



Inclusion of nuclear intron sequence data helps to identify the Asian sister group of New World pitvipers

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ABSTRACT

Despite much effort towards resolving the molecular phylogenetic tree for pitvipers, some aspects remain unresolved. In particular, the sister group of the diverse New World radiation has remained impossible to identify with any certainty. In this study, which for the first time includes nuclear intron data from all major groups of Asian pitvipers as well as representatives of the New World radiation, Bayesian inference allows *Gloydus* to be identified as the most likely sister group to the New World radiation and sheds light on other ambiguous relationships among the Old World pitvipers. The sister group relationship of “*Ovophis*” *okinavensis* and “*Trimeresurus*” *gracilis* is confirmed by the addition of nuclear genes, and we hypothesise that they form a sister group to the *Gloydus* + New World clade, best supported when the phylogenetic signal from gaps is included in the form of a simple-coded matrix.

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1. Introduction

Pitvipers are found through most of Asia and the New World, with one species (*Gloydus halys*) extending its range into south eastern Europe. While it has long been accepted that pitvipers evolved in the Old World and invaded the New World across the Bering Land Bridge, other aspects of the relationship between Old and New World pitvipers have been more contentious. An early division of Asian pitvipers into the *Agkistrodon* and *Trimeresurus* groups, based largely on osteology, led to the view that each of these groups had invaded the New World independently (Brattstrom, 1964; Gloyd and Conant, 1990). The *Agkistrodon* group consisted of pitvipers possessing enlarged scutes on the crown of the head, and included the genus *Agkistrodon* (formerly thought to be distributed in both the Old and New World) as well as the genera *Calloselasma*, *Deinagkistrodon*, and *Hypnale* in the Old World (Gloyd and Conant, 1990). The idea of two or more separate invasions of the New World was finally laid to rest by molecular studies, which unambiguously showed that the New World radiation was monophyletic (Kraus et al., 1996; Parkinson et al., 1997, 2002; Parkinson, 1999; Malhotra and Thorpe, 2004; Castoe and Parkinson, 2006). This work also led to a general acceptance of the genus *Gloydus*

(Hoge and Romano-Hoge, 1981) for the Old World species formerly placed in *Agkistrodon*.

Despite much subsequent effort towards resolving an accurate molecular phylogenetic tree for all pitvipers, which has involved increasing taxon sampling and number of characters included, as well as use of increasingly more sophisticated models of sequence evolution and methods of analysis (Malhotra and Thorpe, 2004; Castoe and Parkinson, 2006), some parts of the tree have resisted resolution. This includes an identification of the Asian sister group to the New World radiation. The studies above, all of which have been based on mitochondrial genes, have at best restricted the likely sister group to among the following taxa: *Protobothrops* (sensu Guo et al., 2007), *Gloydus*, *Ovophis* sensu stricto (excluding *Ovophis okinavensis*), and the enigmatic clade consisting of the sister species “*T*”. *gracilis* and “*O*”. *okinavensis* (both formerly placed in different genera and whose generic placement is currently unclear).

Castoe and Parkinson (2006) stated that “sequences of nuclear genes may hold valuable synapomorphies required to solidify estimates of relationships at deeper nodes that are not (yet) confidently resolved”. So far, nuclear intron data has been used effectively to increase resolution in part of the pitviper tree (Creer et al., 2003, 2006). For example, Creer et al. (2006) used the seventh intron from the β fibrinogen gene (7 β FIB) and the third intron from the TATA box-binding protein (3ITBP) to provide support for the inclusion of the subclade consisting of *Trimeresurus macrops*

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and *T. venustus* within the “*albolabris*” group. This inclusive group is also supported by a morphological synapomorphy, and has now been described as a distinct genus, *Cryptelytrops* (Malhotra and Thorpe, 2004). The present study builds on Creer et al. (2006) by adding data from an additional nuclear intron and from a larger number of taxa, including representatives of the majority of Asian pitviper genera as well as of the New World radiation, in order to explore the additional information that the nuclear data might contribute towards resolving the phylogenetic history of the pitviper radiation in its entirety.

2. Materials and methods

2.1. DNA isolation, amplification and sequencing

Sequences used in this study are detailed in Table 1. All sequences for four North American pitvipers were downloaded from GenBank. Tissue samples of South American pitvipers were provided by Wolfgang Wüster, who also provided the corresponding unpublished mitochondrial sequences. Sequences of four regions of the mitochondrial genome encoding portions of 12S rRNA (12S), 16S rRNA (16S), Cytochrome *b* (CYTB) and NADH dehydrogenase subunit 4 (NADH4) were mostly obtained from previous studies while a few were sequenced specifically for this study following protocols outlined in Malhotra and Thorpe (2000, 2004). The correct sequences for NADH4 primers used were reported in Dawson et al. (2008). Some nuclear sequences were obtained from previous studies (Creer et al., 2005, 2006). All other nuclear sequences were generated according to the following protocol. Whole genomic DNA was extracted from muscle tissue stored in 80% ethanol using a Sigma GenElute™ mammalian Genomic DNA Miniprep Kit. Intron sequences for 71βFIB and 3ITBP were obtained as reported in Creer et al. (2006). Following identification of additional introns containing potentially valuable phylogenetic information (Creer et al., 2005), we also sequenced an additional intron for this study, i.e., the third intron of the *lamin A* gene (3ILMNA). The primers for amplification of 3ILMNA were as in Friesen et al. (1999), and PCR conditions were an initial denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 47–60 °C (1 min), extension at 72 °C (1 min), and a final extension of 72 °C (15 min). PCRs were carried out in 25 μl total volume (1× PCR buffer, 0.8 mM total dNTPs, 0.4 μM primers, 0.5–3.5 mM MgCl₂, and 0.4 U Sigma JumpStart™ Taq DNA polymerase), on an Applied Biosystems GeneAmp_PCR System 3700, including a negative control (upH₂O) to exclude the possibility of contamination. Double stranded PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Appropriately sized discrete bands were then excised and cleaned using Qiaquick columns (QIAGEN). Single stranded sequencing was performed using dye-labelled terminators (ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit), and subsequently run on an ABI Prism 377 DNA sequencer (Creer et al., 2005).

Nuclear sequences present in two copies in a single individual may differ in length yet not be distinguishable as separate bands on an agarose gel (Creer et al., 2005, 2006). PCR products that were suspected to contain length-variant heterozygotes (LVHs), as indicated by premature termination of readable traces in sequencing chromatograms, were cloned using an Invitrogen™ life technologies TOPO TA Cloning kit (incorporating PCR 2.1-TOPO and TOP10F' One Shot Chemically Competent cells) according to the manufacturer's instructions. If a heterozygote was suspected, five individual transformed One Shot colonies were sequenced using combinations of standard M13 forward and reverse primers to ensure that both alleles of the intron would be detected (Creer et al., 2005, 2006).

2.2. Sequence alignment and analysis

Mitochondrial alignments were as in Malhotra and Thorpe (2004). Nuclear introns were aligned and analysed using the recommendations of Creer et al. (2006), who explored various alignment and gap treatment strategies. Introns were aligned using Clustal W2 (Larkin et al., 2007) with a gap-opening penalty of 5 and a gap extension penalty of 1, and subsequently manually edited to remove obvious errors (e.g., identical sequences which had been aligned differently) using Jalview (Waterhouse et al., 2009). In order to include all the detected allelic size variants, all possible combinations of alleles from each specimen were included as independent terminals in the analyses (Creer et al., 2006), potentially up to 8 (2³) combinations if LVHs are present in all three introns. We treated gaps in a number of ways (see Creer et al. (2006) for a more complete discussion of this issue). First, gaps were converted into a simple-coded binary matrix using FASTGAP v1.1 (Borchsenius, 2009), hereafter identified as “gc”. We also analysed data with the gaps omitted (“nogaps”).

Phylogenetic analysis was performed using maximum parsimony and Bayesian inference approaches. Parsimony analyses were performed using a parsimony ratchet implemented in PRAP v2 (Müller, 2005), which generates a command file for execution in PAUP v4.0b10 (Swofford, 2002). Bootstrapping was performed using an efficient heuristic search strategy (Müller, 2005) with simple addition sequence, TBR branch swapping, and holding only one tree at each step.

Prior to Bayesian analysis, the sequence data were partitioned in a number of ways (see below). The optimal model of evolution for each partition was investigated using MrModeltest 2.3 (Nylander, 2004). Bayesian Markov Chain Monte Carlo (MCMC) analysis was run using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001), with *Daboia russelii* selected as the outgroup. We ran four independent iterations for 10,000,000 generations, using four chains (one cold and three heated) and only retained every 5000th iteration of the chain to reduce the autocorrelation between successive samples. Convergence and mixing of the independent runs were explored using Tracer v1.4.1 (Rambaut and Drummond, 2007) and the burn-in cut-off points determined. All post-burn samples from converged runs were combined and checked for adequate sampling of important parameters (i.e., ESS > 200), and the maximum clade credibility tree was calculated using BayesTrees v1.0 (downloaded from <http://www.evolution.reading.ac.uk/BayesTrees.html>).

Brown and Lemmon (2007) showed that errors in posterior probability estimates, resulting from assuming an incorrect partitioning strategy, were greatest when the assumed model was under-partitioned. Thus, we first partitioned by gene (for mitochondrial rRNA genes and nuclear introns), or by gene and codon position (for mitochondrial protein-coding genes), to give a total of eleven partitions (3ITBP, 71βFIB, 3ILMNA, 12S, 16S, CYTB codon position 1, CYTB pos 2, CYTB pos 3, NADH4 pos 1, NADH4 pos 2 and NADH4 pos 3). In the gap-coded matrices, the gaps formed an additional partition. These fully partitioned models are referred to, respectively, as the part11 or part12 models. However, they result in the definition of several quite small partitions, and errors are also known to be induced by over-partitioning, probably due to the fact that adding a new partition causes a large increase in the number of parameters to be estimated while decreasing the amount of sequence data available to do so (Brown and Lemmon, 2007). Hence, several alternative, less partitioned, schemes were also investigated and compared by calculating the Bayes Factor using the marginal likelihood estimator of Suchard et al. (2001) based on a harmonic mean estimation procedure (implemented in Tracer). Kass and Raftery (1995) include tables to interpret the Bayes Factor; these assume that the Bayes Factor has been estimated accurately. However, stable estimates of the harmonic mean

Table 1

List of specimens used in the study, locality information, and GenBank accession numbers for all sequences in this study. "Code" corresponds to the author's catalogue identification numbers (except WW: samples loaned by Wolfgang Wüster) or museum voucher number. Locality prefixes are N-north, S-south, E-east, W-west, and C-central. Two GenBank accession numbers for the intron partitions represent different alleles of heterozygous individuals. Species marked with an asterisk were represented by composite haplotypes; intron and mtDNA sequences are from different individuals (from the same population or region as far as possible). More detailed information may be available about the specimen locality from the individual GenBank records.

Species	Code	Origin	GenBank Accession Nos.						
			7I/ β FIB	3ITBP	3ILMNA	CYTB	NADH4	12S	16S
<i>Azemiops feae</i>	B108/B499*	N Viet Nam/ China	GQ428348	GQ428442	GQ428391	AY352747	AY352808	AY352774	AY352713
				GQ428443					
<i>Agkistrodon contortrix</i> *	–	N America	–	FJ660410	FJ660054	AF039268	AF156576	AF057229	AF057276
<i>Agkistrodon piscivorus</i> *	–	N America	FJ660000	FJ660411	FJ660055	EU483476	AF156578	AF057231	AF057278
<i>Bothrops atrox</i>	WW741	Ecuador	GQ428366	GQ428458	GQ428428	GQ428476	GQ428485	GQ428495	GQ428470
					GQ428429				
<i>Bothrops atrox</i>	WW742	Ecuador	GQ428367	GQ428459	GQ428430	GQ428477	GQ428486	GQ428496	GQ428471
<i>Bothrops atrox</i>	WW537	Suriname	–	–	GQ428426	AF246267	AF246277	GQ428494	GQ428469
					GQ428427				
<i>Calloselasma rhodostoma</i>	A54	S Thailand	GQ428344	GQ428438	DQ131673	AF171918	AY352813	AY352779	AY352718
<i>Cryptelytrops albolabris</i>	B6	W Java	AF517209	DQ117521	GQ428422	AF517186	AF517213	AF517158	AF517171
<i>Cryptelytrops albolabris</i>	B22	C Thailand	AF517205	DQ117520	GQ428404	AF517189	AF517221	AF517165	AF517178
<i>Cryptelytrops albolabris</i>	A165	NE Thailand	AF517196	DQ117522	GQ428371	AF517185	AF517214	AF517169	AF517182
<i>Cryptelytrops albolabris</i>	A229	N Thailand	DQ116985	DQ117507	GQ428380	AY059566	AY059583	AY059544	AY059560
					GQ428381				
<i>Cryptelytrops albolabris</i>	B20	S Thailand	DQ116965	DQ117487	GQ428401	GQ428474	GQ428481	GQ428491	GQ428465
			DQ116966	DQ117488					
<i>Cryptelytrops albolabris</i>	B32/B47*	W Thailand	GQ428359	–	–	AF517187	AF517216	AF517160	AF517173
<i>Cryptelytrops albolabris</i>	B47	W Thailand	DQ131656	GQ428453	DQ131666	AF517187	AF517216	AF517160	AF517173
<i>Cryptelytrops albolabris</i>	B48/B47*	W Thailand	GQ428363	–	–	AF517187	AF517216	AF517160	AF517173
<i>Cryptelytrops albolabris</i>	B410	SE Myanmar	GQ428361	GQ428451	–	–	GQ428483	–	GQ428467
<i>Cryptelytrops albolabris</i>	A157	Hong Kong	GQ428335	GQ428432	–	AF171884	AY352839	AY352805	AY352744
<i>Cryptelytrops albolabris</i>	B191	N Viet Nam	GQ428352	–	GQ428397	GQ428473	GQ428480	GQ428490	GQ428464
<i>Cryptelytrops albolabris</i>	B183	C Viet Nam	–	GQ428444	–	AY352770	AY352838	AY352804	AY352743
<i>Cryptelytrops albolabris</i>	B117	S Viet Nam	AF517202	DQ117519	GQ428393	AF517190	AF517222	AF517166	AF517179
<i>Cryptelytrops erythrurus</i>	B220	Bangladesh	DQ116984	DQ117506	–	AY352768	AY352834	AY352800	AY352739
			DQ116986	DQ117508					
<i>Cryptelytrops fasciatus</i>	B212	Tanadjampea Isl., Indonesia	DQ116993	DQ117515	GQ428403	GQ428475	GQ428482	GQ428492	GQ428466
<i>Cryptelytrops insularis</i>	A96	Indonesia	GQ428347	–	–	AF171882	AF517215	AF517159	–
<i>Cryptelytrops insularis</i>	B7	Indonesia	DQ116978	DQ117500	GQ428424	AY059568	AY059586	AY059534	AY059550
									AF517172
<i>Cryptelytrops macrops</i>	B27	C Thailand	AF517206	DQ117523	GQ428407	AF517184	AF517219	AF517163	AF517176
<i>Cryptelytrops macrops</i>	B72	S Viet Nam	DQ116987	DQ117509	GQ428423	–	GQ428484	GQ428493	GQ428468
			DQ116988	DQ117510					
<i>Cryptelytrops purpureomaculatus</i>	A83	S Thailand	DQ116967	DQ117489	GQ428386	AF517188	AF517218	AF517162	AF517175
			DQ116968	DQ117490	GQ428387				
<i>Cryptelytrops septentrionalis</i>	A99/B487*	Nepal	–	–	GQ428390	AY352755	AY352818	AY352784	AY352724
<i>Cryptelytrops septentrionalis</i>	A100	Nepal	AF517194	DQ117517	GQ428369	AF171909	AY059592	AY059543	AY059559
<i>Cryptelytrops venustus</i>	A241	S Thailand	DQ116994	DQ117516	GQ428382	AF171914	AY293930	AY293931	AY35272
<i>Cryptelytrops spp.</i>	A209	S Myanmar	AF517210	DQ117518	GQ428374	AF171900	AF517217	AF517161	AF517174
<i>Daboia russelii</i>	A4	C Thailand	–	–	GQ428383	AY165090	AY165065	AY352773	AY352712
<i>Deinagkistrodon acutus</i>	A223	Taiwan	GQ428341	GQ428436	GQ428379	AF171919	AY352811	AY352777	AY352716
<i>Garthius chaseni</i>	B306	E Malaysia	DQ131653	GQ428448	DQ131672	AY352760	AY352825	AY352791	AY352729
<i>Gloydus blomhoffi</i>	B524	Teuri Isl., Japan	GQ428364	GQ428454	GQ428420	AY352751	AY352814	AY352780	AY352719
					GQ428421				
<i>Gloydus brevicaudus</i>	B525	China	–	–	–	AY352752	AY352815	AY352781	AY352720
<i>Gloydus halys</i>		Khazakstan	–	–	–	AY223564	AY223621	AF057191	AF057238
<i>Gloydus shedaoensis</i>	ROM 20468	Liaoning, China	–	–	–	AY223566	AY223623	AF057194	AF057241
<i>Gloydus strauchi</i>	ROM 20473	China	–	–	–	AY223563	AY223620	AF057192	AF057239
<i>Gloydus ussuriensis</i>	ROM 20452	China	–	–	–	AY223565	AY223622	AF057193	AF057240
<i>Himalayophis tibetanus</i>	B253/B258*	Nepal	–	–	GQ428405	AY352749	AY352810	AY352776	AY352715
					GQ428406				
<i>Hypnale hypnale</i>	A53	S India	GQ428343	GQ428437	GQ428384	AY352750	AY352812	AY352778	AY352717
<i>Ovophis monticola</i>	A87	Taiwan	GQ428346	GQ428440	GQ428389	AF171907	AY059582	AY059545	AY059561
" <i>Ovophis</i> " <i>okinavensis</i>	B1	Okinawa, Japan	GQ428354	GQ428445	GQ428400	AF171915	AY352824	AY352790	–
<i>Parias flavomaculatus</i>	B3	Luzon Isl., Philippines	DQ116980	DQ117502	GQ428417	AF171916	AY059584	AY059535	AY059551
<i>Parias hageni</i>	A224	S Thailand	GQ428342	–	–	AF171911	–	GQ428488	GQ428462
<i>Parias hageni</i>	B33	S Thailand	DQ116981	DQ117503	GQ428410	AY059567	AY059585	AY059536	AY059552
<i>Parias hageni</i>	B390/B364*	Sumatra, Indonesia	DQ116991	DQ117513	GQ428416	AY371825	AY371863	AY371763	AY371790
			DQ116992	DQ117514					
<i>Parias malcolmi</i>	B349	Sabah, E Malaysia	DQ116982	DQ117504	GQ428413	AY37183	AY371861	AY371757	AY371786
			DQ116983	DQ117505					

Table 1 (continued)

Species	Code	Origin	GenBank Accession Nos.						
			7IβFIB	3ITBP	3ILMNA	CYTB	NADH4	12S	16S
<i>Parias schultzei</i>	B210	Palawan, Philippines	DQ116979	DQ117501	GQ428402	AY352756	AY352819	AY352785	AY352725
<i>Parias sumatranus</i>	B347/B348*	E Malaysia	–	–	GQ428412	AY371823	AY371859	AY371759	AY371788
<i>Parias sumatranus</i>	B367	Sumatra, Indonesia	–	GQ428450	GQ428415	AY371824	AY371864	AY371765	AY371791
<i>Porthidium nasutum</i>	WW751/ WW1010*	Ecuador	GQ428368	GQ428460	GQ428431	AF292574	AF292612	EU624243	EU624277
<i>Popeia nebularis</i>	B246	W Malaysia	–	GQ428446	–	AY059570	AY059589	AY059540	AY059556
<i>Popeia sabahi</i>	A202	S Thailand	GQ428339	–	–	AF171904	AY371840	AY371739	AY371770
<i>Popeia sabahi</i>	A203	S Thailand	DQ116975	DQ117497	GQ428372	AY371796	AY059588	AY059537	AY059553
					GQ428373				
<i>Popeia sabahi</i>	B339/B338*	E Malaysia	–	–	GQ428409	AY371802	AY371835	AY371735	AY371785
<i>Popeia sabahi</i>	B344	E Malaysia	DQ116976	DQ117498	GQ428411	AY371815	AY371842	AY371736	AY371771
<i>Popeia popeiorum</i>	B52	W Thailand	GQ428365	–	–	AY371800	AY371836	AY371754	AY371768
<i>Popeia popeiorum</i>	B34	W Thailand	DQ116977	DQ117499	DQ131668	AY059572	AY059591	AY059542	AY059558
			DQ116974						
<i>Popeia popeiorum</i>	B196	N Laos	GQ428353	–	GQ428398	AY059571	AY059590	AY059538	AY059554
					GQ428399				
<i>Protobothrops cornutus</i>	B350	C Viet Nam	GQ428360	GQ428449	GQ428414	AY294272	AY294262	AY294276	AY294267
<i>Protobothrops mangshanensis</i>	B300	Hunan, China	GQ428355	GQ428447	GQ428408	AY352758	AY352821	AY352787	AY352726
<i>Protobothrops mucrosquamatus</i>	A211/A223*	Taiwan	GQ428340	GQ428433	GQ428375	AF171897	AY294265	AY294279	AY294270
					GQ428376				
<i>Protobothrops mucrosquamatus</i>	B106	N Viet Nam	–	GQ428441	DQ131671	AY294275	AY294266	AY294280	AY2942721
<i>Protobothrops sieversorum</i>	B162	Laos	DQ116971	DQ117493	GQ428394	AY352753	AY352816	AY352782	AY352721
<i>Sistrurus catenatus</i> *	–	N America	FJ659992	FJ660402	FJ660048	AY223610	AY223648	AF057227	AF057274
<i>Sistrurus miliarius</i> *	–	N America	FJ659996	FJ660406	FJ660050	AY223611	U41889	AF057228	AF057275
<i>Trimeresurus borneensis</i>	B301	Sabah, E Malaysia	DQ116973	DQ117495	DQ131669	AY352754	AY352817	AY352783	AY352722
" <i>Trimeresurus</i> " <i>gracilis</i>	A86	Taiwan	GQ428345	GQ428439	GQ428388	AF171913	AY352823	AY352789	AY352728
<i>Trimeresurus gramineus</i>	A220	S. India	DQ116972	DQ117494	GQ428378	AY352761	AY352827	AY352793	AY352731
<i>Trimeresurus malabaricus</i>	A218	S India	–	GQ428434	GQ428377	AY059569	AY059587	AY059548	AY059564
					GQ428435				
<i>Trimeresurus puniceus</i>	B213	Indonesia	AF517212	DQ117524	–	AF517192	AF517220	AF517164	AF517177
<i>Trimeresurus trigonocephalus</i>	A58	Sri Lanka	DQ116969	DQ117491	GQ428385	AF171890	AY059597	AY059549	AY059565
			DQ116970	DQ117492					
<i>Tropidolaemus subannulatus</i>	B311	E Malaysia	GQ428357	–	–	AY352759	AY352822	AY352788	AY352727
			GQ428358						
<i>Tropidolaemus wagleri</i>	B132	W Malaysia	GQ428350,	–	DQ131670	AF517191	AF517223	AF517167	AF517180
			GQ428351						
<i>Tropidolaemus wagleri</i>	B133	W Malaysia	DQ131655	–	–	AF517191	AF517223	AF517167	AF517180
<i>Viridovipera gumprechtii</i>	B163/B174*	N Viet Nam	DQ116962	DQ117484	GQ428395	AY059573	AY059595	AY059547	AY059563
			DQ116963	DQ117485	GQ428396				
<i>Viridovipera gumprechtii</i>	A164	NE Thailand	DQ116964	DQ117486	GQ428370	AY352766	AF517224	AF517168	AF517181
<i>Viridovipera gumprechtii</i>	A174/A181*	NE Thailand	GQ428336	–	–	AF171898	GQ428478	GQ428487	GQ428461
<i>Viridovipera gumprechtii</i>	A176/A181*	NE Thailand	GQ428337	–	–	AF171898	GQ428478	GQ428487	GQ428461
<i>Viridovipera gumprechtii</i>	A178/A181*	NE Thailand	GQ428338	–	–	AF171898	GQ428478	GQ428487	GQ428461
<i>Viridovipera medoensis</i>	B416	N Myanmar	GQ428362	GQ428452	–	AY352765	AY352831	AY352797	AY352735
<i>Viridovipera vogeli</i>	B125	S Laos	GQ428349	–	–	AY059581	AF517225	AF517170	AF517183
<i>Viridovipera vogeli</i>	B97	NE Thailand	DQ116989	DQ117511	DQ131667	AY059574	AY059596	AY059546	AY059562
			DQ116990	DQ117512					
<i>Viridovipera stejnegeri</i>	T112/T23*	Taiwan	–	GQ428456	GQ428425	AF277689	EU443799	EU443800	EU443801
				GQ428457					
<i>Viridovipera stejnegeri</i>	B109	N Viet Nam	–	–	GQ428392	AF278709	GQ428479	GQ428489	GQ428463

are difficult to obtain, and Lartillot and Philippe (2006) showed that this estimator tends to favour parameter-rich models. Thus, rather than employing a strict interpretation, we employed a rough rule of thumb whereby $2\log_e B_{01} < 100$ was taken as indicating insignificant difference between runs.

An alternative mixture modelling approach, in which the number of appropriate partitions is also defined as part of the optimisation process using reversible-jump MCMC, rather than defined a priori, was also implemented using BayesPhylogenies (Pagel and Meade, 2004), using the GTR model and 4 gamma rate categories. Gaps were excluded in this analysis, referred to hereafter as BP. A single chain was run for 100 million generations, sampling every 10,000 generations, and only results from chains in which at least two independent runs converged were used.

3. Results

The final dataset consisted of 5516 basepairs made up of the following: 7IβFIB = 1127, 3ITBP = 885, 3ILMNA = 1113, 12S = 420, 16S = 510, CYTB = 798, NADH4 = 663. Of these, 2176 characters were variable (7IβFIB = 340, 3ITBP = 315, 3ILMNA = 329, 12S = 199, 16S = 177, CYTB = 429, NADH4 = 387) and 1658 were parsimony informative (7IβFIB = 219, 3ITBP = 216, 3ILMNA = 213, 12S = 157, 16S = 135, CYTB = 383, ND4 = 335). The simple gap-coded matrix added a further 419 variable and 249 parsimony informative characters. Several taxa were represented by multiple OTUs where LVHs were present at intron loci. Both hLRT and AIC criteria in MrModeltest supported the GTR + I + Γ model for all sequence partitions, apart from 7IβFIB (GTR + Γ) and 3ILMNA (SYM + Γ). However, in the latter two cases, the GTR + I + Γ model

was not significantly worse than the selected model, and since a slightly over-specified model has been shown to be beneficial in Bayesian analysis (Nylander et al., 2004), this was applied to all sequence partitions for simplicity. Parameters were unlinked across all partitions. The M1 model was applied to the gapcoded partition, where it was included. After inspecting the estimated parameters and their ESS in the fully partitioned model using Tracer v1.4.1, we combined first and second codon positions for the two coding genes, but left their third positions as separate unlinked partitions. This gave the following models: Part12gc, Part11nogaps, Part9gc, and Part8nogaps. For both the nogaps and gc matrices, $2\log_e B_{10}$ was less than 100, indicating little preference for models with the reduced number of partitions (part8nogaps, part9gc) over fully partitioned models (part11nogaps, part12gc). The topology of consensus trees differed not at all (part9gc and part12gc) or very little (two taxa differed in position in trees resulting from analysis of part8nogaps and part11nogaps matrices). However, posterior probabilities (PP) were generally higher in analyses of less partitioned models. The reversible-jump BayesPhylogenies analyses converged on five patterns. The topology of the BP consensus tree was most similar to that of part8nogaps but differed in the relative position of relatively few clades whose position was not generally well supported in either analysis.

Bayesian analyses found *Gloydus* to be the sister group of the New World Clade (Fig. 1), regardless of data matrix or model. PP values range from 0.96 and 0.99 in the nogaps and gc matrices in MrBayes, to 0.89 in BP). However, a notable feature of all trees was the inclusion of *okinavensis/gracilis* as the sister group to the *Gloydus* + New World clade in all analyses, with very short branch lengths and very low support values in many cases (PP = 0.5 for the BP analysis). We therefore investigated what effect removing the *okinavensis/gracilis* group had on support values in this region of the tree. In all cases the support for the node defining the *Gloydus* + New World clade increased dramatically (PP > 0.95 for all analyses apart from part12gc where PP increased from 0.77 to 0.87). The support for a clade including *Gloydus*, New World species and *Protobothrops* also increased in most analyses (e.g., from 0.70 to 0.99 in BP). Interestingly, the position of the *okinavensis/gracilis* group appeared to be least labile in the analyses of the gc matrix, where the removal of the group caused little change in the support values of the remaining clades (e.g., the node defining the *Gloydus* + New World + *Protobothrops* clade changed no more than 0.02) and the position of *okinavensis/gracilis* as sister group to the *Gloydus* + New World clade received relatively high support (0.74 and 0.91 for part12gc and part9gc, respectively). Thus, insertion-deletion events seem to contain valuable signal regarding the phylogenetic position of this enigmatic group.

All analyses also strongly supported (PP = 1.0) the inclusion of *P. mangshanensis* and *P. sieversorum* (formerly both placed in monotypic genera) within *Protobothrops* as proposed by Guo et al. (2007). The position of *Ovophis monticola* as sister group to *Protobothrops* was identical in all analyses and support values indicate a strong belief in this position (PP = 0.74–0.88).

Other relationships did vary among model/matrix combinations, for example *Deinagkistrodon* appeared as the sister group of either *Tropidolameus* or *Garthius* but usually with poor support (0.50 < PP < 0.60) with the exception of BP where its sister relationship with *Garthius* was supported by a PP of 0.98. Another notable variation among analyses was the relationship of *Viridovipera*, which was placed in a sister relationship with *Cryptelytrops* in the BP and nogaps analyses (PP > 0.95) but appeared as the sister group of *Popeia* in the gc analyses (PP = 0.97). However, all analyses unequivocally supported the newly-defined genera formerly assigned to *Trimeresurus* (PP = 1.0). The position of *Himalayophis*, which is placed as the sister group to *Popeia* in the nogaps and BP analyses (PP > 0.80), also differed in the gc analyses where it ap-

pears as a sister group to the clade consisting of *Viridovipera*, *Cryptelytrops*, *Parias* and *Popeia*.

Tree topologies of consensus trees from parsimony ratchet (PRAP) on the nogaps and gc matrices (eight equally parsimonious trees of 9181 steps and 13 equally parsimonious trees of 10,044 steps, respectively) were identical to each other (although bootstrap support [BS] varied slightly) and, in most details, to the Bayesian tree shown in Fig. 1. In contrast to the Bayesian analyses, the parsimony analysis did not resolve the sister group of the New World clade, but instead placed this clade in an unresolved polytomy with *Gloydus*, *Protobothrops*, “*T. gracilis* + “*O. okinavensis*, and *Ovophis monticola*. The analysis was repeated with the *okinavensis/gracilis* group deleted in order to investigate whether the lack of resolution was attributable to the lability of this group. However, bootstrap support for other clades did not change.

Differences in bootstrap proportions between the nogaps and gc analyses did not usually change the interpretation of the node as being strongly, weakly or un-supported. Within the *Trimeresurus* radiation, all genera were strongly supported in both analyses, and both nogaps and gc analyses also supported the sister relationship of *Himalayophis* and *Popeia* (BS = 60 and 64%, respectively). The inclusion of *C. macrops* and *C. venustus* within *Cryptelytrops*, which received no support in the mitochondrial tree alone but was found to be weakly supported (BS = 52%) when two nuclear intron partitions were added by Creer et al. (2006), shows substantially increased support in this analysis (BS = 85% in parsimony analyses of both matrices). The sister-group relationship between *Cryptelytrops* and *Viridovipera* was only weakly supported by the nogaps analysis (BS = 55%), and other intergeneric relationships were unresolved. The sister relationship between *Deinagkistrodon* and *Garthius* found in some Bayesian analyses was weakly supported in the gc parsimony analysis (BS = 51%).

4. Discussion

This study has resolved a long-standing question in snake systematics: from which group of Asian pitvipers were the ancestors of all the New World pitvipers derived? The identification of *Gloydus* as the sister group to the New World radiation is not altogether a surprise, since members of this genus currently occupy the more northerly parts of the Eurasian continent closest to Beringia. However, in view of the success of the nuclear intron data in resolving this node, it is perhaps somewhat surprising that it was less successful in resolving other parts of the pitviper phylogeny. For example, the relationships among a relatively small lineage arising from the most basal split in pitvipers (containing several monotypic taxa such as *Garthius*, *Calloselasma* and *Deinagkistrodon* as well as genera with a limited number of extant species, such as *Tropidolaemus* and *Hypnale*) are still inconsistent between different datasets and analyses, apart from the sister-group relationship of *Calloselasma* and *Hypnale*, which has been well established in the past by morphological as well as molecular studies. However, for the first time a very strongly supported relationship between *Deinagkistrodon* and *Garthius* is suggested by the reversible-jump MCMC analysis (also weakly supported by some alternative analyses).

The inclusion of gaps in the analysis also appears to have added useful signal in some regions, but seems noisier in other regions, of the tree. However, this gain in resolution is only seen in the Bayesian analyses and not in parsimony analyses. In fact, few of the relationships which were previously unsupported by the mitochondrial data alone have gained any further support by inclusion of the nuclear intron data in parsimony analysis, which perhaps reflects the greater power of model-based methods in analysing complex datasets.

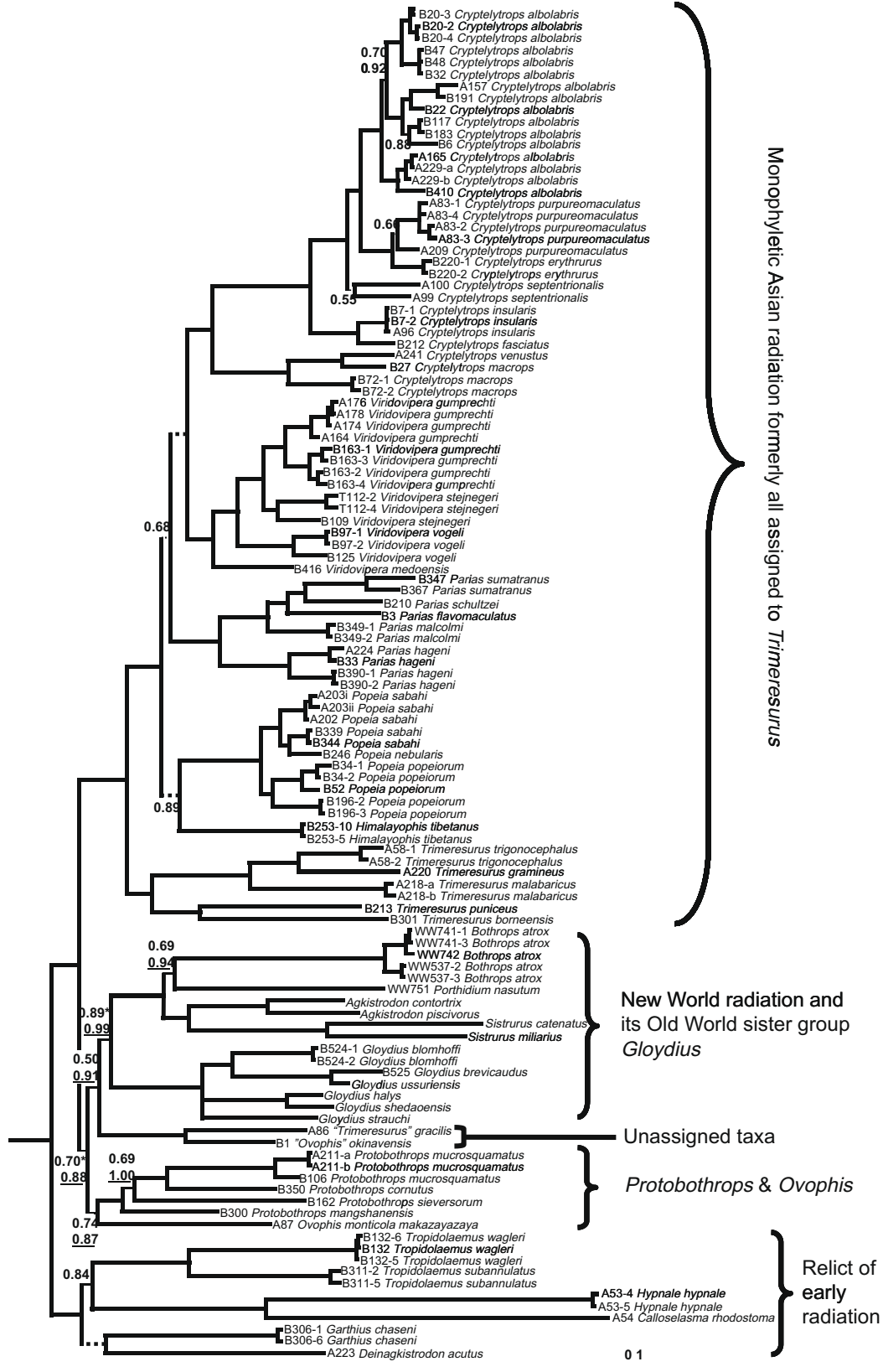


Fig. 1. Bayesian inference tree based on four mtDNA genes and three nuclear introns analysed using reversible-jump MCMC (five patterns, gaps excluded, see text for more details). Very strongly supported parts of the tree (PP > 0.95) are shown in bold. Posterior probabilities for clades are also given where 0.95 > PP > 0.50 (on thin branches). Posterior probabilities from alternative manually partitioned analyses of matrices with gaps deleted (part8nogaps) are given in italics and of simple-coded gaps (part9gc) underlined, where they are substantially different only. Asterisks indicate nodes with large increase in support value when the *okinavensis*/*gracilis* group is deleted and further information is given in the text. Branches that do not appear in at least one of the alternative analyses are shown in dashed type. A more detailed description of the differences between the maximum clade credibility trees from the different Bayesian analyses can be found in Section 3.

This study has also shed some light on the relationships of the enigmatic sister taxa *Ovophis okinavensis* and *Trimeresurus gracilis*. Recent detailed studies of cranial morphology (Guo et al., 2009) has confirmed that *Ovophis okinavensis* is very similar to *Ovophis monticola* in skull osteology (the original basis for inclusion in the genus *Ovophis*), raising the possibility that their inconsistent morphological and molecular classifications reflects lineage sorting of the linked mitochondrial genes (i.e., unrepresentative of the species tree). Guo et al. (2009) also showed that *Trimeresurus gracilis* groups with the above two species with respect to its skull osteological characteristics. However, here we have reconfirmed the sister-group relationship and misclassification of *Ovophis okinavensis* and *Trimeresurus gracilis* using several unlinked nuclear genes. Moreover, as a result of these analyses we have shown that the best supported hypothesis is that the “O”. *okinavensis* + “T”. *gracilis* clade forms the sister group to the *Gloydus* + New World clade. Both “O”. *okinavensis* and “T”. *gracilis* have small and isolated current ranges in the Ryukyu islands and Taiwan, respectively, adjacent to the current range of a number of *Gloydus* species. The long period of isolation and extinction of closely related species during the repeated cycles of land bridge formation and inundation that is thought to have taken place in the Ryukyu islands (Ota, 1998; Toda et al., 1999) may have erased additional phylogenetic signal. Rather than continue the present uncertainty regarding their generic nomenclature and relationships, we recommend that these two species now be placed in their own genus, a full description of which is in preparation.

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