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A phylogenetic analysis of variation in reproductive mode within an Australian lizard (*Saiphos equalis*, Scincidae)

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Saiphos equalis, a semi-fossorial scincid lizard from south-eastern Australia, is one of only three reptile species world-wide that are known to display geographic variation in reproductive mode. Uniquely, *Saiphos equalis* includes populations with three reproductive modes: oviparous with long (15-day) incubation periods; oviparous with short (5-day) incubation periods; and viviparous (0-day incubation periods). No *Saiphos* populations show 'normal' scincid oviparity (>30-day incubation period). We used mitochondrial nucleotide sequences (*ND2* and *cytochrome b*) to reconstruct relationships among populations from throughout the species' distribution in New South Wales, Australia. Under the phylogenetic species concept, phylogenetic analyses are consistent with the oviparous and viviparous populations of *S. equalis* being conspecific. Phylogenetic analyses suggest that the long incubation period oviparous lineage is the sister group to all other populations; and that the viviparous populations belong to a cluster of weakly supported clades basal to the short-incubation-period oviparous clade. These clades correspond to variation in reproductive mode and geographic location.

ADDITIONAL KEY WORDS: viviparity – Scincidae – reproductive mode – phylogeny – reptiles – Australian lizards.

INTRODUCTION

The evolutionary transition from oviparity (egg-laying) to viviparity (live-bearing) has occurred frequently within squamate reptiles (>100 times: Bustard, 1964; Greer, 1989; Shine, 1985). The large number of independent origins of viviparity within reptiles makes this group an ideal model system in which to investigate the evolution of reproductive modes. Additionally, some transitions in reproductive mode within reptiles have occurred quite recently, resulting in the occurrence of reproductively bimodal genera and, in a very few cases, reproductively bimodal species. Although conspecific oviparous and viviparous individuals have been reported in several taxa, detailed investigation has shown that some of these records reflect taxonomic confusion (Shine, 1985; Tinkle & Gibbons, 1977). Nonetheless, three cases of reproductively bimodal species have been substantiated by more recent studies. Both oviparous and viviparous populations are known to occur within the lacertid lizard *Lacerta vivipara* (Arrayago, Bea & Heulin, 1996), and the scincid lizards *Lerista bougainvillii* (Qualls *et al.*, 1995) and *Saiphos equalis* (Smith & Shine, 1997). These reproductively bimodal species are especially significant as they allow comparison between viviparous and oviparous organisms that are similar to each other in virtually all respects except their mode of reproduction, thereby providing an opportunity for robust testing of evolutionary theories. Although phylogenetic effects are minimized by conspecific comparisons, a sound understanding of the taxonomic status and phylogenetic relationships of populations remains essential (Harvey & Pagel, 1991).

Two of the three reproductively bimodal reptile taxa

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(*Le. bougainvillii* and *La. vivipara*) have been the focus of several phylogenetic studies. For example, allozyme and mitochondrial DNA (mtDNA) studies of *Le. bougainvillii* have established the conspecific status of all populations, and have suggested that there may be two separate viviparous lineages within this species (Qualls *et al.*, 1995; Fairbairn *et al.*, 1998). Allozyme and hybridization studies of *La. vivipara* suggest that oviparous and viviparous populations are conspecific (Arrayago *et al.*, 1996), and mtDNA studies have generated biogeographical hypotheses about the divergence between these two forms (Heulin *et al.*, 1999). Both *La. vivipara* and *Le. bougainvillii* have also been used as model systems to test theoretical models on the evolution of squamate viviparity (concerning for example developmental timing (Heulin, Osenegg & Lebouvier, 1991; Fairbairn *et al.*, 1998); eggshell morphology (Qualls, 1996); costs of reproduction (Qualls & Shine, 1998a); and comparative geographic distribution (Qualls & Shine, 1998b)).

In contrast, the third reproductively bimodal species (*S. equalis*) has attracted less scientific attention. Although variability of reproductive modes within *S. equalis* was reported by Shine (1985) and Greer (1989), the detailed reproductive biology of *Saiphos* was not described until recently (Smith & Shine, 1997). Detailed studies of the reproductive biology of this species have revealed two distinct types of 'oviparity', with parturition occurring at different stages of embryonic development. Both of these developmental stages are intermediate between those characteristic of 'normal' oviparous and viviparous reptiles (Smith & Shine, 1997, and in prep.; Blackburn, 1995).

Saiphos equalis is a medium-sized (to ~120 mm snout–vent length, ~210 mm total length) semi-fossorial scincid lizard distributed through coastal south-eastern Australia (Cogger, 1992). Reproductive mode in this species varies geographically, although in contrast to *Lacerta* and *Lerista*, the distribution of reproductive mode forms has not been fully documented. Lizards from high elevation sites (>1000 m altitude) in north-eastern NSW are viviparous: females from these populations give birth to fully-formed offspring in transparent membranes (Smith & Shine, 1997). In contrast, low-elevation populations from both northern and southern NSW display a mode of reproduction that is intermediate between viviparity and 'normal' oviparity (Bustard, 1964; Smith & Shine, 1997). In these oviparous populations, females lay partly-shelled eggs that contain well-developed embryos. However, embryogenesis is not complete at oviposition; instead, the embryo continues to develop for some time prior to hatching. The duration of this post-laying (incubation) period differs significantly among populations. Lizards from coastal northern NSW have relatively long incubation periods of approximately 15 days (Smith & Shine, unpub. data), whereas south-coastal lizards have much briefer incubation of approximately 5 days (Smith & Shine, 1997). These differences in incubation period also correspond to discrete differences in embryonic development at parturition and eggshell thickness between populations, and represent distinct states in the oviparity–viviparity continuum. We refer to both of these reproductive types as 'oviparous', because they retain a (partly-) shelled egg within the life cycle (Blackburn, 1993). Nonetheless, we note that even the 'long-incubation-period' reproductive mode of *Saiphos* involves far briefer incubation than is the case for any other 'oviparous' scincid lizards so far studied in this respect (>30 days at equivalent temperature: Greer, 1989).

A previous study based on limited morphological data suggested that oviparous and viviparous populations of *S. equalis* are conspecific, but resolution of the intraspecific phylogeny was poor (Smith, 1996). A detailed phylogenetic hypothesis for this species may clarify the evolution of reproductive mode in a reptile species that displays a previously unrecorded range of stages of embryonic development at parturition. In addition, further studies based on this phylogeny will allow independent tests of the conclusions drawn from studies of *Le. bougainvillii* and *La. vivipara*. In this paper we present a phylogenetic analysis of *S. equalis* populations based on mitochondrial DNA sequences. We use this phylogenetic hypothesis to provide a more robust test of the species status of populations with different reproductive modes, and to reconstruct the direction and number of evolutionary transitions of reproductive mode within the species.

MATERIALS AND METHODS

SPECIMENS AND TISSUES

Tissue samples were available from 25 individuals from 13 localities (Fig. 1, Appendix) spanning the distribution of *S. equalis* in NSW. Although the species' distribution extends further north, material from this area was not available. Muscle and liver tissue samples were dissected from freshly sacrificed specimens, and stored at −80°C. The reproductive mode of most populations was inferred from direct observation, published reports (Bustard, 1964; Greer, 1989), unpublished data (A. Greer, unpub. field notes), or the condition of late-stage gravid females in the collection of the Australian Museum. Late embryonic stages were used to avoid errors in inferring reproductive mode from preserved specimens (Blackburn, 1993). Two populations with unverified reproductive mode were also included, as follows: (1) Comerong Island is the southernmost known population, and was classed as oviparous because all geographically close populations exhibit this reproductive mode (Smith & Shine, 1997); and (2) a

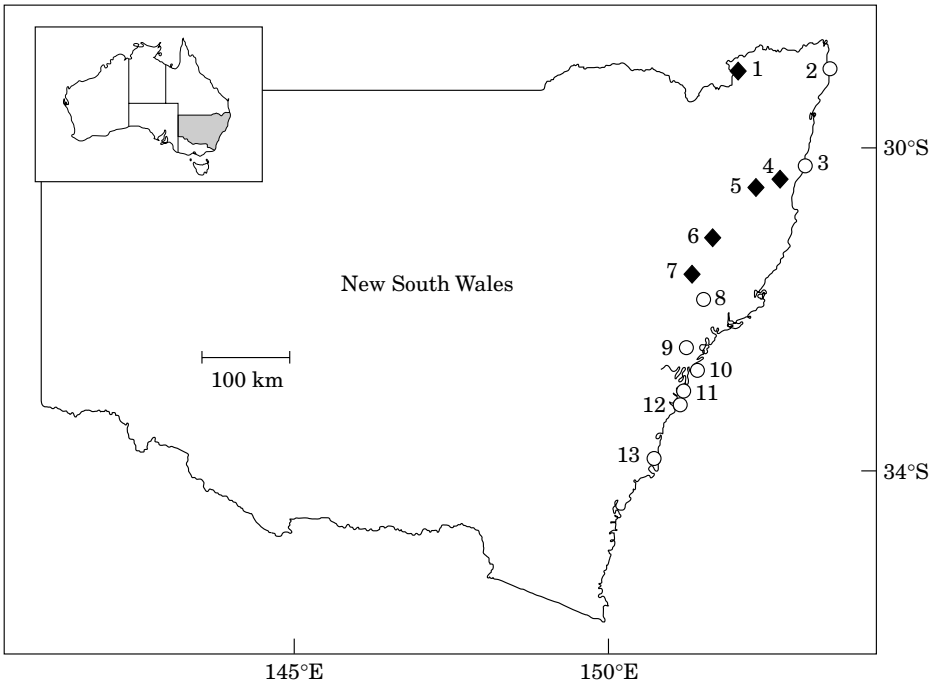

Figure 1. Map of New South Wales, Australia showing the locations of *Saiphos equalis* populations included in this study. (○) Oviparous and (◆) viviparous populations. Population numbers correspond to those in the Appendix and Figure 2.

single individual from Amosfield, in the collection of the Australian Museum, contains an embryo which is completely pigmented and has a small amount of unincorporated yolk, as would be expected of a viviparous individual. However, the embryo is surrounded by a thick opaque membrane, as occurs in oviparous taxa (Smith & Shine, 1997). Scanning electron microscopy of the thickened membrane around the embryo revealed no calcium crystal layer. As this layer occurs on all oviparous *Saiphos* eggshells that we have examined, we have classed the Amosfield animal as viviparous.

Saiphos is currently recognized as a monotypic genus belonging to the *Sphenomorphus* group of lygosomine skinks. Trees were rooted using *Eugongylus albofasciolatus*, a member of the *Eugongylus* group, another lygosomine lineage (Greer, 1974; Hutchinson, 1993). Four other *Sphenomorphus* group taxa were included as closer outgroups (*Calyptotis scutirostrum*, *Gnypetoscincus queenslandiae*, *Sphenomorphus fasciatus* and *Sphenomorphus leptofasciatus*), based on their overall morphological similarity to *Saiphos*, as well as inferences about phylogenetic relationships from previous studies (e.g. Greer, 1989).

DNA ISOLATION, AMPLIFICATION, AND SEQUENCING

DNA was isolated from muscle or liver tissue following the protocols of Hillis *et al.* (1996). Tissues (~100–200 mg) were digested with proteinase K for 3 h. The polymerase chain reaction (PCR) was used to amplify a 309 base-pair region of the *cytochrome b* (*cyt b*) gene and a 400 base-pair region of the *ND2* gene. The *cyt b* primers were: L14841 and H15149 (Kocher *et al.*, 1989). The *ND2* primers are: H715 5′-CGT GTY TGT GTY TGG TTT ADK CC-3′ and either L305 5′-CAC TGA CTT CTT GCC TGA WTY GG-3′ or L390 5′-CAK ARK CCG CRA CAA AAT ACT TC-3′. The protocols of Palumbi *et al.* (1991) were used to amplify double-stranded PCR products. The specific thermal cycle used was as follows: (a) one cycle at 94°C for 3 min, 47°C for 1 min, and 72°C for 1 min; (b) 34 cycles at 94°C for 45 s, 47°C for 45 s, and 72°C for 1 min; (c) one cycle at 72°C for 6 min. PCR products were sequenced using ABI Prism dRhodamine terminator cycle sequencing kit and run on an ABI 377 automated sequencer. To test the potential of these primers to amplify nuclear paralogues rather than mitochondrial copies of the genes, products were sequenced from amplifications of total cellular DNA and dilute mitochondrial enriched DNA from a single individual following the method of Donnellan, Hutchinson & Saint (1999). Indistinguishable sequences from products from total cellular and mitochondrial DNA indicate that the primers being used are unlikely to amplify nuclear paralogues.

PHYLOGENETIC ANALYSIS

Maximum parsimony (MP) and maximum likelihood (ML) optimality criteria were used to assess phylogenetic relationships (Edwards, 1972; Felsenstein, 1981). All phylogenetic analyses were carried out using PAUP* 4.0b2a (Swofford, 1999). Modeltest v3.3 (Posada & Crandall, 1998) was used to perform likelihood ratio tests to determine an appropriate model of nucleotide substitution for ML analyses. The ILD partition homogeneity test (Farris *et al.*, 1995; Mickevich & Farris, 1981) was used to assess whether data from both genes should be combined in a single analysis. All searches were done using the heuristic search option in PAUP* with 20 random addition sequences. Initial trees were obtained by stepwise addition, followed by branch swapping using the tree bisection-reconnection (TBR) method. The bootstrap, with 100 pseudoreplicates (with model parameters fixed at values estimated from the best tree) for ML and 500 pseudoreplicates for parsimony, was used to assess confidence for particular nodes (Felsenstein, 1985; Hillis & Bull, 1993).

EVOLUTION OF REPRODUCTIVE MODE

The ability of our data to reject alternative (more conservative) phylogenetic hypotheses was assessed using Templeton's (1983) implementation of the Wilcoxon signed ranks test and the Kishino–Hasegawa test (Kishino & Hasegawa, 1989). The hypotheses tested were that: (1) all oviparous populations form a single monophyletic group, (2) all viviparous populations form a single monophyletic group, and (3) oviparous and viviparous populations belong to two reciprocally monophyletic clades. Reconstruction of the evolution of reproductive mode onto the best tree, and the most conservative tree that could not be rejected statistically, was carried out using MacClade v 3.08 (Maddison & Maddison, 1992).

RESULTS

Sequences from mitochondrial and total cellular DNA of each of the two individuals tested were identical for both *cyt b* and *ND2*, indicating that primers amplified mitochondrial DNA only. All sequences used in this study are available from GenBank (accession numbers AF373232–AF373279).

In order to sample within- and between-population variation in haplotype diversity, two and in some cases more individuals from each population were sequenced for *cyt b* (Stewart's Brook and Dorrigo were represented by a single individual each). Three hundred and one aligned sites, including 61 parsimony informative sites from *cyt b* were included, uncorrected divergence between haplotypes ranged from 0.33% to 11.96%. In four cases both individuals from a population had identical sequences; in addition, one individual from Mt. Wilson had a sequence identical to those from Forsyth Park. A ML search using the HKY model (Hasegawa, Kishino & Yano, 1985) with an estimated gamma-shaped parameter (Γ) and proportion of invariant sites (i) (parameters estimated from ML analysis are: base frequencies A = 0.2700, C = 0.3561, G = 0.1451, T = 0.2287; transition:transversion ratio = 3.23406; $\Gamma = 1.23605$; i = 0.512180) resulted in a single tree (not shown). Overall haplotypes strongly cluster within locations, or pairs of close locations such as Kurnell and Comerong Island. The results of the *cyt b* analysis were used to select representative of haplotypes to be sequenced for *ND2* to attempt to improve resolution among lineages.

Six hundred and seventy unambiguously aligned sites for 18 haplotypes were used in the phylogenetic analysis (369 sites *ND2*). Of these, 200 sites were parsimony-informative and there were no insertions or deletions. An open reading frame for both genes was observed by translating DNA sequence into protein sequences using Se-Al version 1.0a1 (Rambaut, 1995). The partition homogeneity test was not significant ($P = 0.95$), indicating that datasets from the two genes should be combined. Uncorrected '*P*' distances between mitochondrial haplotypes ranged between 0 and 9%, with Forsyth Park and Mt. Wilson having identical sequences (Table 1). Hierarchical likelihood ratio tests suggest that the general time reversible model with equal transition rates and four transversion rates (Rodríguez *et al.*, 1990) and estimated gamma-shape parameter and proportion of invariable sites, is an appropriate model of nucleotide substitution for these data. Parameters estimated from ML analysis are: base frequencies A = 0.3250, C = 0.3552, G = 0.1138, T = 0.2059; substitution rates A⇔C = 0.7738, A⇔T = 0.9837, C⇔G = 0.8139, A⇔G & G⇔T = 6.7459; proportion of invariable sites = 0.4956; and gamma-shape parameter = 1.4543.

Maximum parsimony MP and maximum likelihood ML methods each produced a single fully resolved best tree with identical topologies, although the level of bootstrap support differs between the methods (Fig. 2, Table 2). Both reconstruction methods agree with the generic level branching pattern (*Gnypetoscincus*, (*Sphenomorphus*, (*Calyptotis*, *Saiphos*))). Both ML and MP analyses suggest that the southern (short incubation) and northern ('long' incubation) oviparous haplotypes belong to monophyletic clades and that the viviparous haplotypes form a weakly supported series of sister clades to the southern oviparous clade. The clades that are strongly supported by all analyses are: the northern oviparous (ML and MP bootstrap support 89% and 94% respectively), southern oviparous (98% and 100%), and Riamukka + Stewart's Brook (97%

Table 1. Uncorrected '*P*' distances between populations of *Saiphos equalis*

	1	2	3	4	5	6	7	8	9	10	11	12	13
Amosfield (1)	–												
Byron Bay (2)	0.09	–											
Emerald Beach (3)	0.09	0.07	–										
Dorrigo (4)	0.08	0.10	0.10	–									
Styx River (5)	0.07	0.11	0.09	0.08	–								
Riamukka (6)	0.08	0.10	0.08	0.07	0.06	–							
Stewart's Brook (7)	0.08	0.10	0.09	0.08	0.08	0.03	–						
Barrington (8)	0.07	0.11	0.09	0.08	0.07	0.07	0.08	–					
Olney (9)	0.08	0.10	0.09	0.07	0.07	0.06	0.08	0.02	–				
Mt. Wilson (10)	0.08	0.11	0.09	0.08	0.07	0.07	0.08	0.02	0.02	–			
Forsyth Park (11)	0.08	0.11	0.09	0.08	0.07	0.07	0.08	0.02	0.02	0.00	–		
Kurnell (12)	0.09	0.11	0.10	0.09	0.07	0.07	0.09	0.03	0.02	0.03	0.03	–	
Coomerong Island (13)	0.08	0.11	0.10	0.09	0.07	0.07	0.09	0.03	0.02	0.03	0.03	0.01	–

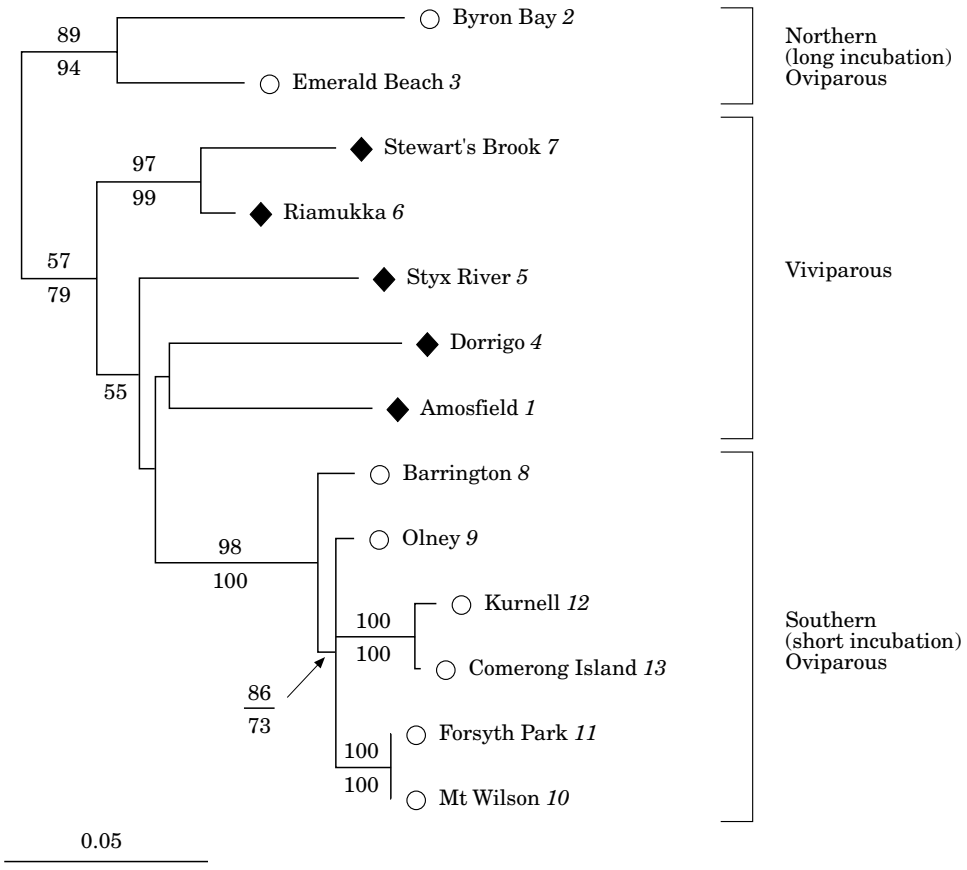

Figure 2. Best tree topology from ML and MP analysis. Branch lengths are from the ML analysis. Figures above branches are bootstrap support from ML analysis, those below the branches are support from MP. Numbers in italics following species correspond to locations in Figure 1 and the Appendix.

Table 2. Results of Templeton (MP) and Kishino–Hasegawa (ML) tests of monophyly of oviparous and viviparous populations

Hypothesis	Alt. tree length	Templeton			Alt. tree likelihood	Kishino–Hasegawa	
		T_s	z	*P*		T	*P*
Oviparous populations monophyletic	645	111	−1.2140	0.2247	3689	1.0534	0.2925
Viviparous populations monophyletic	642	16.5	−1.2649	0.2059	3687	0.9526	0.3411
Reciprocal monophyly	648	38	−2.3570	**0.0184**	3691	1.3108	0.1904

and 99%) clades. The sister relationship between the northern oviparous haplotypes and the remaining haplotypes is moderately supported by the ML and MP analysis (bootstrap proportions of 57% and 79% respectively).

EVOLUTION OF REPRODUCTIVE MODE

There are three hypotheses about reproductive mode transitions within *S. equalis* we wish to test statistically. First, we can test whether northern and southern oviparous populations belong to a single monophyletic group. Second, we can test whether or not the viviparous populations form a monophyletic group, and third, we can test whether both of these constraints can occur simultaneously, i.e. oviparous and viviparous clades are reciprocally monophyletic. Table 2 shows the results of Templeton and Kishino–Hasegawa tests. Neither of the first two hypotheses is significantly worse than the best unconstrained tree. Therefore, we cannot reject monophyly of either the viviparous populations or the northern + southern oviparous populations. The test of reciprocal monophyly, however, was rejected under parsimony but not likelihood.

DISCUSSION

The relationships among mitochondrial haplotypes of *S. equalis* inferred by maximum parsimony and likelihood analysis are largely concordant with geographic distribution and reproductive mode of populations. Our results do not support the existence of more than a single species of *Saiphos*. However, the relationships among two oviparous lineages and the viviparous populations remain unclear.

If reproductive mode variation in *Saiphos* was between unrecognized species rather than being intraspecific, we would expect to see strongly divergent clades concordant among a number of independent data partitions. According to our analysis of the mitochondrial gene tree of *S. equalis* the only possible species boundary among these populations is between the northern oviparous haplotype lineage and the remaining haplotype lineages. However, the moderate bootstrap support for the position of this clade (MP 57%, ML 79%), and the non-significant hypothesis test results for combining this clade with all other oviparous populations does not support the notion of this clade as a distinct species. Several morphological characteristics (relatively small adult body size and proportionally longer first and second toes on the front feet: Smith, 1996) support the distinctness of the northern populations. However, a phylogenetic analysis incorporating this information resulted in a poorly supported phylogeny, in which the position of the northern oviparous clade is not congruent with its position in the mt gene tree (Smith, 1996). Therefore, despite the occurrence of reproductive mode variation, the null hypothesis that the sampled populations of *S. equalis* belong to a single species cannot be rejected. However, further information regarding the affinities of populations north of those we have sampled is required before the status of *S. equalis* throughout their geographic distribution can be resolved. More northern populations are particularly important in light of major phylogeographic breaks in similarly distributed frog species north of the sampled range of *S. equalis* (James & Mortiz, 2000; McGuigan *et al.*, 1998).

Our data suggest that the two forms of oviparity occurring in *S. equalis* represent two distinct lineages. The relationships between these lineages, and the evolutionary history of reproductive mode in *Saiphos* is difficult to reconstruct beyond this. On the best tree, reproductive mode can be reconstructed in several ways. The most parsimonious reconstruction is a single origin of viviparity in a long-incubation-period oviparous lineage followed by a reversal from viviparity to oviparity (Fig. 3A). Transitions from oviparity to viviparity in squamates have traditionally been viewed as irreversible (e.g. Neill, 1964; Blackburn, 1992), and recent investigations have largely supported this view (De Fraipont, Clobert & Barbault, 1996; Lee & Shine, 1998). In *Saiphos*, however, the reverse transition

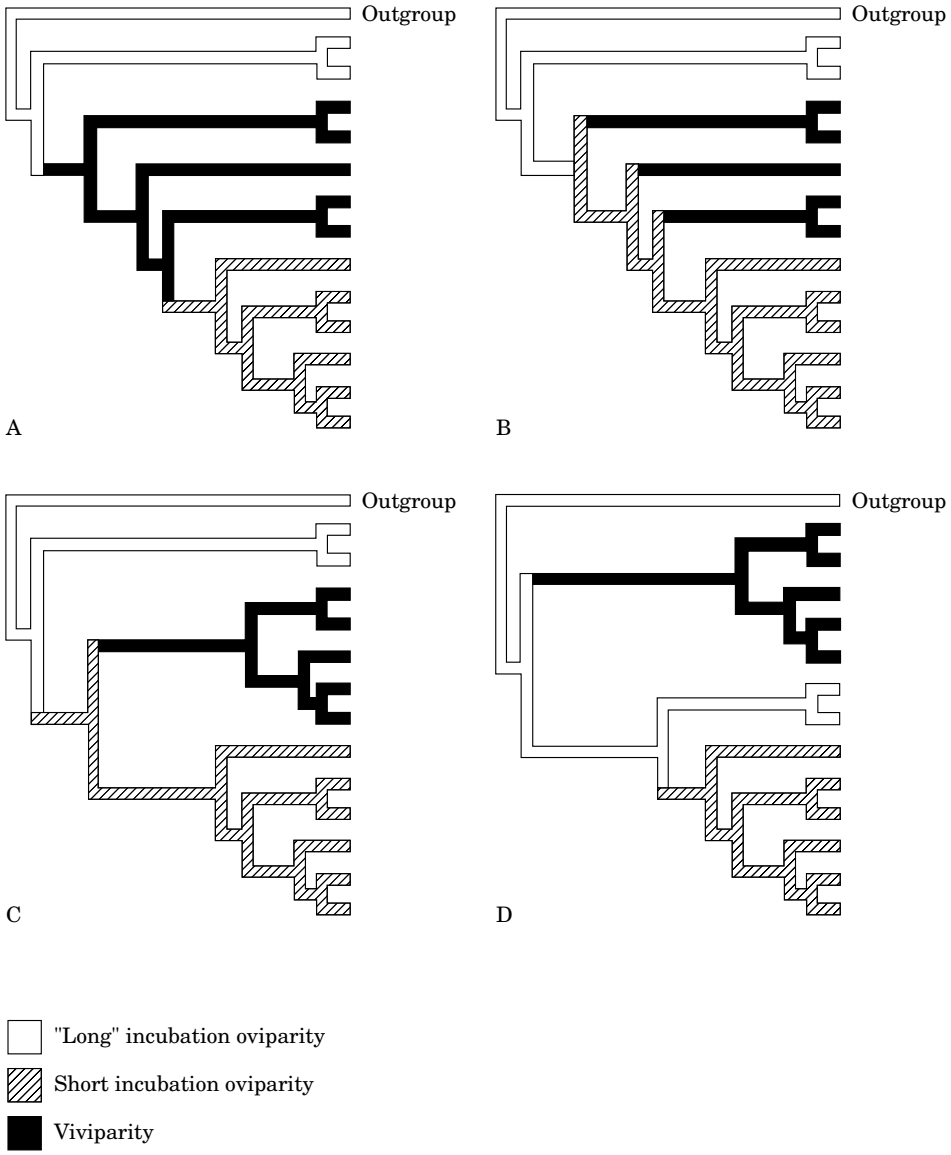

Figure 3. Alternative reconstructions of the evolution of reproductive mode onto the best tree (A and B), or the best trees obtained under constraint 2 (viviparous monophyly, C), or constraints 1 and 3 (oviparous monophyly, reciprocal monophyly, D).

inferred is a relatively small step: that is, a shift in incubation period from 0 days (viviparity) to 5 days (short-incubation-period oviparity). In this case, the arguments supporting irreversibility (most importantly that physiological or genetic characteristics essential for oviparity have been irredeemably lost in viviparous populations) are relatively weak. The alternative optimization of reproductive mode onto the best tree is a shift from long- to short-incubation-period oviparity early in the tree, followed by an independent transition to viviparity in each viviparous lineage (Fig. 3B). This hypothesis does not require any 'reverse' transitions, but increases the total number of reproductive mode changes inferred (from 2 to 4) and the number of homoplasious changes. In any case, the results of our hypothesis tests suggest that the most conservative interpretation of relationships that cannot be rejected is simply that each reproductive mode group is a monophyletic lineage, either arrangement of these lineages infers two reproductive mode changes (Fig. 3C,D).

Regardless of the remaining uncertainty surrounding the history of reproductive mode evolution in *S. equalis*, it is clear that this small Australian

lizard species offers an important model system to further tease apart the microevolutionary processes involved in the phylogenetic shift between oviparity and viviparity.

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APPENDIX

SPECIES, MUSEUM IDENTIFICATION NUMBER, LOCALITY AND REPRODUCTIVE MODE FOR SPECIMENS USED IN THIS STUDY

All locations for *Saiphos equalis* are in New South Wales, Australia; *S. equalis* population numbers (in parentheses) correspond to locations in Figure 1. Institution codes are: NR – Australian Museum, Sydney, Australia; SAMA – South Australian Museum, Adelaide, Australia; and CCA specimens are currently being catalogued into the Texas Memorial Museum. See text for discussion of reproductive mode in Amosfield and Comerong Island.

Eugongylus albofasciolatus NR 480, Papua New Guinea, Oviparous; *Gnypetoscincus queenslandiae* NR 2134, Australia, Viviparous; *Sphenomorphus fasciatus* CCA 1254, Philippines, Oviparous; *Sphenomorphus leptofasciatus* AMS R124195, Papua New Guinea, Oviparous; *Calyptotis scutirostrum* NR 246, Australia, Oviparous; *Saiphos equalis*: (1) NR 3926, NR 3927 Amosfield, Viviparous; (2) NR 4982, NR 3171, NR 3172 Byron Bay, Oviparous; (3) NR 5027, NR 5029 Emerald Beach, Oviparous; (4) NR 695, Dorrigo, Viviparous; (5) NR 714, NR 715 Styx River, Viviparous; (6) NR 3991, NR 3992 Riamukka State Forest, Viviparous; (7) NR 3147, Stewart's Brook State Forest, Viviparous; (8) NR 6095, NR 6097 Barrington House, Oviparous; (9) NR 3972, NR 3973 Olney State Forest, Oviparous; (10) NR 4868, NR 4867 Mt. Wilson, Oviparous; (11) NR 3128, NR 3129 Forsyth Park, Sydney, Oviparous; (12) NR 3332, NR 3333 Kurnell, Sydney, Oviparous; (13) NR 4366, NR 4367 Coomerong Island, Oviparous.