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Uncovering cryptic diversity in *Aspidomorphus* (Serpentes: Elapidae): Evidence from mitochondrial and nuclear markers

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ABSTRACT

The Papuan region, comprising New Guinea and nearby islands, has a complex geological history that has fostered high levels of biodiversity and endemism. Unfortunately, much of this diversity remains undocumented. We examine the evolutionary relationships of the venomous snake genus *Aspidomorphus* (Elapidae: Hydrophiinae), a Papuan endemic, and document extensive cryptic lineage diversification. Between *Aspidomorphus* species we find 22.2–27.9% corrected *cyt-b* sequence divergence. Within species we find 17.7–23.7% maximum sequence divergence. These high levels of genetic divergence may have complicated previous phylogenetic studies, which have had difficulty placing *Aspidomorphus* within the subfamily Hydrophiinae. Compared to previous studies, we increase sampling within Hydrophiinae to include all currently recognized species of *Aspidomorphus* and increase species representation for the genera *Demansia* and *Toxicocalamus*. We confirm monophyly of *Aspidomorphus* and resolve placement of the genus utilizing a set of seven molecular markers (*12S, 16S, cyt-b, ND4, c-mos, MyHC-2, and RAG-1*); we find strong support for a sister-group relationship between *Aspidomorphus* and a *Demansia/Toxicocalamus preussi* clade. We also use one mitochondrial (*cyt-b*) and one nuclear marker (*SPTBN1*) to document deep genetic divergence within all currently recognized species of *Aspidomorphus* and discuss the Solomon Island Arc as a potential center of divergence in this species. Lastly, we find high levels of concordance between the mtDNA and nuDNA markers used for inter-species phylogenetic reconstruction.

1. Introduction

With continuing advances in molecular systematics, large amounts of genetic diversity are being uncovered in nearly all taxonomic groups (e.g. Elejalde et al., 2008; Hellborg et al., 2005; Thum and Harrison, 2009). The prevalence of unexpected levels of variation in many species has made it clear that reproductive isolation and molecular divergence are not necessarily accompanied by morphological change (Bickford et al., 2007; Bond et al., 2001; Darda, 1994; Highton and Macgregor, 1983; Narang et al., 1993; Sturmbauer and Meyer, 1992). Research on cryptic diversity has focused primarily on temperate areas and it remains undetermined how much may be present in the tropics, which are home to more than two-thirds of the world's described diversity (Bickford et al., 2007).

The Papuan region—which includes New Guinea and immediately adjacent islands, the Solomon Islands, and the Admiralty and Bismarck archipelagos—has a complex geological and biological history. New Guinea is the largest and highest tropical island in the world (Allison, 2007b). The Central Cordillera, a large mountain range that forms the backbone of the island, divides the island into northern and southern versants and provides a wealth of different habitat types (Beehler, 2007). This range of habitats, combined with a geologic history of island-arc accretions and the relative isolation of the Papuan region from all large land masses except Australia, has fostered high levels of biodiversity (Allison, 2007b; Flannery, 1995; Takeuchi, 2007). The herpetofauna of the Papuan region alone includes more than 800 species, of which over 100 have been identified since 2000 and many remain to be described (Allison, 2007a; Allison and Kraus, 2009; F.K., unpubl. data). Approximately 80% of described species of herpetofauna in the Papuan region are endemic and most nonendemics are shared only with Australia (Allison, 2007a; Allison and Kraus, 2009).

Of the three families of front-fanged venomous snakes found worldwide, only one, Elapidae, occurs in the Papuan region. Hydrophiinae is one of two major subfamilies of elapid snakes and comprises all marine and terrestrial Australopapuan elapids (Slowinski and Keogh, 2000; Slowinski et al., 1997). This subfamily represents an adaptive diversification that occurred after the arrival of an elapid ancestor in the Australopapuan region approximately 14–30 million years ago (MYA; Keogh et al., 1998; Sanders and Lee, 2008; Sanders et al., 2008). There are 27 genera and 100 species of terrestrial hydrophiines currently recognized (Keogh et al.,

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1998; Slowinski et al., 1997), including 20 genera (86+ species) found only in Australia or in both Australia and New Guinea, and 6 genera (16 species) endemic to the Papuan region. Additional species remain to be described (F.K., unpubl. data).

The hydrophiine genus *Aspidomorphus*, which occupies lowland forests of New Guinea and some of its adjacent islands (Fig. 1; McDowell, 1967), contains considerable genetic variation (Keogh et al. 1998) and has the potential for cryptic variation. Morphologically, *Aspidomorphus* appears most closely related to the venomous Austro-Papuan whip snakes in the genus *Demansia*, based on skull structure, head musculature, and hemipene morphology (McDowell, 1967). However, in external appearance the two genera differ. Compared to *Demansia, Aspidomorphus* is smaller, more compact, has a shorter tail with fewer subcaudals, more posterior scale rows, and smaller eyes with more elliptical pupils; it lacks a preorbital fossa and canthus rostralis (McDowell, 1967).

The most recent taxonomic revision of *Aspidomorphus* excluded Australian species that had previously been assigned to *Aspidomorphus* (including species now classified within the genera *Furina* and *Demansia*) and restricted the group to three species endemic to New Guinea and associated islands (McDowell, 1967). The three species can be distinguished from each other on the basis of hemi-

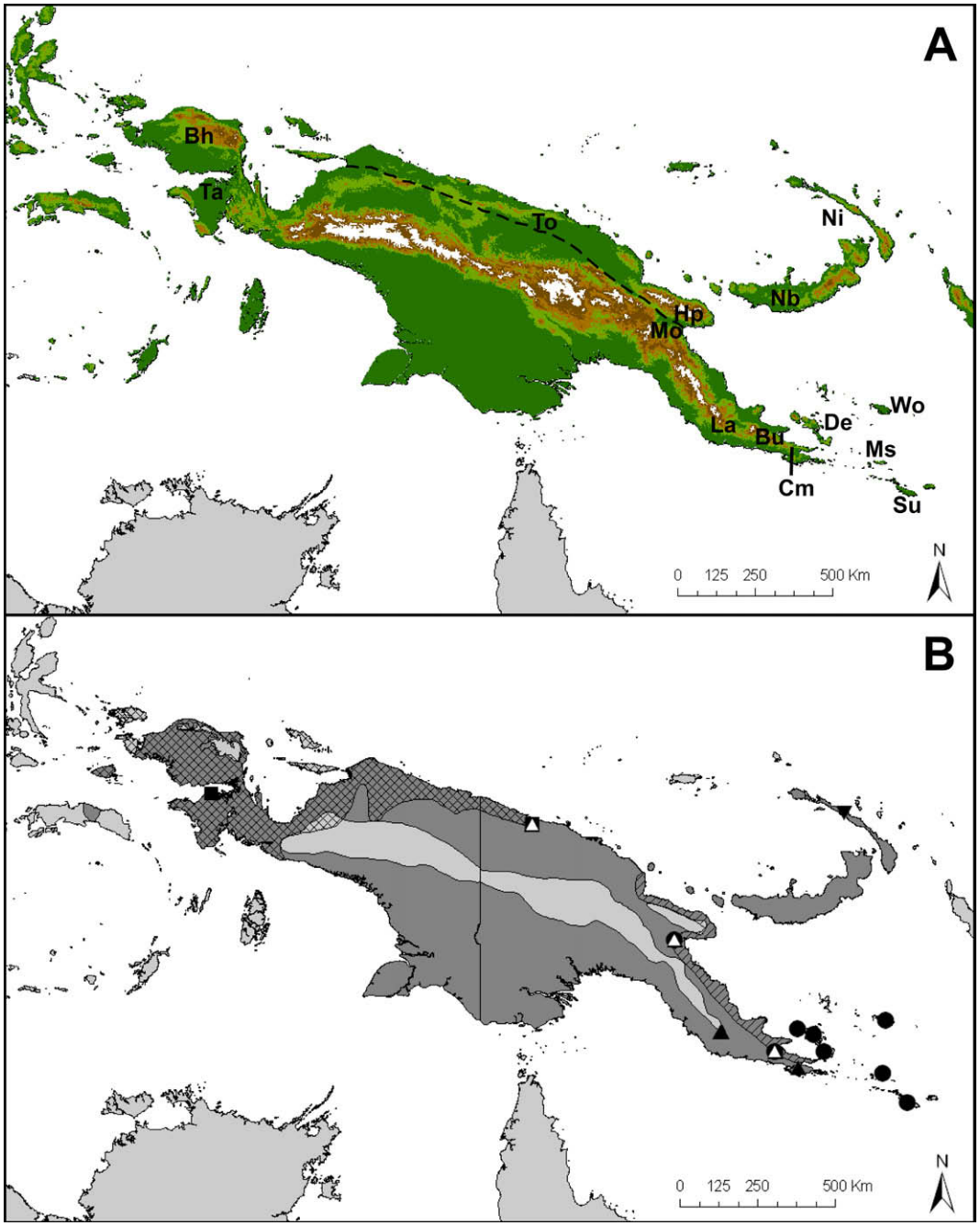

Fig. 1. (A) Topographic map of Papuan region (excluding Solomon Islands) with important features and locations indicated. Abbreviations from west to east are: Bh = Bird's Head Peninsula, Ta = Tangguh, To = Torricelli Mtns., Mo = Morobe, Hp = Huon Peninsula, La = Laronu, Bu = Bunisi, Nb = New Britain, Cm = Cloudy Mtns., De = D'Entrecasteaux Archipelago, Ni = New Ireland, Ms = Misima Island, Wo = Woodlark Island, Su = Sudest Island. Dashed line indicates portion of northern New Guinea belonging to the Solomon Island Arc system. (B) Range maps and collection localities for *Aspidomorphus* species. Dark gray and triangles indicate *A. muelleri*; crosshatching and squares indicate *A. schlegelii*; hatching and circles indicate *A. lineaticollis*. Inverted triangle is *A. muelleri* from Sanders et al. (2008).

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pene morphology, skull structure, and color pattern (McDowell, 1967). Of the three *Aspidomorphus* species, *A. muelleri* is the largest and is widely distributed across mainland New Guinea and the islands of the Bismarck Archipelago (Fig. 1B; McDowell, 1967). *Aspidomorphus schlegelii* occurs in the northwestern portion of New Guinea and *A. lineaticollis* occupies the eastern portion of New Guinea and a number of the offshore islands of Milne Bay Province (Fig. 1B).

There is considerable intraspecific morphological variation in scale counts and color pattern within *Aspidomorphus* (McDowell, 1967). When considered in combination with molecular divergence within *A. muelleri* documented by Keogh et al. (1998) this variation suggests that there may be patterns of diversity not captured by current taxonomy. Nevertheless, McDowell (1967) could not find geographically consistent patterns in morphological variation in *A. muelleri* and *A. schlegelii* and he was not convinced that the geographic variation present in *A. lineaticollis* merited a revised taxonomy. Additionally, recent expeditions to New Guinea have recovered snakes that appear to represent new species (Austin et al., 2008; F.K., pers. obs.).

Despite repeated attempts, studies on the Hydrophiinae combining molecular and morphological data or using molecular data alone have failed to resolve the phylogenetic position of *Aspidomorphus* (Keogh et al., 1998; Sanders et al., 2008; Scanlon and Lee, 2004; Slowinski and Keogh, 2000). These studies have presented several hypotheses, including sister-group relationships with *Cacophis, Acanthophis, Micropechis, Demansia,* a *Demansia/Toxicocalamus* clade or a *Demansia/Ogmodon* clade. Support for each of these hypotheses has been weak, with varying taxon sampling among studies combined with sparse sampling within studies. Additionally, these studies have assumed the monophyly of *Aspidomorphus,* using only *A. muelleri* to represent the entire genus. Monophyly of *Aspidomorphus* has never been explicitly tested using molecular data, and molecular studies of other hydrophiine genera have revealed instances of paraphyly (*Pseudechis*, Keogh et al., 1998; *Hydrophis*, Lukoschek and Keogh, 2006; *Neelaps*, Sanders et al., 2008).

In addition to the taxonomic issues highlighted above, the phylogeography of *Aspidomorphus* has not been addressed. Recently, several authors have suggested that it is inappropriate to use mtDNA for taxonomic or phylogeographic studies (Ballard and Whitlock, 2004; Bazin et al., 2006; Edwards et al., 2005; Hudson and Coyne, 2002) unless the results are validated by multiple nuclear markers. In contrast, Zink and Barrowclough (2008) argue that mtDNA is an appropriate molecular marker for phylogeographic studies, and that due to long coalescence times and low mutation rates, nuclear markers are likely to be uninformative or misleading. Many inter-specific snake phylogeographic and systematic studies to date have relied heavily on mtDNA (Burbrink et al., 2000; Castoe et al., 2007; Daza et al., in press; Guiher and Burbrink, 2008; Lukoschek and Keogh, 2006; Rawlings and Donnellan, 2003; Wüster et al., 2005); in this study we conduct both separate and combined analyses of mitochondrial and nuclear markers to evaluate their reliability and phylogeographic utility.

Here we address four important phylogenetic questions about diversification in *Aspidomorphus.* First, we combine four mitochondrial and three nuclear markers plus the most complete taxonomic sampling of Hydrophiinae to date to address two questions: (1) Is *Aspidomorphus* monophyletic? and (2) What is the sister group to *Aspidomorphus*? We also combine a mitochondrial gene and a nuclear intron with extensive sampling within *Aspidomorphus* to address two additional questions: (3) Do the three species currently identified within *Aspidomorphus* represent genetically distinct lineages? and (4) Does current taxonomy reflect the evolutionary history of this genus?

2. Materials and methods

2.1. Sampling

We acquired 51 blood or liver tissue samples, from all three nominal species of *Aspidomorphus* as well as from the hydrophiine genera *Demansia, Micropechis,* and *Toxicocalamus* (Appendix A) for use as outgroups in our analyses. Sequences for 48 additional outgroups (33 genera) were obtained from Kate Sanders (Sanders et al. 2008), allowing us to include representatives of nearly all terrestrial hydrophiine genera and all major lineages of sea snakes. For *A. lineaticollis,* we sampled 30 individuals, including three individuals from the mainland and others from the islands of Sudest, Misima, Woodlark, Goodenough, Fergusson, and Normanby. Kiriwina was the only island with a known population of *A. lineaticollis* that we were unable to sample. For *A. muelleri* we collected 15 individuals from five mainland localities representing the northeastern quarter of the species range. For *A. schlegelii*, historically the least collected member of this genus (McDowell, 1967; Shine and Keogh, 1996), we were able to obtain three individuals: two from the Torricelli Mtns. in Papua New Guinea (PNG) and one from Tangguh in Papua Province of Indonesia (Fig. 1).

2.2. Laboratory methods

We extracted total genomic DNA from tissue samples using the DNEasy Blood and Tissue Kit (Qiagen). To address our first two questions we amplified seven loci for ten individuals selected to represent the geographic and taxonomic range present in our sampling (Fig. 1, Appendix A): cytochrome *b* (*cyt-b*), 12S rRNA (*12S*), 16S rRNA (*16S*), NADH dehydrogenase and associated tRNAs (*ND4*), myosin heavy chain 2 intron (*MyHC-2*), oocyte maturation factor (*c-mos*), and recombination activating gene 1 (*RAG-1*). We call this the Q12 dataset. To address questions 3 and 4 we amplified the mitochondrial gene *cyt-b* and one nuclear locus, β-spectrin nonerythrocytic intron 1 (*SPTBN1*; Matthee et al., 2001) for all 51 samples used in this study. We call this the Q34 dataset. PCR conditions and primers for all loci used in this study are given in Table 1. For eleven degraded samples of *A. lineaticollis* (from the islands of Misima, Woodlark, and Goodenough), we designed internal primers to amplify the *cyt-b* gene in three segments (Table 1) and were unable to amplify *SPTBN1*. Sequencing of PCR products was done by the Nevada Genomics Center on an ABI sequencer and chromatograms were edited using Sequencher 4.7 (Gene Codes Corp.). For *SPTBN1* we found several heterozygotes with alleles that differed by a single nucleotide. These sites were coded using appropriate ambiguity codes. We performed sequence alignment in MEGA 3.1 (Kumar et al., 2004) using Clustal W, and we refined alignments by eye. Ambiguous sections were identified by eye and excluded from analysis. GenBank accession numbers for all *cyt-b* and *SPTBN1* sequences generated for this study are given in Appendix A. Accession numbers for all other sequences generated for this study are given in Appendix B. Accession numbers for the previously published sequences are in Sanders et al. 2008.

2.3. Phylogenetic analysis

For questions 1 and 2, we combined our Q12 dataset with the data from Sanders et al. (2008) and recovered the phylogeny through Bayesian inference (BI) following the Sanders et al. methodology. We used MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with seven data partitions: mitochondrial protein-coding genes partitioned by codon position, mitochondrial RNA, nuclear protein-coding codon positions 1 and 2, nuclear protein-coding codon

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Table 1
Primers and PCR conditions for all genes used in this study.

Gene	Primers	Temp (°C)	MgCl (mM)	Reference
RAG-1	G396 (R13) 5′-TCT GAA TGG AAA TTC AAG CTG TT-3′ G397 (R18) 5′-GATGCTGCCTCGGTCGGCCACCTTT-3′	57	3	Groth and Barrowclough (1999)
C-MOS	G303 5′-ATT ATG CCA TCM CCT MTT CC-3′ G74 5′-TGA GCA TCC AAA GTC TCC AAT C-3′ G708 5′-GCT ACA TCA GCT CTC CAR CA-3′	53	4	Saint et al. (1998) and Hugall et al. (2008)
MyHC-2	G240 5′-GAA CAC CAG CCT CAT CAA CC-3′ G241 5′-TGG TGT CCT GCT CCT TCT TC-3′	61	1.5	Lyons et al. (1997)
12S rRNA	tRNA-Phe 5′-AAA GTA TAG CAC TGA AAA TGC TAA GAT GG-3′ tRNA-Val 5′-GTC GTG TGC TTT AGT CTA AGC TAC-3′	45	1.25	Keogh et al. (1998)
16S rRNA	5′-CGC CTG TTT ATC AAA AAC AT-3′ 5′-CCG GTC TGA ACT CAG ATC ACG T-3′	48	1	Kocher et al. (1989)
ND4 + tRNA	ND4 5′-TGA CTA CCA AAA GCT CAT GTA GAA GC-3′ tRNA-Leu 5′-TAC TTT TACC TTG GAT TTG CAC CA-3′	48	2	Arévalo et al. (1994)
cyt-b + tRNA	L14910 5′-GAC CTG TGA TMT GAA AAA CCA YCG TTG T-3′ H16064 5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′	48	3	Burbrink et al. (2000)
cyt-b (fragment 1)	L14910 5′-GAC CTG TGA TMT GAA AAA CCA YCG TTG T-3′ Asp_linCTB348 5′-CCA AAG AAG GCT GTT GC-3′	48	3	This study and Burbrink et al. (2000)
cyt-b (fragment 2)	Asp_linCTB308 5′-TAC GGC CTA TAC CTT AAC AAA G-3′ Asp_linCTB768 5′-CGG GTT TAA TRT GTT GAG G-3′	48	3	This study
cyt-b (fragment 3)	Asp_linCTB758 5′-AAC TTC ACT AAA GCG AAY CC-3′ H16064 5′-CTT TGG TTT ACA AGA ACA ATG CTT TA-3′	48	3	This study and Burbrink et al. (2000)
SPTBN1	SPTBN1-F1 5′-TCT CAA GAC TAT GGC AAA CA-3′ SPTBN1-R1 5′-CTG CCA TCT CCC AGA AGA A-3′	55	3	Matthee et al. (2001)

position 3, and *MyHC-2*. Models were selected for each partition using Mr. ModelTest 2.2 (Nylander et al., 2004).

For questions 3 and 4, we used our Q34 dataset and inferred the phylogeny using BI and maximum likelihood (ML). We checked for concordance among loci by running separate preliminary Bayesian analyses (following the procedures outlined below for the concatenated dataset) of each gene and compared the topologies. We implemented BI in MrBayes 3.1 and used GARLI 0.96 (Zwickl, 2006) to implement ML analysis. Based on the results of our analysis of the Q12 dataset (see Fig. 2), *Micropechis ikaheka* was chosen as the outgroup for both analyses.

For BI we implemented three partitioning strategies. For the first, we partitioned our data by locus only. For the second and third, we partitioned by locus and also partitioned the *cyt-b* data using two codon-based methods: (1) all codon positions partitioned separately and (2) first and second codon positions together with third codon positions partitioned separately. We used Mr. ModelTest 2.2 to select a best-fit model for each partition based on Akaike Information Criteria (AIC) and ran preliminary Bayesian analyses on the partitioned data sets. Relative Bayes Factors were used to compare partitioning strategies. As there were no significant differences in Relative Bayes Factors, we implemented the simplest strategy, partitioning by locus only, with the selected models in MrBayes (GTR+I+Γ for *cyt-b* and HKY+Γ for *SPTBN1*). We initiated two independent runs (each with 1 cold and 3 heated chains) with random starting trees and ran them for a total of 3×10^6 generations with trees sampled every 100 generations. We discarded the first 7.5×10^5 generations based on stationarity, assessed using the program Tracer (Rambaut and Drummond, 2007). A 50% majority-rule phylogram was constructed from the combined runs.

As GARLI does not support the use of mixed models we used Mr. ModelTest 2.2 on the unpartitioned data set and implemented ML analysis using the best fit model, GTR +I+Γ. We used default parameter settings in GARLI and terminated each run after 20,000 generations elapsed without improvement in the likelihood score of the topology. Four runs were initiated to ensure that convergence had been reached. We then evaluated bootstrap support using 1000 replicates, each terminated after 10,000 generations elapsed with no improvement in the likelihood score of the topology as recommended by the program authors.

To aid comparison of *Aspidomorphus* to other closely related taxa, we calculated genetic distances for *cyt-b* in the program MEGA using a Kimura 2-parameter model and gamma-distributed rate variation.

3. Results

For our analysis of the Q12 dataset, we used a total of 5771 bp of sequence data, including 1031 bp of *12S*, 507 bp of *16S*, 854 bp of *ND4* + tRNAs (histidine, serine, and leucine), 1145 bp of *cyt-b* + tRNA, 523 bp of *MyHC-2*, 641 bp of *c-mos*, and 1068 bp of *RAG-1*. Using BI we recovered a monophyletic *Aspidomorphus* with 100% posterior probability (*Pp*) support. *Aspidomorphus* formed the sister taxon to a clade comprising *Demansia* and *Toxicocalamus preussi* (100% *Pp*). *Toxicocalamus loriae* and *Micropechis ikaheka* (77% *Pp*) are sister species, making *Toxicocalamus* nonmonophyletic (Fig. 2). The *D. psammophis* sample added in this study clustered with the two *Demansia* species from the previous study (100% *Pp*).

Each currently recognized species within *Aspidomorphus* was recovered as genetically distinct (100% *Pp*). The *A. muelleri* sample from New Ireland (Sanders et al., 2008) clusters with our sample from north of the Huon Peninsula (100% *Pp*) and is the sister taxon to our samples of *A. muelleri* collected south of the Huon Peninsula (100% *Pp*).

The Q34 dataset had a length of 2291 bp of which a 17-bp section of tRNA at the 3′ end of *cyt-b* was excluded from analysis due to ambiguity in the alignment. Within *Aspidomorphus*, we identified 44 unique *cyt-b* haplotypes and 13 unique *SPTBN1* genotypes. The *cyt-b* alignment of 1117 bp had a total of 523 variable characters, of which 422 were parsimony-informative. The *SPTBN1* alignment of 1174 bp had 103 bp of the β-spectrin nonerythrocytic exon 1 at the 5′ end and 106 bp of the β-spectrin nonerythrocytic exon 2 at the 3′ end. There were 110 variable characters and 58 parsimony-informative characters. Analysis of each gene region independently resulted in only two instances of a well supported (⩾95% posterior probability in both trees; Fig. 3) difference in the topologies, both within species, therefore we concatenated the data sets for all further analyses according to recommendations by Wiens (1998).

Analysis of the concatenated Q34 dataset with both BI and ML methods also yielded nearly identical topologies, differing only in

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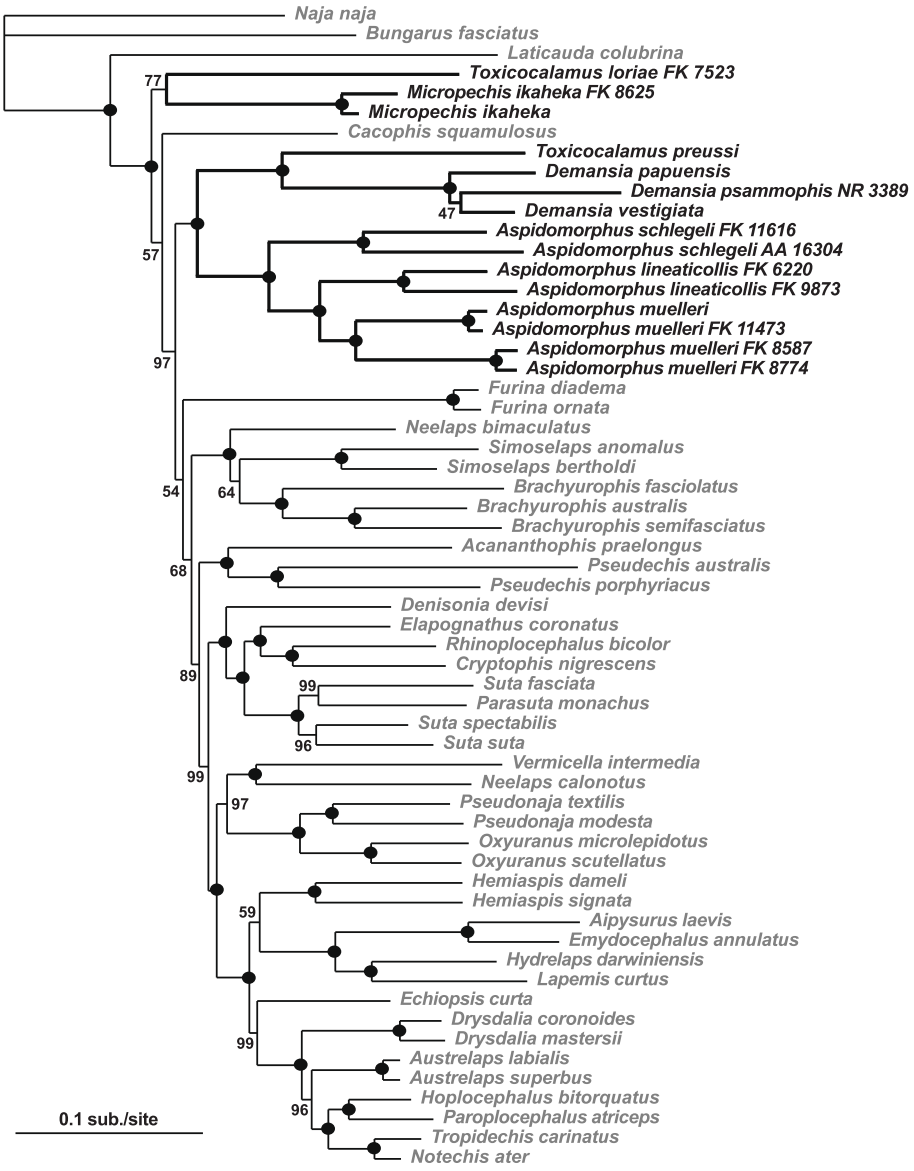

Fig. 2. Bayesian phylogram of hydrophiine relationships. Numbers represent posterior probabilities and dots represent nodes with 100% posterior probability support. Samples with field tags are new to this study. Clades shown in bold are highlighted in the text.

placement of some recently diverged lineages (Fig. 4). Resolution was higher in the Bayesian analysis but support values for these regions were low in both analyses. *Aspidomorphus* again formed a monophyletic group with 100% *Pp* and 83% bootstrap (*Bs*) support and there was strong support (99% *Pp*, 95% *Bs*) for its sister-group relationship with *Demansia psammophis*. Within *Aspidomorphus* the three currently recognized species formed three monophyletic groups (100% *Pp* + *Bs*), with *A. schlegelii* being the sister taxon to a clade containing *A. muelleri* and *A. lineaticollis*. Within *A. lineaticollis*, there was 100% *Pp* and *Bs* support for six distinct groupings of DNA sequences: (1) Kamiali, (2) mainland Milne Bay Province, (3) the D'Entrecasteaux Archipelago, (4) Woodlark, (5) Misima, and (6) Sudest. Within *A. muelleri*, there was a deep divergence between the sample collected in the Torricelli Mtns. and all other samples of *A. muelleri* (100% *Pp* + *Bs*). Similarly, within *A. schlegelii* there was deep divergence between the two samples from the Torricellis and the single Indonesian sample.

Based on the *cyt-b* dataset (including the *A. muelleri* sample from Sanders et al., 2008), the maximum corrected distance between two *A. muelleri* samples was 23.7%, between the sample from the Torricelli Mtns. and another from Bunisi. The average genetic distance between the two lineages of *A. muelleri* was 23.1%. The maximum genetic distance between *A. lineaticollis* samples was 17.7% between a sample from Misima Island and one from Normanby Island. The average genetic distance between distinct lineages of *A. lineaticollis* ranged from 10.5% to 16.5%. The average *cyt-b* distance between the two geographic samples of *A. schlegelii* was 19.1%. The average distance between *A. schlegelii* and *A. lineaticollis* was 24.7%, the average between *A. schlegelii* and *A. muelleri* was 27.9%, and the average between *A. muelleri* and *A. lineaticollis* was 22.2%. Including the two *Demansia* species used in Sanders et al. (2008), the average distance between species in *Demansia* was 14.8%, and the average distance between *Demansia* and *Aspidomorphus* was 27.5%.

4. Discussion

4.1. Monophyly and relationships of Aspidomorphus and sister genera

Relative to Sanders et al. (2008) our results increase support for a sister-group relationship between *Aspidomorphus* and a clade

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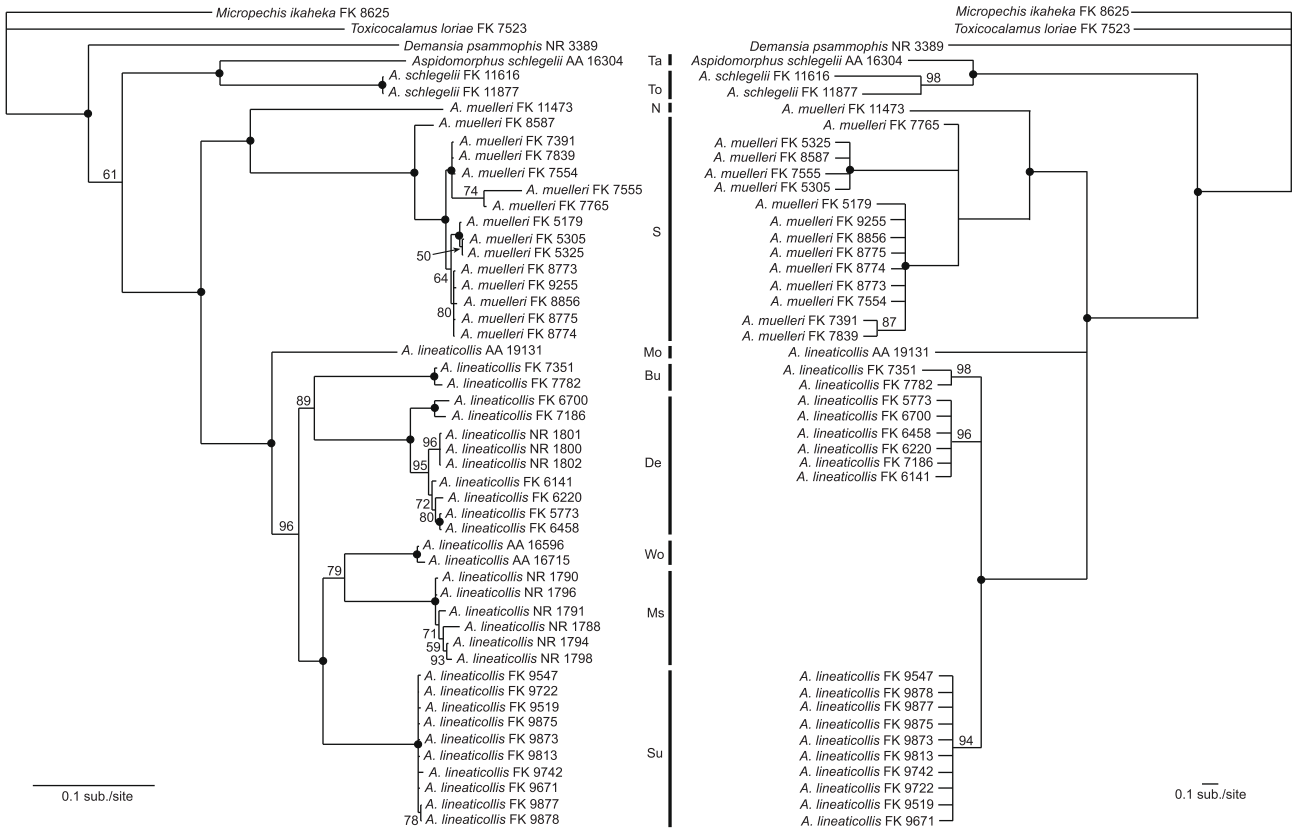

Fig. 3. Bayesian phylograms of *cyt-b* (left) and *SPTBN1* (right) analyzed independently, with posterior probabilities ⩾ 50% shown. Dots indicate 100% posterior probability. Named lineages are indicated: (Ta) Tanguhh, (To) Torricelli Mtns., (N) North of Huon Peninsula, (S) South of Huon Peninsula, (Mo) Morobe, (Bu) Bunisi, (De) D'Entrecasteaux Archipelago, (Wo) Woodlark Island, (Ms) Misima Island, (Su) Sudest Island.

comprising *Toxicocalamus preussi* and *Demansia*, with 100% posterior probability for that node (compared to 55% in Sanders et al.). A close relationship between *Aspidomorphus* and *Demansia* had previously been suggested on the basis of morphological and mtDNA similarities (Keogh et al., 1998; McDowell, 1967; Slowinski and Keogh, 2000). A close relationship between *Aspidomorphus* and *Toxicocalamus* is a recent hypothesis introduced by Sanders et al. (2008). Morphological evidence does not suggest a particularly close association, but *Toxicocalamus* is a diverse and poorly collected genus, and it is possible that further research will reveal additional similarities with *Aspidomorphus* and *Demansia*. Our denser sampling strategy provides the strongest test of *Aspidomorphus* and *Demansia* monophyly to date because of our inclusion of *Demansia psammophis, A. schlegelii* and *A. lineaticollis*. This has provided dense lineage sampling within *Aspidomorphus* as well as including the species of *Demansia* considered the most similar to *Aspidomorphus* (McDowell, 1967).

The New Guinea endemic *Toxicocalamus* is not monophyletic in the family-level analysis, with our sample of *T. loriae* forming a clade with the monotypic endemic *Micropechis ikaheka* while the *T. preussi* sample from Sanders et al. (2008) clusters with *Demansia*. When McDowell (1969) reviewed *Toxicocalamus*, he synonymized *Apistocalamus* and *Ultrocalamus* into *Toxicocalamus* and retained the old generic names as subgenera. Prior to this reclassification, *Toxicocalamus preussi* was classified as *Ultrocalamus preussi* while *Toxicocalamus loriae* was classified as *Apistocalamus loriae*. This prior generic division, and the considerable morphological variation within this genus (F.K., pers. obs.), lend support to the idea that the species currently classified under *Toxicocalamus* may not form a monophyletic group. Nevertheless, when only the mitochondrial genes are considered, the two *Toxicocalamus* samples cluster together. This suggests that either genomic variation does not coalesce within species or that phylogenetic signal is insufficient in the more slowly evolving nuclear markers for correct placement of the *Toxicocalamus* samples. Further sampling of *Toxicocalamus* is required to establish monophyly of the genus and to confirm its position within the Hydrophiinae (ongoing-C.L.P. and F.K.).

4.2. Relationships within Aspidomorphus and concordance between datasets

Within the genus *Aspidomorphus*, we confirm the presence of three major lineages that exactly coincide with the three currently recognized species (Figs. 2–4). Our sampling in three areas of sympatry confirms that the species represent distinct evolutionary units. This represents the first molecular test of McDowell's morphological species concepts within *Aspidomorphus*.

There was very little discordance between the mtDNA and nuDNA data sets used for this study (Fig. 3). Although the nuclear data set provided lower resolution than the mitochondrial data, it helped to resolve the relationship between *Aspidomorphus* and *Demansia*, providing strong support for a node that was highly unstable in analyses using only mtDNA. Deeper relationships within the Hydrophiinae have proven difficult to resolve in previous studies using mitochondrial data alone, mitochondrial and morphological data, and mitochondrial and nuclear markers (Keogh et al., 1998; Sanders et al., 2008; Scanlon and Lee, 2004; Slowinski and Keogh, 2000). It has been hypothesized that this uncertainty may represent a rapid splitting of lineages after the invasion of Australia, resulting in short internal branches that are difficult to completely resolve. Alternatively, substitutional saturation of

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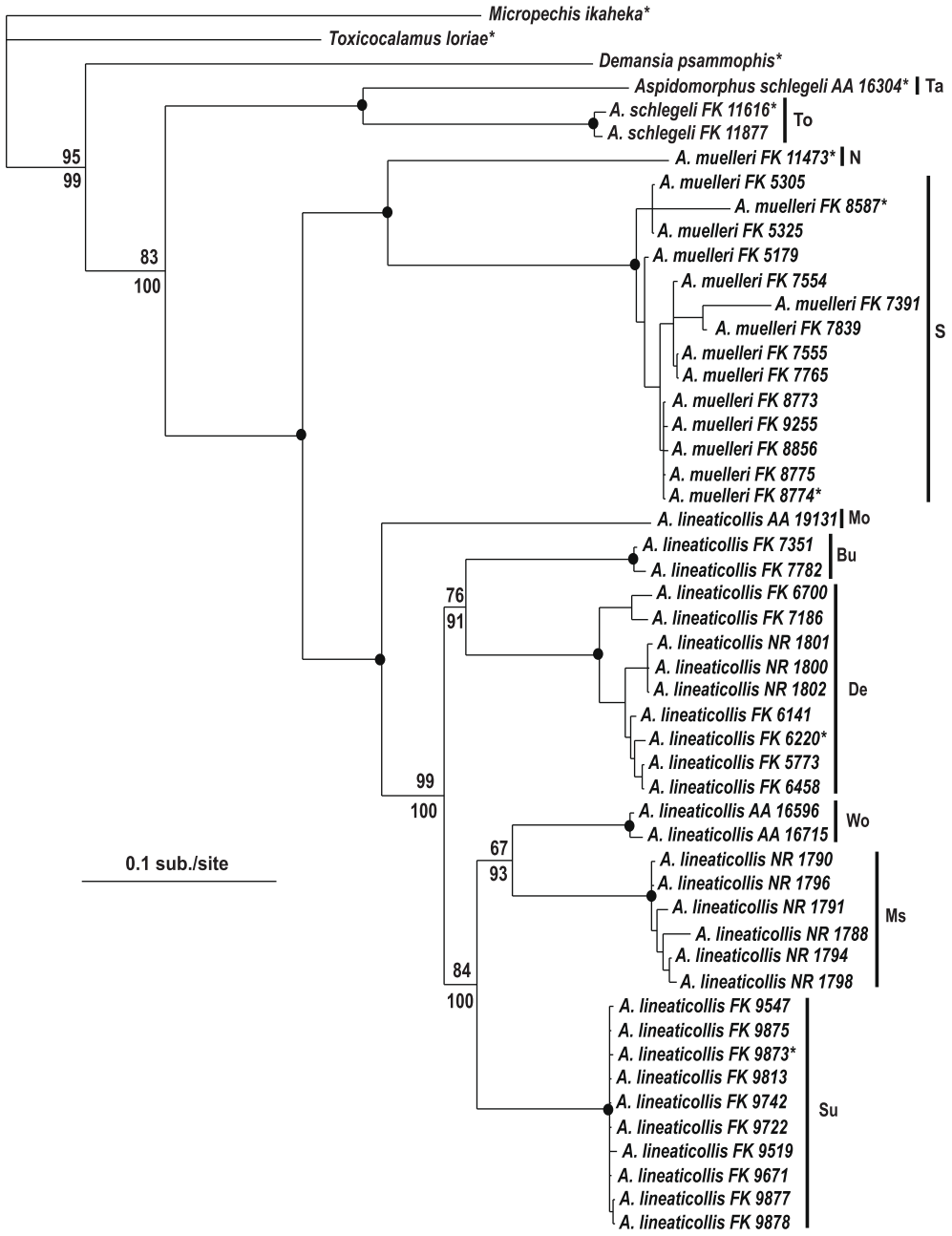

Fig. 4. Bayesian phylogram compiled from analyses of combined data, with posterior probability (above node) and ML bootstrap (below node) values >65% shown. Dots indicate 100% posterior probability and bootstrap support. Named lineages are indicated: (Ta) Tanguhh, (To) Torricelli Mtns., (N) North of Huon Peninsula, (S) South of Huon Peninsula, (Mo) Morobe, (Bu) Bunisi, (De) D'Entrecasteaux Archipelago, (Wo) Woodlark Island, (Ms) Misima Island, (Su) Sudest Island. Asterisks indicate samples used in the Q12 dataset.

mitochondrial markers might reduce phylogenetic signal for deep divergences and currently used nuclear markers may be too slowly evolving to have sufficient phylogenetic signal. Our results suggest that *SPTBN1* may evolve at a rate that permits resolution of divergences occurring in the last 10–30 million years. The *SPTBN1* locus, along with other nuclear loci with sufficient phylogenetic signal, may be an important tool for addressing deeper elapid relationships that have been difficult to resolve with current markers. These results highlight the utility of mtDNA for phylogeographic studies and provide justification for its continued use in species delimitation and taxonomic investigations. However, the addition of independent lines of evidence, in our case an appropriately chosen nuclear marker, can be vital in addressing species-level evolutionary questions.

4.3. Cryptic variation in Aspidomorphus

This study has confirmed that each of the three *Aspidomorphus* species represents a genetically distinct entity, with deep molecular divergences. This finding is consistent with the possibility that each of these nominal species contains multiple cryptic species. In recent years, cryptic species diversification has been shown to be far more common than previously expected (Sáez and Lozano, 2005), and there is considerable evidence for this phenomenon within *Aspidomorphus*. At least six deeply divergent, well-supported lineages occur within *A. lineaticollis*, two within *A. muelleri* and two within *A. schlegelii*. These lineages are supported by both nuclear and mitochondrial markers, suggesting that gene flow between lineages has been limited for a considerable period of time.

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The populations of *A. lineaticollis* on the isolated islands of Sudest, Misima, and Woodlark form well-supported and divergent clades, while the islands of the D'Entrecasteaux Archipelago have populations that are closely related to each other, but still divergent from mainland *A. lineaticollis* (Figs. 4 and 5). Our sample of *A. lineaticollis* from Morobe Province on the mainland is the sister lineage to all conspecifics. Several of these patterns make geological sense, and the overall pattern may be consistent with an isolation-by-distance model. Misima and Woodlark islands were adjacent until the rifting of the Woodlark Basin began approximately 8 MYA (Little et al., 2007), so sister-group relationships between their biotic elements is tobe expected. Misima and Sudest belong to the Louisade Archipelago, the southeasternmost extension of the Owen Stanley Terrane, that had been continuous with the mainland during the distant past (B. Taylor, University of Hawaii, pers. comm., 2006). The D'Entrecasteaux islands have never been connected to the New Guinea mainland or to other island groups, but have been drawing nearer to New Guinea with time (H. Davies, University of Papua New Guinea, pers. comm., 2004). The herpetofauna of these islands is largely shared with the mainland, and endemic elements appear to be derived from sister taxa inhabiting that source (F.K., unpubl. data).

In *A. muelleri*, based on available sampling, major lineage divergence appears on either side of the Huon Peninsula (Figs. 1 and 2). This break may be linked to the fact that the northern portion of New Guinea, including the Torricelli Mtns. and the Bismarck Islands, belongs to the Solomon Island Arc and has been accreting onto mainland New Guinea within the last 5–6 million years (Fig. 1; Davies et al., 1998; Hall, 2002; Pigram and Davies, 1987; Pigram and Symonds, 1991; Polhemus and Polhemus, 1998). This possibility is supported by the clustering of our sample of *A. muelleri* from the Torricellis with the Sanders et al. (2008) sample from the island of New Ireland in our outgroup analysis (Figs. 1 and 2). These two samples are separated by approximately 1000 km but show only 2.5% *cyt-b* sequence divergence (data not shown), compared to 15.5% *cyt-b* sequence divergence between our samples from the Toricellis and Morobe, which are separated by only 500 km. This pattern suggests a biogeographic break, rather than simple isolation-by-distance. Further evidence of a biogeographic break between the Solomon Island Arc system and the rest of New Guinea occurs within *A. schlegelii,* where there is more than 19% sequence divergence in the *cyt-b* gene between samples collected in the Torricelli Mtns. (Solomon Arc system) and the sample collected in Indonesia (south of the Solomon Arc system). Evidence from other organisms also suggests that populations derived from this arc system are more closely related to each other than they are to the lineages inhabiting the Southeast Peninsula (Polhemus and Polhemus, 1998; van Welzen, 1998).

The high genetic distances between individuals within *A. schlegelii* are echoed in its congeners, and represent a level of divergence comparable to or greater than that seen within other hydrophiine genera (Fig. 2 and Supp. Table 1; Lukoschek and Keogh, 2006; Sanders et al., 2008; Wüster et al., 2005). In addition to the previously hypothesized problem with short internal branches, the genetic divergence found within each lineage and within *Aspidomorphus* as a whole may explain some of the difficulty encountered by previous studies in placing the taxon accurately within Hydrophiinae. The poor lineage sampling within *Aspidomorphus* in those studies may have caused problems with long-branch attraction, confusing the placement of the genus. Our improved support for the sister-group relationship between *Aspidomorphus* and *Demansia* vis a vis Sanders et al. (2008) likely derives from our improved taxon-sampling scheme. Because similar high levels of sequence divergence characterize other hydrophiine genera (Lukoschek and Keogh, 2006; Sanders et al., 2008; Wüster et al., 2005) a focus on more thorough taxon representation may help to resolve other relationships as well (Hillis, 1998; Rannala et al., 1998).

Even with limited sampling, our study has uncovered deep molecular divergences within *Aspidomorphus* species. Further sampling of this genus is likely to uncover additional deeply divergent lineages, particularly wherever populations are isolated, for exam-

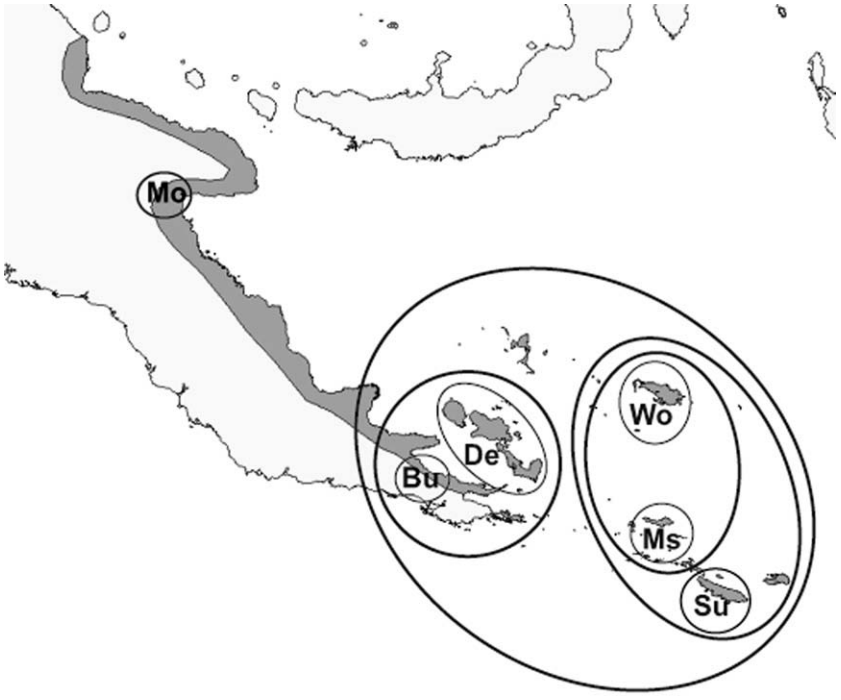

Fig. 5. Map showing clade relationships found in *A. lineaticollis*. Named lineages are indicated: (Mo) Morobe, (Bu) Bunisi, (De) D'Entrecasteaux archipelago, (Wo) Woodlark Island, (Ms) Misima Island, (Su) Sudest Island.

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ple in the southern portion of mainland New Guinea (*A. muelleri*) or the Bird's Head Peninsula at the northwest extreme of the mainland (*A. schlegelii* and *A. muelleri*). Furthermore, it is likely that barriers to gene flow within *Aspidomorphus* may be acting as barriers for other organisms on New Guinea; this is especially likely among the islands of the Milne Bay Province, but will probably apply elsewhere as well. Our study lends additional support to a growing body of research suggesting the existence of considerable cryptic diversity in the Papuan region specifically (Baker et al., 2008; Kraus, 2007; Kraus, 2008; Rawlings and Donnellan, 2003; Zug, 2004), and the tropics as a whole (Duda et al., 2008; Helgen, 2005; Moritz et al., 1993; Padial and de la Riva, 2009) and provides the phylogenetic background for a comparative analysis to look for factors driving cryptic diversification.

4.4. Conservation implications

New Guinea is the third-largest tract of High Biodiversity Wilderness in the world, behind the Amazon and the Congo (Mittermeier et al., 2003). While approximately 70% of the island's forested areas remain intact, deforestation from logging and agriculture is mounting. As of 2002, the forests in Papua New Guinea are being lost at a rate of 1.41% per year, and commercially accessible forests are being lost at twice the overall rate (Shearman et al., in press). Nearly half of the commercially accessible forests are already under logging concessions. If current trends continue, more than 80% of commercially accessible forest will be gone by 2021 (Shearman et al., 2008). A comprehensive system for conservation and protected areas management is vitally important for New Guinea but is not yet in place. Cryptic diversity and restricted range endemism, both of which occur frequently on New Guinea, must be considered during the establishment of any conservation and management system. Our study highlights the importance of augmenting morphological taxonomic descriptions with molecular analysis when cataloging diversity and determining conservation priorities.

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Appendix A

Localities and identification tags for samples used in this study and GenBank accession numbers for sequences generated for ingroup analysis. PNG = Papua New Guinea, NSW = New South Wales, F = Fergusson Island, G = Goodenough Island, N = Normanby Island; AMS = Australia Museum, BPBM = Bishop Museum, PNGNM = Papua New Guinea National Museum.

Taxon	Location	Catalogue No.	Field No.	*cyt-b*	SPTBN1
Aspidomorphus lineaticollis	Kamiali, PNG	BPBM 31736	AA 19131	GQ397128	GQ397173
A. lineaticollis	Bunisi, PNG	BPBM 17282	FK 7351	GQ397135	GQ397175
A. lineaticollis	Bunisi, PNG	BPBM 17283	FK 7782	GQ397136	GQ397176
A. lineaticollis	D'Entrecasteaux, F	BPBM 16540	FK 5773	GQ397129	GQ397174
A. lineaticollis	D'Entrecasteaux, F	BPBM 16541	FK 6141	GQ397130	GQ397174
A. lineaticollis	D'Entrecasteaux, F	uncat. PNGNM	FK 6220	GQ397131	GQ397174
A. lineaticollis	D'Entrecasteaux, F	BPBM 16542	FK 6458	GQ397132	GQ397177
A. lineaticollis	D'Entrecasteaux, N	BPBM 16543	FK 6700	GQ397133	GQ397174
A. lineaticollis	D'Entrecasteaux, N	BPBM 17281	FK 7186	GQ397134	GQ397174
A. lineaticollis	D'Entrecasteaux, G	AMS 125028	NR 1800	GQ397141	—
A. lineaticollis	D'Entrecasteaux, G	AMS 125029	NR 1801	GQ397140	—
A. lineaticollis	D'Entrecasteaux, G	AMS 125030	NR 1802	GQ397141	—
A. lineaticollis	Woodlark	BPBM 17860	AA 16596	GQ397137	—
A. lineaticollis	Woodlark	BPBM 17862	AA 16715	GQ397138	—
A. lineaticollis	Misima	AMS 125020	NR 1788	GQ397139	—
A. lineaticollis	Misima	AMS 125021	NR 1790	GQ397142	—
A. lineaticollis	Misima	AMS 125022	NR 1791	GQ397143	—
A. lineaticollis	Misima	AMS 125023	NR 1794	GQ397144	—
A. lineaticollis	Misima	AMS 125024	NR 1796	GQ397145	—
A. lineaticollis	Misima	AMS 125025	NR 1798	GQ397146	—
A. lineaticollis	Sudest	uncat. PNGNM	FK 9519	GQ397152	GQ397178
A. lineaticollis	Sudest	BPBM 20782	FK 9547	GQ397147	GQ397178
A. lineaticollis	Sudest	BPBM 20783	FK 9671	GQ397148	GQ397178
A. lineaticollis	Sudest	BPBM 20786	FK 9722	GQ397148	GQ397178
A. lineaticollis	Sudest	BPBM 20784	FK 9742	GQ397149	GQ397179
A. lineaticollis	Sudest	BPBM 20785	FK 9813	GQ397148	GQ397178

(*continued on next page*)

Appendix A (*continued*)

Taxon	Location	Catalogue No.	Field No.	*cyt-b*	SPTBN1
A. lineaticollis	Sudest	uncat. PNGNM	FK 9873	GQ397148	GQ397179
A. lineaticollis	Sudest	BPBM 20787	FK 9875	GQ397150	GQ397179
A. lineaticollis	Sudest	BPBM 20788	FK 9877	GQ397151	GQ397180
A. lineaticollis	Sudest	BPBM 20789	FK 9878	GQ397151	GQ397179
Aspidomorphus muelleri	Torricelli Mtns, PNG	BPBM 23453	FK 11473	GQ397153	GQ397183
A. muelleri	Apele, PNG	BPBM 18929	FK 8587	GQ397161	GQ397187
A. muelleri	Laronu, PNG	BPBM 19496	FK 8773	GQ397162	GQ397184
A. muelleri	Laronu, PNG	BPBM 19497	FK 8774	GQ397163	GQ397184
A. muelleri	Laronu, PNG	BPBM 19498	FK 8775	GQ397164	GQ397188
A. muelleri	Laronu, PNG	BPBM 19500	FK 9255	GQ397166	GQ397184
A. muelleri	Bunisi, PNG	BPBM 17284	FK 7391	GQ397157	GQ397181
A. muelleri	Bunisi, PNG	BPBM 19030	FK 7554	GQ397158	GQ397184
A. muelleri	Bunisi, PNG	BPBM 17285	FK 7555	GQ397159	GQ397185
A. muelleri	Bunisi, PNG	BPBM 17286	FK 7765	GQ397160	GQ397186
A. muelleri	Bunisi, PNG	BPBM 17287	FK 7839	GQ397157	GQ397181
A. muelleri	Mt. Obree, PNG	BPBM 19499	FK 8856	GQ397165	GQ397184
A. muelleri	Cloudy Mtns, PNG	BPBM 15154	FK 5179	GQ397154	GQ397182
A. muelleri	Cloudy Mtns, PNG	BPBM 15155	FK 5305	GQ397155	GQ397187
A. muelleri	Cloudy Mtns, PNG	BPBM 19029	FK 5325	GQ397156	GQ397187
Aspidomorphus schlegelii	Tangguh, Papua, Indonesia	uncatalogued	AA 16304	GQ397169	GQ397189
A. schlegelii	Torricelli Mtns., PNG	BPBM 23434	FK 11877	GQ397168	GQ397191
A. schlegelii	Torricelli Mtns., PNG	BPBM 23433	FK 11616	GQ397167	GQ397190
Demansia psammophis	NSW, Australia	AMS 147748	NR 3389	GQ397172	GQ397192
Micropechis ikaheka	Mt. Shungul, PNG	BPBM 18936	FK 8625	GQ397171	GQ397194
Toxicocalamus loriae	Mt. Simpson, PNG	BPBM 17987	FK 7523	GQ397170	GQ397193

Appendix B

Locality, identification numbers, and GenBank accession numbers for sequences generated for outgroup analysis. PNG = Papua New Guinea, NSW = New South Wales, F = Fergusson Island; AMS = Australia Museum, BPBM = Bishop Museum, PNGNM = Papua New Guinea National Museum.

Taxon	Location	Catalogue No.	Field No.	12S	16S	ND4	MyHC-2	*c-mos*	RAG-1
Aspidomorphus lineaticollis	d'Entrecasteaux, F	uncat. PNGNM	FK 6220	GQ397245	GQ397237	GQ397205	GQ397217	GQ397227	GQ397198
A. lineaticollis	Sudest	uncat. PNGNM	FK 9873	GQ397247	GQ397239	GQ397212	GQ397219	GQ397229	GQ397199
Aspidomorphus muelleri	Torricellis, PNG	BPBM 23453	FK 11473	GQ397251	GQ397233	GQ397207	GQ397214	GQ397224	GQ397195
A. muelleri	Apele, PNG	BPBM 18929	FK 8587	GQ397250	GQ397241	GQ397213	GQ397221	GQ397231	GQ397202
A. muelleri	Laronu, PNG	BPBM 19497	FK 8774	GQ397249	GQ397242	GQ397206	GQ397222	GQ397232	GQ397203
Aspidomorphus schlegeli	Toricellis, PNG	BPBM 23433	FK 11616	GQ397252	GQ397234	GQ397204	GQ397215	GQ397223	GQ397196
A. schlegeli	Papua, Indonesia	uncatalogued	AA 16304	GQ397246	GQ397238	GQ397210	GQ397218	GQ397228	GQ397200
Demansia psammophis	NSW, Australia	AMS 147748	NR 3389	GQ397248	GQ397240	GQ397209	GQ397220	GQ397230	GQ397201
Micropechis ikaheka	Mt. Shungul, PNG	BPBM 18936	FK 8625	GQ397243	GQ397236	GQ397208	—	GQ397226	—
Toxicocalamus loriae	Mt. Simpson, PNG	BPBM 17987	FK 7523	GQ397244	GQ397235	GQ397211	GQ397216	GQ397225	GQ397197

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.07.027.

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