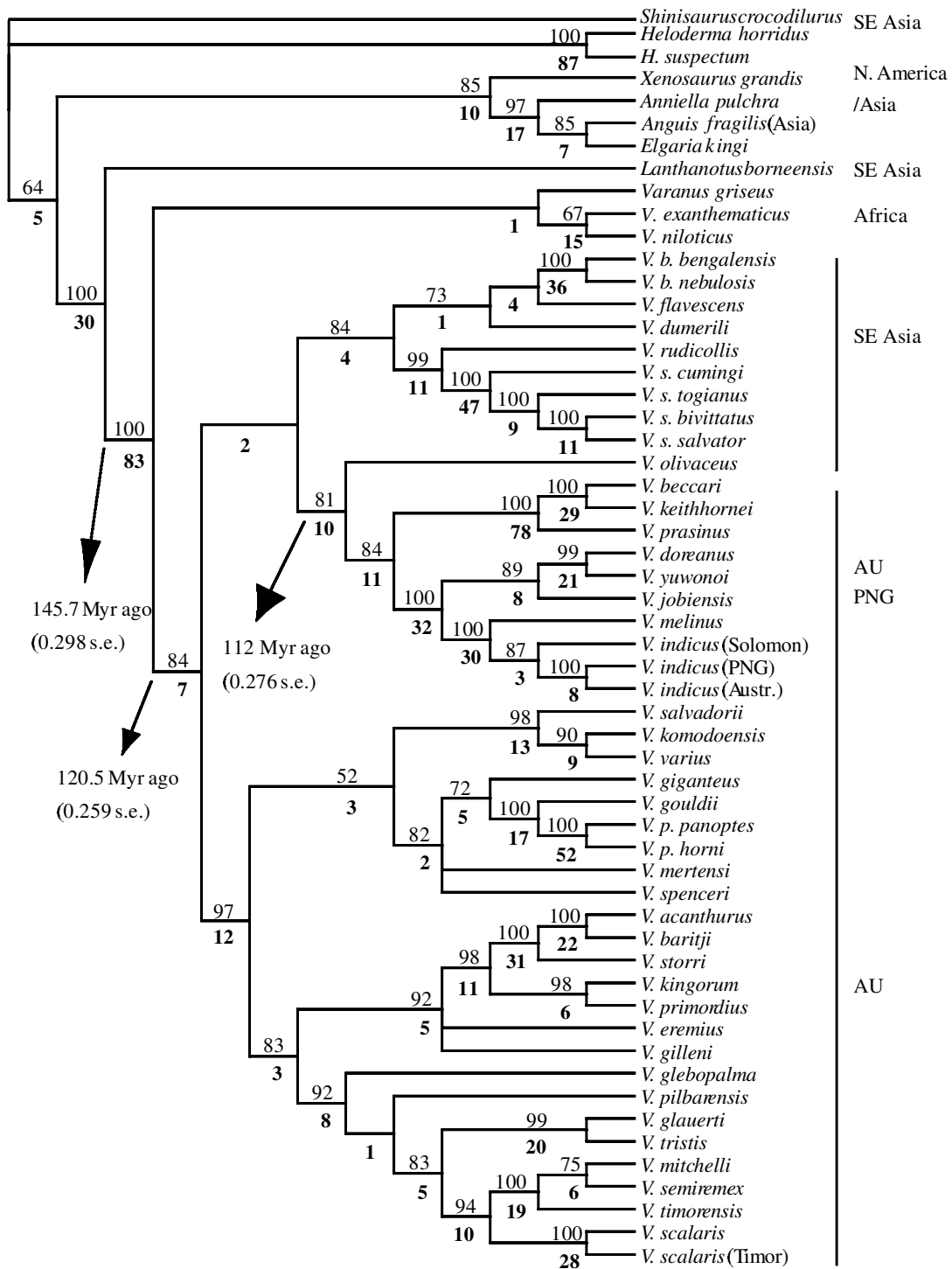
Figure 3. Phylogenetic relationships among varanoid lizards. Strict consensus of two overall most parsimonious trees is presented based on analysis of 2042 aligned base positions (1149 parsimony informative) of mitochondrial DNA sequence data (length = 9253 steps). This tree is identical in topology to the ML topology. Bootstrap values are indicated above the branches and decay indices are denoted in bold below the branches. Abbreviations are as in figure 2.

Philippine taxon, *V. olivaceus*, is the sister taxon to a group of species predominantly from Papua New Guinea and islands east of Wallace's Line. Finally, the fourth major clade contains species found to the east of Wallace's Line distributed throughout Australia and Papua New Guinea.

Divergence analyses indicate that a split occurred between taxa on either side of Wallace's Line 150 Myr ago, with a standard error (s.e.) of 0.452 Myr, for amphibolurine agamids (figure 2). Among varanid lizards, the first major break between the African clade and all remaining species is dated at 145.7 Myr ago (s.e. = 0.298) (figure 3). The second major split, between taxa east of Wallace's Line and a clade with taxa west of Wallace's Line as the basal groups, is dated at 120.5 Myr ago (s.e. = 0.259). Finally, the split between the Philippine taxon *V. olivaceus* and the remaining Australian–New Guinean species is estimated to have occurred 112 Myr ago (s.e. = 0.276).

4. DISCUSSION

Estimated divergence dates presented here are compatible with the date of separation of the Indian subcontinent from western Australia, eastern Africa and Madagascar beginning between 160 and 128 Myr ago. Based on current knowledge of the Jurassic tectonic history of this region, we cannot reject an alternative scenario that South East Asian *Varanus* species and *P. cocincinus* rafted with a smaller Gondwanan fragment that separated from the northern margin of Australia or the Indian subcontinent (Metcalfe 2001). None the less, we can reject the hypothesis of Late Tertiary (less than 25 Myr ago) dispersal events introducing agamid and varanid lizards into the Australia–New Guinea region.

Numerous other animal groups with low dispersal ability show a pattern similar to the one presented here for agamid and varanid lizards. Cicadas exhibit considerable molecular evolutionary divergence between major clades on either side of Wallace's Line (DeBoer & Duffels 1996). Elapid snakes also show significant subdivision of groups from South East Asia and the Australia–New Guinea region (Keogh 1998). Finally, a recent phylogenetic hypothesis for passerine birds suggests a major phylogenetic break between groups found predominantly west of Wallace's Line and those found east of Wallace's Line (Barker *et al.* 2002). Although passerine birds may be considered better dispersers than most insects and squamate reptiles, the imprint of an ancient divergence is reflected in the phylogeny of the group.

A Middle to Late Mesozoic split between lizard taxa on either side of Wallace's Line is consistent with results of recent molecular phylogenetic studies indicating that radiations of modern mammal and bird orders are older than traditionally believed. The timing of mammalian and avian ordinal diversification has been much debated in recent literature (Benton 1999; Easteal 1999). Molecular dating techniques have estimated that modern orders appeared in the Early Cretaceous (100 Myr ago) or earlier, in contrast with the traditional view that these orders radiated around the K–T boundary 65 Myr ago (Hedges *et al.* 1996). This same trend appears for lizard families. Until recently, representatives of modern lizard families were well represented in the Cenozoic fossil record but poorly known in Mesozoic strata. Our results support the hypothesis that modern lizard families had diversified at least by the Late Jurassic (Macey *et al.* 1997*b*; Evans *et al.* 2002) as indicated by numerous recently discovered Mesozoic fossils (see Evans *et al.* 2002).

A recent hypothesis concerning the faunal break across Wallace's Line invokes three major geological events responsible for this division: (i) South East Asian blocks split from the northern margin of Australia hundreds of millions of years ago; (ii) accretion of these blocks to Eurasia occurred 120 and 65 Myr ago; and (iii) accretion of the Indian plate with Asia occurred *ca.* 50 Myr ago (Windley 1988). Our analyses, combined with evidence from extinct terrestrial vertebrate groups (Buffetaut 1987; Evans *et al.* 2002), support the hypothesis that these geological events introduced Gondwanan members into Asia as proposed by Macey *et al.* (2000). Dinosaur fossils of Gondwanan affinities have been found on the Indian subcontinent in Late Jurassic to Early Cretaceous sediments (Buffetaut

1987). The Indian subcontinent approached Eurasia during the Cretaceous, and there was an increase in Laurasian taxa on the Indian subcontinent early in the Tertiary (Buffetaut 1987). Evolution of faunal elements as a result of separation and convergence of tectonic plates has played and continues to play a dynamic role in shaping global communities throughout the Earth's history.

Although Gondwanan fragmentation and associated vicariance are not the exclusive explanations for the current distribution of iguanian lizards in other geographical regions (Raxworthy *et al.* 2002; Townsend & Larson 2002), they provide the primary explanation for the South East Asian phenomenon of Wallace's Line. Our dating of the faunal break known as Wallace's Line to Gondwanan vicariance occurring between 112 and 150 Myr ago may be tested further by applying the methods used here to additional taxa illustrating this faunal division.

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