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Phylogeography, geographic structure, genetic variation, and potential species boundaries in Philippine slender toads

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ABSTRACT

We investigated phylogeography of Philippine slender toads (genus *Ansonia*) and used a temporal framework for diversification, statistical tests of alternate topologies, and Bayesian approaches to test previous hypotheses concerning dispersal to, and colonization routes within, the southern Philippine island of Mindanao. Two species of *Ansonia* previously have been documented, with ranges separated by an east–west split corresponding to the approximate boundaries of Mindanao’s paleoisland precursors. We present new mtDNA sequence data (1946 bp from genes encoding ND1, 16S rRNA and tRNA^{Leu}) for 105 *Ansonia* specimens sampled from 20 localities on Mindanao Island. Our data suggest that Philippine *Ansonia* is composed of at least eight, well-supported population lineages, structured into a minimum of four highly divergent mtDNA clades. One clade corresponds to *Ansonia mcgregori*, a range-restricted species apparently limited to the distal portion of the Zamboanga Peninsula of western Mindanao. Two morphologically indistinguishable, but genetically divergent, lineages possibly are undescribed cryptic species from western Mindanao. We recognize the five remaining lineages as *Ansonia muelleri* pending data from morphology or bioacoustics that might diagnose separate species among these lineages. Regardless of their species status, the five allopatric lineages of *A. muelleri* should be viewed as important genetic units for future genetic conservation planning.

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1. Introduction

The geologically active islands of the Philippines possess high levels of endemic vertebrate biodiversity, which is predominantly partitioned hierarchically into (1) Pleistocene Aggregate Island Complexes (PAICs; Inger, 1954; Heaney, 1985; Voris, 2000; Brown and Diesmos, 2002), (2) individual islands within PAICs, and (3) montane subcenters of diversity within major landmasses (review: Brown and Diesmos, 2009). This nested, highly partitioned nature of the archipelago’s fauna has contributed to the recognition of the Philippines as a global conservation hotspot, with one of the highest concentrations of land-vertebrate diversity on the planet (Mittermeier et al., 1997, 1998, 1999; Reid, 1998; Brooks et al., 2002; Brown and Diesmos, 2009). However, the vast majority of this diversity is based on species boundaries conceived by traditional morphological taxonomy, calling attention to the possibility of hidden or “cryptic” species diversity masquerading in conserva-

tive or possibly outdated taxonomy (Alcala and Brown, 1998; Bickford et al., 2007; Brown, 2006; Brown and Alcala, 1994; Brown et al., 2000, 2008; Brown and Stuart, in press; Stuart et al., 2006).

With robust geographical sampling of genetic material from throughout the archipelago, a new group of studies have drastically increased species diversity in several clades (e.g., Brown and Guttman, 2002; Brown et al., 2009; Evans et al., 2003a; Linkem et al., 2010a, 2010b; Siler et al., 2010; Siler and Brown, 2010; Welton et al., 2010a, 2010b). Analyses of rates of species description through time reveal rates of species discovery and description unparalleled in the history of Southeast Asian biodiversity studies (Brown and Diesmos, 2002; Brown et al., 2002, 2008; Stuart and Bain, 2008; Brown and Stuart, in press; Siler et al., 2010, 2011).

Relative to other parts of the archipelago, the large southern island of Mindanao has not received the same renewed research focus, due principally to the inaccessibility of its many isolated mountains and logistical obstacles to field work. Much of what is known of the island’s high levels of herpetological diversity and endemism (Taylor, 1920, 1928; Inger, 1954; Brown and Alcala, 1970; Alcala and Brown, 1998; review: Brown et al., 2000, 2008) comes from faunal inventories conducted during the early European exploration (e.g., Boulenger, 1882, 1920; Van Kampen, 1923; Smith, 1930, 1935), field work conducted in the early

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1900s (Taylor, 1915, 1918, 1920, 1922a, 1922b), a single major expedition following World War II (Hoogstral, 1951; Inger, 1954), and field work from the 1960s and 1970s (summarized in Leviton (1963), Brown and Alcala (1970, 1978, 1980)). Subsequent works have been scattered, site-specific, and have not yet been synthesized in a biogeographic context (Smith, 1993a, 1993b; Amoroso, 2000; Delima et al., 2006, 2007; Nuñez et al., 2010). Although endemic Mindanao species exemplars have been included in country-wide or regional phylogenetic studies (e.g., McGuire and Alcala, 2000; McGuire and Kiew, 2001; Brown and Guttman, 2002; Evans et al., 2003a; Siler et al., 2011), no densely sampled phylogeographic or population genetic studies of Mindanao endemic vertebrate groups have been conducted.

Here we present a phylogeographic study of the endemic Mindanao slender toads of the genus *Ansonia*. Philippine species of *Ansonia* historically have been divided into two species, *Ansonia muelleri* (Boulenger, 1887, from “Mindanao Island;” presumably eastern Mindanao; Inger, 1954, 1960; Alcala and Brown, 1998), and *Ansonia mcgregori* (Taylor, 1922c; from the southern tip of the Zamboanga Peninsula and nearby Basilan Island; Fig. 1). In his first review of Philippine Amphibia, Inger (1954) questioned the validity of Taylor’s *A. mcgregori*. However, in later works he treated both *A. mcgregori* and *A. muelleri* as valid species (Inger, 1960, 1966); other workers have not questioned this perspective (Alcala and Brown, 1998; Brown, 2007). Finally, one recent species-level phylogenetic study (Matsui et al., 2010) supported the distinctiveness of two species of *Ansonia* on Mindanao and postulated an ancient (~20 mya) invasion of the southern Philippines for the pair of species on Mindanao.

In this paper we ask: (1) do robust genetic sampling and phylogeographic analyses support the recognition of only two species of Philippine *Ansonia*? Alternatively, do phylogeographic patterns indicate the presence of additional divergent lineages that might

warrant recognition as species or Evolutionary Significant Units (ESUs) for conservation planning? (2) Are geographic patterns of genetic variation consistent with stable, geographically structured, populations or can we detect the signature of recent geographic or demographic expansion? (3) Do genetic areas of endemism correspond to the isolated montane areas of Mindanao, as would be predicted by natural history, larval morphology, and torrent-dwelling larval ecology (Inger, 1960; Brown and Alcala, 1982; Alcala and Brown, 1998; Inger, 1992)? (4) Can we reject Matsui et al.’s (2010) recent “early invasion” hypothesis as a general temporal framework for the invasion of the southern Philippines and subsequent diversification of Philippine *Ansonia*? (5) Finally, can we reject hypothesized routes of dispersal along Mindanao’s elongate mountain chains as possible corridors for colonization and population expansions in order to account for the current distribution of *Ansonia* on the island of Mindanao?

1.1. Geological setting and Mindanao biogeography

Because the central portion of the island of Mindanao has been formed by a sequence of collision, accretion, and subduction events that have occurred over the past ten million years (Yumul et al., 2003, 2009; Hall, 1996, 1997), it is conceivable that the highly dynamic history of the southern Philippines in part contributed to diversification of its fauna. An improved knowledge of the extent of land emergence (Lewis, 1997; Hall, 1998), combined with detailed information concerning the timing of landmass collision (Yumul et al., 2003; Hall, 1998), suggests possible dispersal routes for fauna entering the southern portions of the Philippines in its early history. For example, although some components (particularly Zamboanga and extreme eastern Mindanao) may have been land-positive greater than 15 mya, it is clear that they were very far apart, and differed radically from today’s configuration (Hall, 1996, 1997, 1998). By 10–5 mya, the Sulu Archipelago–Mindanao arc was forming as a series of islands distributed west-to-east across the southern Philippines (Yumul et al., 2003, 2009; Hall, 1998, 2002). During this period, with extreme western and eastern Mindanao formed as widely separated islands (Hamilton, 1979; Hall, 2002), a pair of subduction zones, centered on the Cotabato Trench and the Philippine trench, respectively (Yumul et al., 2003), lead to the uplift of portions of central Mindanao. These uplifts, combined with volcanic activity, produced many of the large mountains of the island (Hall, 1996; Yumul et al., 2008, 2009). Today Mindanao’s isolated mountain ranges are each separated from others by wide, expansive, low-elevation plains and valleys. Thus, it is plausible that the temporally variable, dynamic emergence of the major upland montane regions of Mindanao first appeared as an island archipelago that spanned much of today’s central Mindanao. It is also conceivable that *Ansonia* and other taxa may have dispersed through what is today’s Mindanao by island hopping west-to-east across this paleoarchipelago. Many of the foothills of the mountains of central Mindanao are noted for low-elevation bands of marine sediments, suggesting past seashores, and the possibility that the major montane components likely existed as islands, formerly separated by shallow seas (Taylor, 1925, 1975; Hamilton, 1979; Hall, 1998). Thus, an hypothesis of an early invasion of the southern Philippines, followed by coincident or subsequent diversification, may be at least partially consistent with some of the available geological evidence (Yumul et al., 2009) if the temporal framework for diversification was shown to be consistent with these earlier geological events.

Biogeographers have identified the eastern Philippine island arc as a possible dispersal route or entry point into the Philippines (Diamond and Gilpin, 1983; Brown and Guttman, 2002; Brown et al., 2009; Jones and Kennedy, 2008; Oliveros and Moyle, 2010). This hypothesized route of biogeographic dispersion into

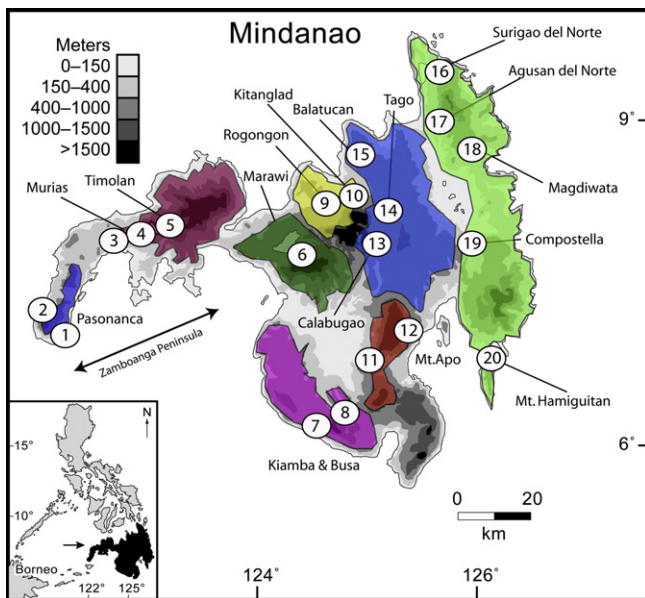


Fig. 1. Map of Mindanao Island, southern Philippines (topographical relief indicated with elevational increments of increasingly dark shading) with 20 sampling localities indicated and approximate expected (on the basis of topographic relief and larval biology of *Ansonia*; see Section 4) distribution of each genetic entity (haplotype clade, population lineage, or species) indicated with differently colored shading. Populations 1–3 correspond to *A. mcgregori*, populations 4 and 5 are *Ansonia* sp. 1, population 6 is *Ansonia* sp. 2, and the remaining localities correspond to the five lineages of central and eastern Mindanao (Fig. 2), referred here to *A. muelleri*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the archipelago has been invoked in conjunction with an “island hopping” mode of dispersal to explain the distribution of Mindanao faunal-region endemics (Brown and Guttman, 2002; Brown et al., 2009; Welton et al., 2010a, 2010b; Roberts et al., 2011). Scenarios of this type typically assume a series of successive dispersal events via the Sulu Archipelago (the small chain of islands between Borneo and Mindanao; Fig. 1), Basilan, Mindanao, Dinagat/Siargao, Bohol, Leyte, and finally Samar islands (e.g., Brown and Guttman, 2002; Brown et al., 2009). However, the curious absence of *Ansonia* on the northern islands of the Mindanao PAIC (e.g., Dinagat, Siargao, Bohol, Leyte, or Samar; Brown and Alcala, 1970; Alcala and Brown, 1998), plus only slight morphological character differences between the two nominal species, suggests at least the possibility of a recent (i.e., Pleistocene) invasion by a Bornean lineage as the basis of distribution of *Ansonia* in the extreme southern Philippines. In a recent species-level phylogenetic analysis including a few samples from Mindanao, Matsui et al. (2010) demonstrated that the two Philippine endemics were each others' closest relatives, moderately divergent (3–4% uncorrected sequence divergence) from one another and from Bornean congeners, and most closely related to *A. fuliginea* and *A. guibei* from Borneo (Inger, 1960, 1966). That study suggested an ancient (~20 mya) invasion of the southern Philippines by way of the Sulu archipelago (Matsui et al., 2010).

1.2. Larval biology, habitat, and ecological preferences of *Ansonia*

The genus *Ansonia* contains 26–32 named and unnamed taxa (Matsui et al., 2010; Quah et al., 2011) distributed from Thailand north of the Isthmus of Kra, through Peninsular Malaysia, and on the islands of Sumatra, Borneo (Indonesia and Malayisa), Basilan and Mindanao (Philippines). An understanding of the larval ecology and morphology of toads of the genus *Ansonia* may provide an explanation for their often patchy and topographically circumscribed pattern of montane endemism. *Ansonia* larvae are torrent specialists that possess a suite of highly derived morphological and ecological specializations (Inger, 1954, 1960, 1966, 1992, 1985, 2005; Duellman and Trueb, 1994; Matsui et al., 1998, 2005, 2007, 2009, 2010) enabling them exclusively to inhabit fast-flowing streams. *Ansonia* larvae possess specialized adhesive oral sucker mouthparts (Inger, 1985, 1992) allowing them to cling to rocks in the most rapid portions of high gradient, highly oxygenated streams (Inger, 1954, 1966, 2005). Philippine *Ansonia* tadpoles are exclusively found adhering to rocks under white-water cascades, around waterfalls, in shoots between boulders of steep, rocky montane habitats, or in foothills or large mountains with topographic relief (RMB, *personal observation*). Unable to survive in slow-moving, low-gradient streams, *Ansonia* larvae and adults are thus elevationally restricted to montane slopes or low-elevation habits at the immediate base of mountains (where water currents are sufficiently swift). Because of this unique larval morphology and natural history, we suspect that the low-lying valleys and inter-montane plains of Mindanao (Fig. 1) have been formidable barriers to dispersal. Thus, past dispersal, population dispersion, and gene flow should have been necessarily restricted to the strips of suitable habitat along the slopes and foothills of major mountains of Mindanao.

2. Materials and methods

2.1. Taxon sampling and data collection

In-group sampling included 105 individuals collected from 20 localities throughout Mindanao Island (Fig. 1; Appendix A). Our sampling maximizes geographical coverage of the island and,

importantly, includes genetic material from each of the isolated montane components of the island (Hall, 1996, 1997; Yumul et al., 2004, 2008). Outgroup samples were selected based on the results of Matsui et al. (2010), which supported *Ansonia guibei* and *A. fuliginea* as the closest relatives of Philippine lineages (Appendix A); accordingly, we used Matsui et al.'s *A. guibei* sequence (Voucher KUHEL06B 054) and two sequences from *A. fuliginea* (BOR 22770 and KUHE 17537) as outgroups for this study.

For our 105 samples, we generated complete or partial sequences for the mitochondrial genes encoding ribosomal RNA 16S (16S), NADH Dehydrogenase Subunit 1 (*ND1*), and a component of a single transfer RNA (tRNA^{leu}) (Appendix A). Only 16S sequences were previously published and available as outgroups (Matsui et al., 2010).

Genomic DNA was extracted from liver tissues stored in 95–100% ethanol following a guanidine thiocyanate protocol (Esselstyn et al., 2008). We used a combination of published and newly developed primers to amplify targeted gene regions. Two primers were used to amplify a ~840 bp region spanning most of the 16S ribosomal RNA gene via polymerase chain reaction: 5' to 3': 16Sc GTRGGCCTAAAAGCAGCCAC and 16Sd CTCGGTCTGAA CTCAGATCACGTAG (Moriarty and Cannatella, 2004); PCR thermal conditions followed Evans et al. (2003a). For *ND1*, we used ND1F2 CTACGTGATCTGAGTTCAGACCG and ND1R2 AAGGAGGTYC YTAWCTTTCGGGC primers and the same thermal profiles.

Amplified products were visualized on 1.5% agarose gels. PCR products were purified with 1 µL of a 20% dilution of ExoSAP-IT (US78201, Amersham Biosciences, Piscataway, NJ) on the following thermal profile: 31 min at 37°, followed by 15 min at 80°. Cycle sequencing reactions were run using ABI Prism BigDye Terminator chemistry (Ver. 3.1; Applied Biosystems, Foster City, CA), and purified with Sephadex (NC9406038, Amersham Biosciences, Piscataway, NJ) in Centri-Sep 96 spin plates (CS-961, Princeton Separations, Princeton, NJ). Purified products were analyzed with an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Continuous gene sequences were assembled and edited using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI). All sequences were deposited in GenBank (Accession Nos. JN314641–JN314839).

2.2. Sequence data, alignment, geographic structure, and phylogeographic analyses

Initial alignments were produced in Muscle (Edgar, 2004), and manual adjustments made in MacClade 4.08 (Maddison and Maddison, 2005). To measure phylogenetic congruence between the two mitochondrial fragments, we inferred the phylogeny for each gene region independently using separate likelihood and Bayesian methods. Following the observation of no incongruence between single-gene region topologies, we concatenated the data for subsequent analyses. Exploratory analyses of the combined dataset of 108 individuals (including 12 individuals with missing data for one of the two genes) and a reduced dataset of individuals with no missing data exhibited identical relationships. We therefore chose to include all available data for 108 individuals in subsequent analyses of the concatenated dataset. After excluding 98 bp of ambiguous 16S rRNA and tRNA, the final dataset totaled 1946 aligned nucleotide positions. Our dataset was complete for 16S and nearly complete for the *ND1* region (missing *ND1* only for the Bornean outgroups, two *A. mcgregori* samples, and seven *A. muelleri* samples).

To assess general patterns of genetic diversity within clades, we calculated the numbers of haplotypes (*N*), haplotype diversity (*h*; Nei, 1987), numbers of polymorphic sites, and nucleotide diversity (π ; Nei and Tajima, 1981) using DNASP 4.0 (Rozas et al., 2003) and

Arlequin 3.1 (Excoffier et al., 2005) for each major lineage of Mindanao *Ansonia* following the methods of Brown et al. (2010).

Parsimony analyses were conducted in PAUP* 4.0b 10 (Swofford, 2002), with gaps treated as missing data and all characters weighted equally. Most-parsimonious trees were estimated using heuristic searches with 1000 random addition-sequence replicates and tree bisection and reconnection (TBR) branch swapping. To assess clade confidence, nonparametric bootstrapping was conducted using 1000 bootstrap replicates, each with 100 random addition-sequence replicates and TBR branch swapping.

Partitioned Bayesian analyses were conducted in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). *ND1* was partitioned by codon position; *16S* and the single tRNA (*tRNA^{Leu}*) were treated as a single partition. The Akaike Information Criterion (AIC), as implemented in jModeltest v0.1.1 (Guindon and Gascuel, 2003; Posada, in press), was used to select the best-fit model of nucleotide substitution for each partition (Table 1). The best-fit model for each data partition was implemented in subsequent Bayesian analyses. A rate multiplier model was used to allow substitution rates to vary among subsets, and default priors were used for all model parameters. We ran four independent Metropolis-coupled MCMC analyses, each with four chains and an incremental heating temperature of 0.05. All analyses were run for 40 million generations, sampling every 5000 generations. To assess stationarity, all sampled parameter values and log-likelihood scores from the cold Markov chain were plotted against generation time and compared among independent runs using Tracer v1.4 (Rambaut and Drummond, 2007). Furthermore, we plotted the cumulative and non-overlapping split frequencies of the 20 most variable nodes, and compared split frequencies among independent runs using Are We There Yet? [AWTY (Wilgenbusch et al., 2004)]. Although all samples showed patterns consistent with stationarity after 2.5 million generations (i.e., the first 12.5%), we conservatively discarded the first 20% of samples as burn-in.

Partitioned maximum likelihood (ML) analyses were conducted in RAXMLHPC v7.0 (Stamatakis, 2006) on the concatenated dataset using the same partitioning strategy as above. The complex GTR + Γ model was used for all subsets, and 100 replicate ML inferences were performed. Each inference was initiated with a random starting tree, and employed the rapid hill-climbing algorithm (Stamatakis et al., 2007). Clade confidence was assessed with 100 bootstrap pseudoreplicates (Stamatakis et al., 2008).

2.3. Hypothesis testing: taxonomy and dispersal corridors

Current taxonomy suggests the occurrence of one species (*A. muelleri*) in central and eastern Mindanao (Boulenger, 1887; Taylor, 1922a, 1922b, 1922c; Inger, 1954, 1960, 1966; Alcalá and Brown, 1998; Brown, 2007) and another (*A. mcgregori*) confined to the extreme western Mindanao Zamboanga Peninsula. We tested this hypothesis by searching the post burn-in sample for topologies consistent with a simple east–west geographic split, corresponding to the suture (Fig. 1) between the Zamboanga Peninsula and the remaining portions of Mindanao.

Additionally, we evaluated five hypotheses of dispersal routes and phylogeographic history (Fig. 3) based on the assumption that

Table 1
Models of evolution selected by AIC and applied for partitioned, model-based phylogenetic analyses.

Partition	AIC model	Model applied	Number of characters
<i>ND1</i> , 1st codon position	TVM + Γ	GTR + Γ	310
<i>ND1</i> , 2nd codon position	F81	HKY	310
<i>ND1</i> , 3rd codon position	TrN + Γ	GTR + Γ	309
<i>16S</i>	TVM + Γ	GTR + Γ	944
<i>tRNA^{Leu}</i>	HKY	HKY	73

larval biology and specialization to torrent habitats has restricted dispersal of Mindanao *Ansonia* to mountain slopes and the foothills of Mindanao's major montane components. We generated hypotheses of phylogeographic history based primarily on the geological history of Mindanao Island (Hamilton, 1979; Yumul et al., 2003, 2004, 2008, 2009; Hall, 1996, 1997, 1998, 2002), and the general expectations that (1) *Ansonia* populations should be confined to strips of suitable habitat along Mindanao's elongate mountain chains and (2) an overall patterns of west-to-east invasion and colonization should have occurred (and, in fact, was detected in the signal of a previous species-level phylogeny for the genus; Matsui et al., 2010). The topological constraints that we formulated to test hypothesized routes of dispersal are illustrated in Fig. 3.

We evaluated hypotheses with two methods, via a Bayesian approach of evaluating alternative topologies contained in post-burnin samples and with Approximately Unbiased tests (AU; Shimodaira and Hasegawa, 2001; Shimodaira, 2002).

We began by constructing constraint trees consistent with our *a priori* hypotheses (see below) in McClade. Post-burnin trees were then filtered (in PAUP) for topologies consistent with these constraint topologies. Constraint topologies were conservatively constructed to address only specific components of each dispersal scenario (Fig. 3). For the Bayesian implementation, we took the percentage of 6400 post-burnin trees consistent with individual constraint hypotheses to represent the posterior probability that explains our data.

AU tests were then performed on the per-site likelihoods using CONSEL v0.1i (Shimodaira and Hasegawa, 2001), following the methods of Siler et al. (2010). The *p*-value reported for a given hypothesis is the largest *p*-value of all the trees inferred under that constraint. We automated the process with Perl and Python scripts (written by J. Oaks and CDS; available by request).

The first hypothesis that we tested addresses the expectation of a west-to-east, stepping-stone mode of dispersal up the Zamboanga Peninsula following presumed dispersal from Basilan Island (Matsui et al., 2010). For this hypothesis (Fig. 3a) we constructed a grade-like constraint topology, with western populations constrained to be the sister taxon to populations from other areas of modern Mindanao. This hypothesis was very similar to the taxonomic hypothesis (*A. mcgregori* + *A. muelleri*), above, except that Lake Lanao populations from extreme western Mindanao were constrained to be the sister taxon to the remaining populations of Mindanao (arranged in a polytomy). For Hypothesis 2 (Fig. 3b), we left Zamboanga and western Mindanao populations free to vary outside the clade comprising 7–15, and tested a hypothesized route of dispersal from west-central highlands to east-central highlands, south via the Apo Massif, with final dispersal events into the southern mountains of Cotabato and Sarangani Provinces. In Hypothesis 2, we did not constrain eastern Mindanao populations (16–20) in any way. For Hypothesis 3 (Fig. 3c), we tested an alternative route of dispersal into central Mindanao, namely the southern route along the Cotabato coast, the Mt. Busa Range, the Apo Massif, and finally the east-central highlands. As before, we did not constrain branches leading to western or eastern Mindanao populations, but allowed these terminals to vary freely outside the clade comprising populations 6–8 + 11–15 in analyses. In Hypotheses 4 and 5, we addressed the origins of the eastern Mindanao populations. For Hypothesis 4 (Fig. 3d) we tested for evidence of a northern entryway into eastern Mindanao (east-central highlands, northeast mountains, southeast mountains), and for Hypothesis 5 (Fig. 3e) we constructed a constraint representing the opposite route, namely dispersal into eastern Mindanao via the Apo Massif, to the southeastern mountains, and finally the northeastern range. As in the case of the previous taxonomic hypothesis of two species, we used the percentage of 6400 post-burnin trees that were consistent with each hypothesis as

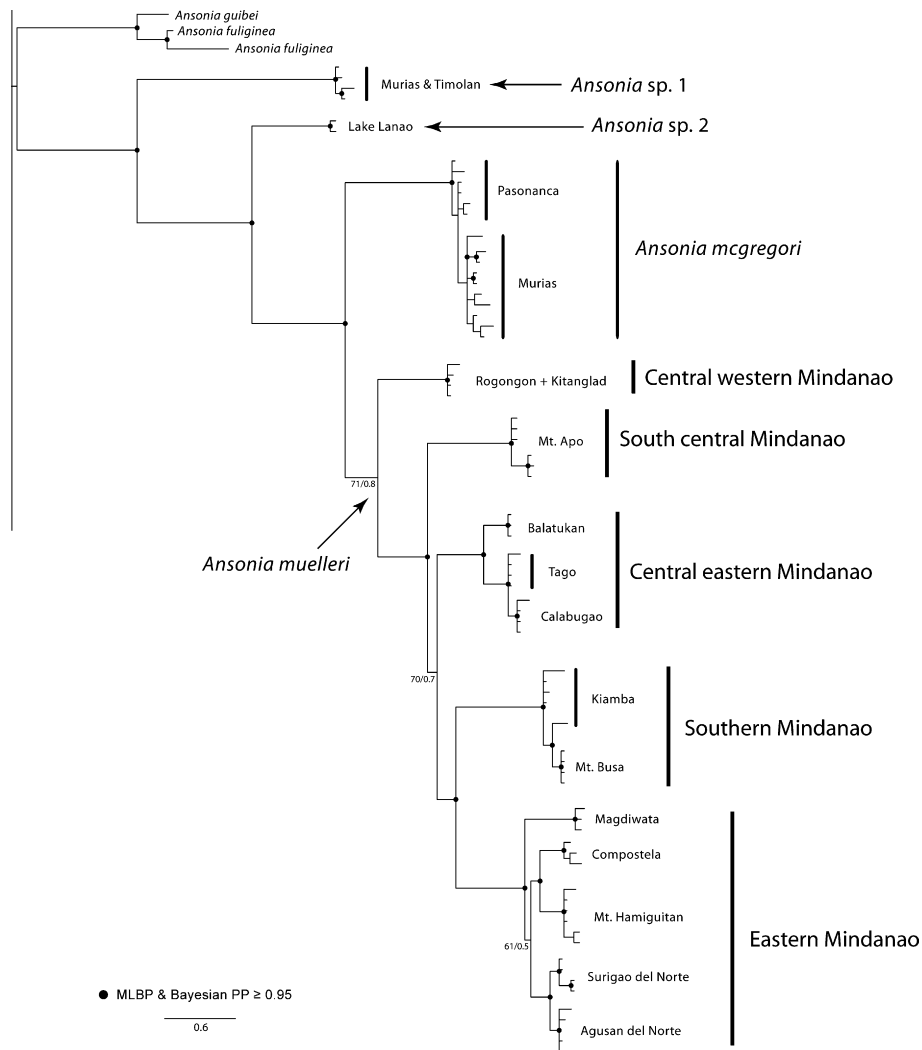


Fig. 2. Preferred phylogenetic estimate inferred from Bayesian, Likelihood, and Parsimony (not shown) analyses of mitochondrial DNA sequence data. Nodes supported by $\geq 95\%$ Bayesian PP and ML bootstrap support were considered significantly supported and are indicated by black circles. Terminals are labeled with abbreviated site names, followed by general geographic distribution (Appendix A).

the posterior probability that an individual hypothesis explained our data.

2.4. Inference of historical demography

Because of the uncertainty with regard to taxonomy of Mindanao *Ansonia* (see Sections 3 and 4), wherever possible, we assessed each of the various lineages of Mindanao *Ansonia* for evidence of recent change in effective population size in nested fashion. First, we performed population genetic and historical demographic analyses at a fine scale, focusing separately on six of the eight (those with sufficient population sampling) empirically observed haplotype groups. These included one haplotype clade clearly assignable to *A. mcgregori* (see below) and also each of the five divergent and allopatric haplotype clades of central and eastern Mindanao (see Section 4). We then combined these five minimally- to moderately-divergent lineages into a single analysis to examine population genetic and demographic parameters under a conservative hypothesis of a single, widespread species (putatively *A. muelleri*).

We calculated mismatch distributions in Arlequin 3.1 (Excoffier et al., 2005), which determines significance via coalescent simulations of a large, neutrally evolving population of constant size in the context of assumed selectively neutral nucleotide substitutions (Slatkin and Hudson, 1992; Rogers and Harpending, 1992). We

qualitatively assessed mismatch distributions for multimodal (which could stem from a structured population) versus smooth unimodal (which may be indicative of possible recent population expansion or sudden panmixia) distributions and calculated raggedness indexes and their significance in Arlequin (Rogers and Harpending, 1992; Harpending et al., 1998). We also calculated Tajima's D (implemented as a test for selective neutrality), and Ramos and Rozas R_2 statistics (Ramos-Onsins and Rozas, 2002), as additional indicators of potential population expansion. Finally, we investigated the possibility of population or demographic expansion using Fu's F_s neutrality test (Fu, 1997). Fu's F assumes neutrality and may diagnose recent population or demographic expansion via a highly negative value of F_s .

Because F_s and R_2 are summary statistics (based on distributions of haplotypes and numbers of segregating sites) they do not use all of the historical information contained in DNA sequence variation (Galbreath et al., 2009). Alternatively, we assessed changes in demographic expansion of estimated effective population size over the history of each major lineage by applying Bayesian skyline procedures (Drummond et al., 2005) in BEAST 1.6.1 (Drummond and Rambaut, 2006, 2007) to the clade comprising *A. mcgregori*, three of Mindanao's five central and eastern gene lineages separately (those with sufficient sampling: Busa–Kiamba, Balatukan–Tago–Calabugao, and Eastern Mindanao), and a final

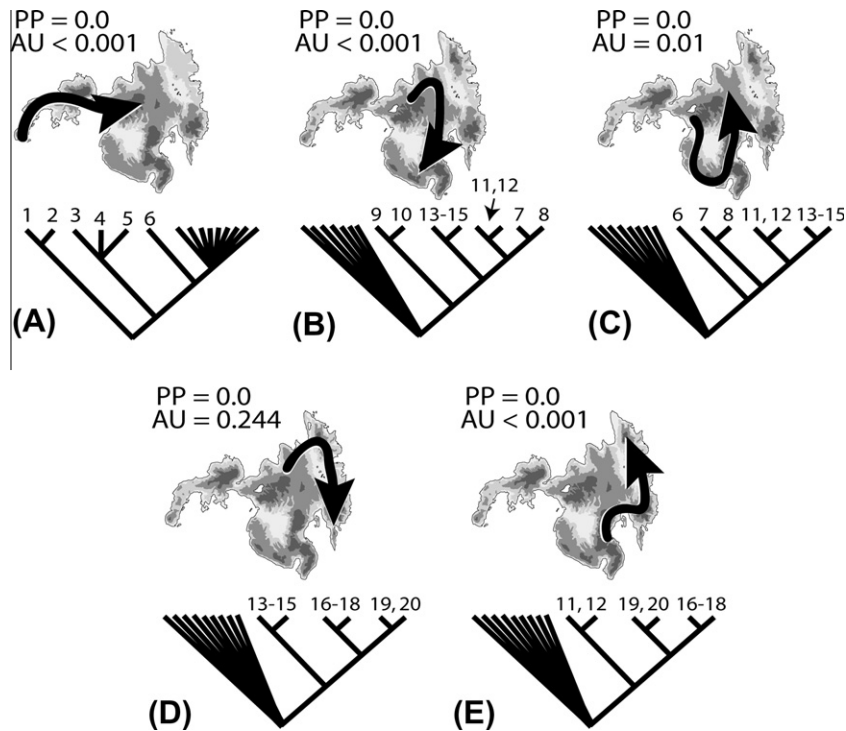


Fig. 3. Five historical hypotheses (constraint topologies) employed to test dispersal routes (montane corridors) to account for the observed pattern of occurrence of *Ansonia* lineages on Mindanao Island. Numbered populations on tree terminals correspond to sampling localities in Fig. 1. See text for detailed explanation of each hypothesis. PP = Posterior Probability; AU = p -value of each hypothesis as inferred from the Approximately Unbiased tests (AU; Shimodaira and Hasegawa, 2001; Shimodaira, 2002).

analysis with these five lineages combined (*A. muelleri*), as described above. We attempted to diagnose fluctuations in population median effective size over time for each population by approximating the posterior distribution of effective population size (from the original, unreduced dataset) over intervals of the phylogeny. For these two analyses we chose the appropriate model of sequence evolution with AIC in jModeltest, and generated input files with BEAUTi, using the same or closest available (and next-most, parameter-rich) model. We ran both analyses for 20 million steps, using default parameters, sampling every 1000 steps, and conservatively discarded the first 10% of samples as burn-in. We employed a fixed substitution rate of 1.4%/my, which was inferred from the entire dataset and the placement of an uncorrelated log-normal prior on a mean substitution rate spanning a range of substitution rates inferred from recent studies involving amphibian mitochondrial DNA (see below).

Analyses were independently replicated four times with different random starting seeds, and results then were combined in LOGCOMBINER 1.4.7 (after burn-in) after examining convergence diagnostics in TRACER 1.4 (Rambaut and Drummond, 2007).

2.5. Timing of diversification

The absence of a fossil record for *Ansonia* or closely related members of the family Bufonidae renders precise and accurate estimation of divergence times difficult. Matsui et al. (2010) extended their analysis of species relationships in *Ansonia* by inclusion of outgroup bufonids from the Pramuk et al. (2007) dataset, permitting two external calibration points for a simultaneous Bayesian estimation of phylogeny and ingroup divergence times. That approach yielded an estimated mean age of 20.2 MY (9.8–31.8 HPD) for the node subtending *A. mcgregori* + *A. muelleri* (Matsui et al., 2010).

The ancient above-water existence of the Zamboanga Peninsula and its proximity to the Sunda Shelf makes plausible a very early

invasion of Mindanao. If early invaders of the Mindanao paleoarchipelago were widespread when these landmasses collided (10–5 mya) and the major period of mountain-building began with subduction at the Cotobato and Philippine trenches (Yumul et al., 2003, 2008, 2009), diversification of Philippine *Ansonia* could have been associated with orogenic vicariance during the uplift and elevational isolation of Mindanao's major mountain chains (Hall, 1996; Yumul et al., 2003). We evaluate this possible scenario in the context of our temporal framework provided by divergence-date estimation.

As a test of the Matsui et al. (2010) calibration, we employed an alternative approach and inferred approximate divergence dates with a Bayesian relaxed molecular clock in BEAST v1.6.1. A variety of recent empirical studies of amphibians (using a variety of calibrations, taxa, and mtDNA gene fragments) have inferred model-corrected mitochondrial sequence divergence rates of between 0.8 and 1.9% total divergence per million years (Tan and Wake, 1995; Macey et al., 1998, 2001; Crawford, 2003a, 2003b; Wang et al., 2008) for various parts of the genome. We used this range and a relaxed clock approach as implemented in BEAST, allowing branch lengths to vary according to an uncorrelated lognormal distribution, employing a Yule process tree prior, with all remaining priors set to default. We selected a rate prior with a normal distribution and a mean of 1.4% (with a CI of 0.008–0.019) and simultaneously estimated divergence dates and chronogram topology. For this analysis we selected the GTR + Γ model and ran four independent analyses of 60,000,000 steps each, reviewed convergence diagnostics in Tracer v1.5 (Rambaut and Drummond, 2007), and combined runs in LogCombiner v1.6.1 after confirming convergence and conservatively excluding 10,000 trees as burn-in. Finally, we summarized results as a maximum clade credibility tree (Drummond and Rambaut, 2006, 2007) in TreeAnnotator v1.6.1.

We recognize that any approach lacking multiple reliable internal calibration points is subject to a variety of sources of error (Graur and Martin, 2004). However we proceeded with the analysis

presented here to provide an approximate estimate for diversification of Mindanao *Ansonia* and to provide an alternative to past analyses (Matsui et al., 2010), which we suspect presented unrealistically old dates for some of the same nodes in our tree. Additionally, this approach allows us to consider the speciation-by-orogenic-vicariance scenario, described above.

3. Results

3.1. Taxon sampling, sequence data, and alignment

The complete, aligned matrix contains 105 newly sequenced samples of Mindanao slender toads. Three additional samples were included as outgroups, consisting of representatives of *Ansonia guibei* and *A. fuliginea*. Following initial unrooted analyses, and the results of a recent phylogenetic study of *Ansonia* (Matsui et al., 2010), we rooted the tree using samples of *A. guibei* and *A. fuliginea*. Within each gene region, variable and parsimony-informative characters were observed as follows: 174 and 147 out of 944 (16S); 166 and 155 out of 929 (ND1); 10 and 10 out of 73 (tRNA^{leu}).

3.2. Phylogeography and analyses of population structure

We identified 58 unique haplotypes among the Philippines *Ansonia* (Table 2). These form four major clades, corresponding to *A. mcgregori* (including the type locality; sixteen distinct haplotypes and 25 polymorphic sites), a clade from Murias and Timolan (4 haplotypes and 33 polymorphic sites), a clade represented by two samples from Lake Lanao (2 haplotypes and 3 polymorphic sites), and five divergent mitochondrial gene lineages, forming a clade we putatively assign to *A. muelleri* (see Section 4; 42 haplotypes with 224 polymorphic sites). Within these four clades, mean number of pairwise nucleotide differences (k), haplotype diversity (h), and percent nucleotide diversity (π) are all relatively low (Table 2). As expected, pairwise nucleotide differences (k) and nucleotide diversity (π) were highest in the large geographic sample referred to *A. muelleri* (especially the Rogongon, Balatukan, and Eastern Mindanao lineages; Table 2), but interestingly k and π were also relatively high in the Murias/Timolan lineage, despite the fact that only four individuals were sequenced in this group. All measures of sequence diversity were lowest in *A. mcgregori* and in the Lake Lanao clade (Table 2).

Phylogenetic analyses of the combined ND1 + 16S data yielded well-resolved topologies with high bootstrap support (MP and ML) and posterior probabilities throughout most trees (Fig. 2; MP results not shown). Topologies were not statistically incongruent regardless of the analytical method, suggesting strong

phylogenetic signal and a lack of any conflict among optimality criteria.

All analyses suggest the existence of eight markedly divergent population lineages diagnosed by mitochondrial haplotype clades (Figs. 1 and 2). Each of these eight haplotype clades is strongly supported with likelihood bootstrap values of ≥ 95 and posterior probabilities of ≥ 0.95 (Fig. 2). With one exception, relationships among these eight clades are also strongly supported (MLBP ≥ 95 ; PP ≥ 0.95). The exception involved moderate likelihood support (MLBP = 71) and Bayesian support (PP = 0.80) for the placement of the Rogongon and Kitanglad lineage as the sister taxon to the remaining samples referred to *A. muelleri* in our preferred phylogenetic hypothesis (Fig. 2). Mindanao's eight divergent genetic lineages of *Ansonia* exhibit pronounced geographical structure (Fig. 2) and are clearly associated with the major upland montane components of this island (Fig. 1).

Specimens referable to *A. mcgregori* were represented by 16 unique haplotypes from numerous specimens from the type locality at the tip of the Zamboanga Peninsula (Nancy + Baluno; Pasonanca Natural Park, Zamboanga City area), and samples from Murias (a locality located midway along the Zamboanga Peninsula). The remaining central and eastern Mindanao sampling consists of five divergent lineages, representing 42 unique haplotypes, which we tentatively assign to *A. muelleri* (see Section 4). These geographically structured clades are moderately divergent (separated by uncorrected p -distances of 1.6–3.8%) and each appears restricted to geographically distinct, upland areas of Mindanao (Fig. 1). Within the five clades putatively assigned to *A. muelleri*, a strongly supported (MLBP ≥ 95 ; PP ≥ 0.95) eastern and eastern clade of Magdiwata, Compostela, Hamiguitan, Surigao del Norte, and Agusan del Norte populations is the sister taxon to a divergent lineage from southern Mindanao (Kiamba and Mt. Busa populations; Figs. 1 and 2). These two clades are moderately supported (MLBP < 70; PP = 0.70) as most closely related to a clade from east-central Mindanao (Balatukan, Tago, and Calabugao populations), and the weakly supported cluster containing these three clades is strongly supported as most closely related to specimens from the Mt. Apo Massif (Figs. 1 and 2). The Rogongon and Kitanglad clade is only moderately supported (MLBP 71; PP = 0.80) as the sister clade to the remaining clades referred to *A. muelleri* (Fig. 2).

A lineage represented by two unvouchered genetic samples from the Lake Lanao (Marawi) area is the sister taxon to *A. mcgregori* + *A. muelleri* and separated from samples of these two species by 3.8–5.0% uncorrected sequence divergence (Table 3). A second highly divergent lineage from Murias and Timolan is the sister taxon to the (Lake Lanao (*A. mcgregori* + *A. muelleri*)) clade and separated from these lineages by 6.2–7.8% uncorrected sequence divergence (Table 3).

Table 2

Summary of southern Philippine *Ansonia* sampling, major lineages/haplotype clades, numbers of individuals (N), numbers of mtDNA haplotypes (N_h), numbers of polymorphic sites (P_N), mean number of pairwise nucleotide differences (k), haplotype diversity (h), and nucleotide diversity (π). See Appendix A for full details of sampling and a list of all samples included.

Region/Clade	N	N_h	P_N	k	h	π
<i>Ansonia</i> sp. 1	4	4	33	16.667 ± 9.46	1.000 ± 0.18	0.0086 ± 0.0058
<i>Ansonia</i> sp. 2	2	2	3	3.000 ± 2.45	1.000 ± 0.50	0.0037 ± 0.0042
<i>A. mcgregori</i>	19	16	25	4.614 ± 2.37	0.983 ± 0.02	0.0024 ± 0.0014
Rogongon–Kitanglad	7	6	8	3.333 ± 1.98	0.933 ± 0.12	0.0017 ± 0.0012
Apo	8	4	1	0.500 ± 0.52	0.500 ± 0.27	0.0003 ± 0.0003
Balatukan–Tago	26	13	40	9.822 ± 4.65	0.89 ± 0.04	0.0050 ± 0.0027
Kiamba–Busa	17	7	23	4.542 ± 2.36	0.68 ± 0.11	0.0023 ± 0.0014
Eastern Mindanao	22	12	82	22.808 ± 10.60	1.00 ± 0.02	0.0117 ± 0.0061
All <i>A. muelleri</i> samples	80	42	224	47.586 ± 20.86	0.966 ± 0.01	0.0245 ± 0.0119
All samples	105	64	1269	110.671 ± 47.93	0.981 ± 0.01	0.0569 ± 0.0273

Table 3
Uncorrected *p*-distances (16S + NAD1 gene sequence divergence, expressed as percentages) among and within mitochondrial gene lineages of Mindanao river toads, genus *Ansonia*. Percentages on the diagonal represent within-clade genetic diversity (bolded for emphasis).

	A. sp 1	A. sp 2	<i>A. mcgregori</i>	Rogongon	Apo	Balatukan	Kiamba	E. Mindanao
1	0.1–0.3							
2	5.5–6.0	0.0						
3	6.9–7.3	3.7–4.0	0.0–0.3					
4	6.3–6.7	4.1–4.3	3.0–3.3	0.0–0.2				
5	6.2–7.8	4.3–4.4	2.9–3.9	2.7–3.4	0.0–0.1			
6	7.4–7.7	3.8–4.1	3.4–3.8	2.7–2.9	1.6–2.8	0.0–0.8		
7	7.1–7.7	4.4–5.0	4.0–4.4	3.2–3.6	2.3–3.4	2.6–3.0	0.0–0.6	
8	6.1–7.8	4.3–4.5	3.0–4.3	2.7–3.8	1.9–3.8	2.1–3.1	2.3–3.5	0.0–1.4

3.3. Hypotheses tested: previous taxonomy and dispersal corridors

Our data reject the hypothesis that Philippine *Ansonia* consists of only two species, separated geographically by the suture between the Zamboanga Peninsula and the remaining portions of Mindanao. The hypothesis that all Zamboanga populations (*A. mcgregori*) are the sister taxon to all others (*A. muelleri*) was rejected by both Bayesian (PP = 0.0) and likelihood-based methods (AU *p*-value < 0.001). Our Bayesian tests of dispersal via montane corridors strongly reject the simple east-to-west dispersion hypothesis and all other hypothesized dispersal routes, with posterior probabilities indicating a complete absence of any of the *a priori* topologies in our post burn-in sample of trees. However, the AU test indicated a significant but marginal (AU = 0.01) rejection of the “southern route” (Fig. 3c) hypothesis, and failed to reject (AU = 0.244) the “east-central to northeastern” hypothesized dispersal route (Fig. 3d).

3.4. Historical demography

With the caveat that our sample sizes vary, mismatch distributions showed striking differences between the two described species (Fig. 4). When mismatch distributions and associated statistics were calculated individually for the five divergent gene lineages of central and eastern Mindanao, these all appeared qualitatively ragged, with the exceptions of the Rogongon and Apo haplotype clades in which sample sizes were low (Fig. 4). Given the lack of statistically significant differences between these individual gene lineages (excepting the Rogongon and Eastern Mindanao clades where Fu’s *F_s* was significant; Table 2) further discussion will focus on the combined analysis in which we pooled these data and putatively consider all of these lineages to be members of a single species, *A. muelleri*. Whereas sequence data of *A. mcgregori* exhibited a smooth, unimodal distribution of pairwise differences suggesting a lack of geographical structure or a population expansion event, the five gene lineages we refer to *A. muelleri* were characterized by a relatively ragged and multimodal distribution, suggesting structured, demographically stable populations. The *T* statistic and Rogers and Harpending’s Raggedness Index are nonsignificant for *A. mcgregori* but significant for *A. muelleri* (Table 4), indicating a failure to reject the sudden expansion model in *A. mcgregori* but a demographically stable population inferred in *A. muelleri*. Tajima’s *D* indicated no departures from expectations of neutrality and demographic stability for either lineage (*p* > 0.05; Table 4). Fu’s *F_s* is positive, close to zero, and nonsignificant in *A. muelleri* (consistent with an hypothesis of constant population size), but large in magnitude, negative, and significant (*p* < 0.001) in *A. mcgregori*, rejecting the hypothesis of demographic stability and consistent with an interpretation of recent range expansion. Ramos-Onsins and Rozas *R₂* statistics, said to be a superior indication of range or demographic expansion in small populations (Ramos-Onsins and Rozas, 2002), were small and positive (Table 4), and the ob-

served value statistically significant (*p* < 0.05), consistent with the hypothesis of population growth in both species.

Bayesian skyline plots revealed qualitatively different demographic histories when considering the three central and eastern Mindanao clades (where sample sizes were sufficient), or when these lineages were pooled and assumed to represent *A. muelleri* (Fig. 5). When individual skyline analyses were conducted on the three central and eastern Mindanao gene lineages for which sampling was sufficient, each one revealed slight demographic or range contraction, followed by expansion after a longer period of demographic stability. When we pooled data and compared putative species (Fig. 5), *A. mcgregori* appears to have experienced gradual population expansion over the past ~1.0–0.7 my, but *A. muelleri* may have undergone more rapid and recent demographic or range contraction (~0.3 mya) following a period of relatively stable demographic history.

3.5. Timing of diversification

As expected, our estimate of the temporal framework for diversification within Mindanao *Ansonia* provided much younger dates than those postulated by Matsui et al. (2010) for the approximate root age and oldest divergences within this clade (Fig. 6). Most divergences associated with recognized and hypothesized species boundaries appear to have occurred well within the Pleistocene (<1.8 mya; Fig. 6). The highest posterior density intervals around our estimates are admittedly wide, but even with substantial uncertainty our analysis places the estimated root age of Mindanao *Ansonia* at approximately 2.3 mya (HPD = 1.19–3.40 mya), far younger (an order of magnitude; HPD intervals non-overlapping) than 20.2 (HPD = 9.8–31.8 mya) estimated by Matsui et al. (2010). Our estimates suggest that *A. mcgregori* diverged 0.87 mya (HPD = 0.42–1.53 mya) and that the node subtending the five lineages that we conservatively assigned to *A. muelleri* originated 1.77 (HPD = 0.99–2.78) mya. Most of the geographically structured clades (Fig. 6) include mean estimated divergences during a period of approximately 1.0–0.5 mya, suggesting that geographic structure was in place well before the last glacial maximum (12–10 kya). We consider these findings to refute both an “early-invasion” hypothesis (~20 mya; Matsui et al., 2010) and a “speciation-by-orogenic-vicariance” scenario (~10–5 mya).

4. Discussion

4.1. Taxonomic implications

How many species of Mindanao Island slender toads are implied by our data? First, we collected the morphologically distinct *A. mcgregori* at the species’ type locality (Pasonanca Natural Park, at the western tip of the Zamboanga Peninsula; Taylor, 1922c) so we can be certain of this species’ identity; our data strongly support its

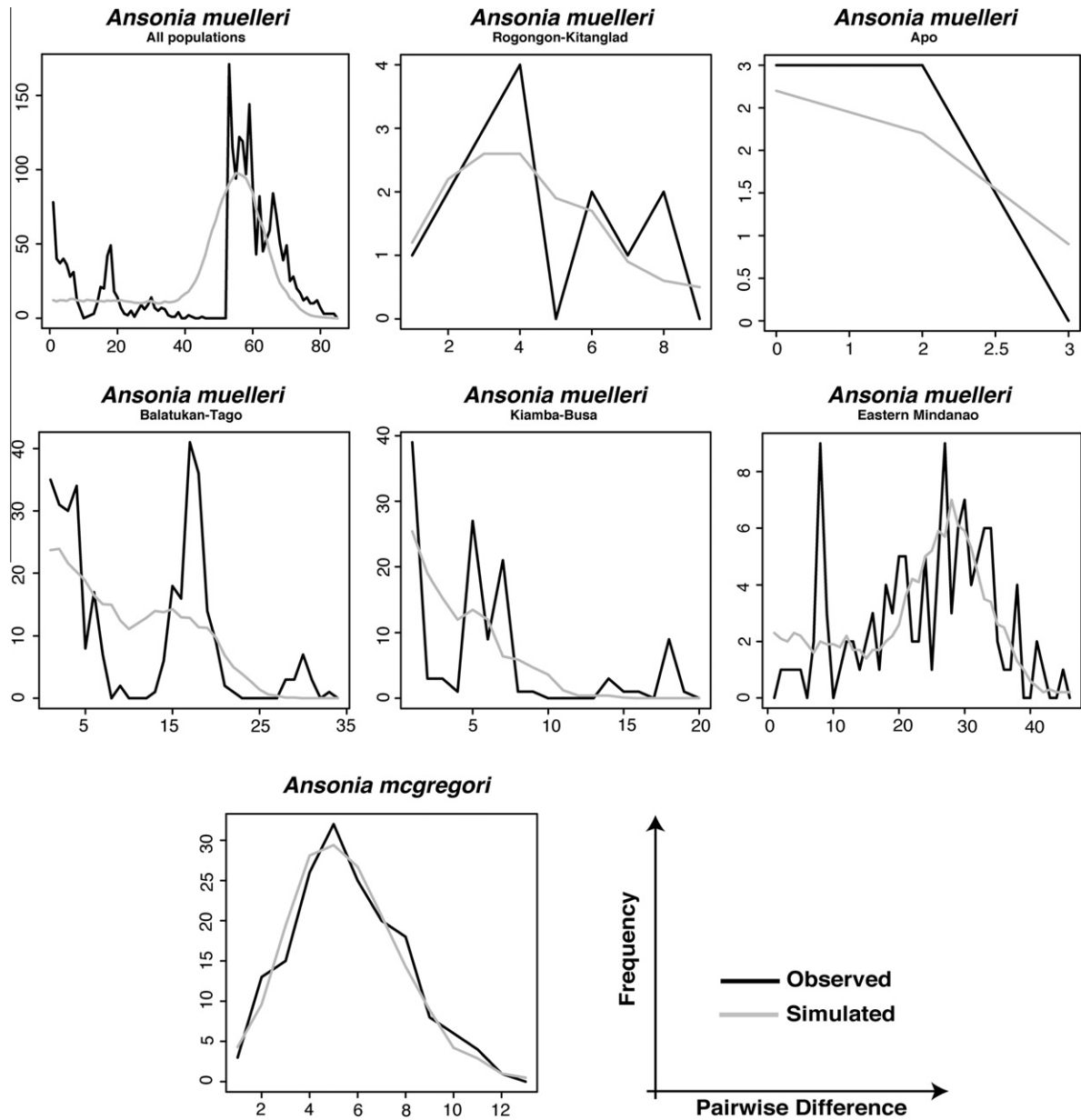


Fig. 4. Observed frequencies of pairwise nucleotide differences among sequences (see key; black lines) and expected frequencies under a model of sudden population expansion (gray lines) (Rogers and Harpending, 1992) for all populations referred to *Ansonia muelleri*, each of the five clades of *A. muelleri* from central and eastern Mindanao (see Figs. 1 and 2: Rogongon–Kitanglad, Apo, Balatukan–Tago–Calabugao, Kiamba–Busa, and eastern Mindanao), and *A. mcgregori*. The relatively smooth and unimodal plot for *A. mcgregori* matches predictions for a demographically expanding population; the ragged and multimodal plots for *A. muelleri* indicate long-term demographic stability.

Table 4

Summary statistics and results of tests of population expansion in Philippine *Ansonia* (*A. mcgregori* and the gene lineages referred to *A. muelleri*; divergent lineages *A. sp. 1* and *A. sp. 2* excluded); analysis of mismatch distributions, and substitution model applied to the Bayesian skyline analysis of demographic history. For mismatch distributions, *T* is presented along with *p*-values for rejection of the sudden expansion model, based on a comparison of the sum of squares of expected and observed distributions (using parametric bootstrapping with 10,000 replicates; Rogers and Harpending, 1992; Excoffier et al., 2005). Additional entries include Harpending’s Raggedness Index (*RI*) and *p*-values for rejection of the goodness of fit test comparing simulated vs. observed distribution raggedness, Tajima’s *D*, Fu’s *F_s*, and Ramos-Onsins and Rozas *R₂* statistics. All tests were implemented separately for the two species *A. mcgregori* and *A. muelleri* as defined in the text.

Species ^a	<i>T</i>	<i>RI</i>	Tajima’s <i>D</i> (<i>p</i> -value)	<i>F_s</i> (<i>p</i> -value) ^b	<i>R₂</i> (<i>p</i> -value) ²	Skyline Model
<i>A. mcgregori</i> (19/16)	3.954 (0.930)	0.016 (0.889)	−1.396 (0.081)	−10.953 (0.000)	0.137 (0.019)	HKY
Rogongon–Kitanglad (7/6)	1.680 (0.510)	0.129 (0.620)	−0.185 (0.477)	−3.174 (0.001)	0.052 (0.341)	HKY
Apo (8/4)	0.768 (0.320)	0.250 (0.890)	−0.612 (0.414)	0.172 (0.339)	0.052 (0.454)	HKY
Balatukan–Tago (26/13)	17.438 (0.460)	0.022 (0.640)	0.897 (0.194)	−0.164 (0.496)	0.101 (0.111)	GTR
Kiamba–Busa (17/7)	6.134 (0.150)	0.209 (0.140)	−0.740 (0.251)	0.778 (0.691)	0.039 (0.09)	HKY
Eastern Mindanao (22/12)	27.166 (0.780)	0.021 (0.540)	−0.178 (0.449)	−5.6178 (0.012)	0.051 (0.01)	GTR
All <i>A. muelleri</i> (80/42)	59.229 (0.040)	0.009 (0.000)	0.385 (0.342)	0.013 (0.590)	0.162 (<0.001)	GTR + Γ

^a Numbers of individuals/numbers of haplotypes in parentheses.

^b Statistical significance for rejecting a null model of constant population size included in parentheses.

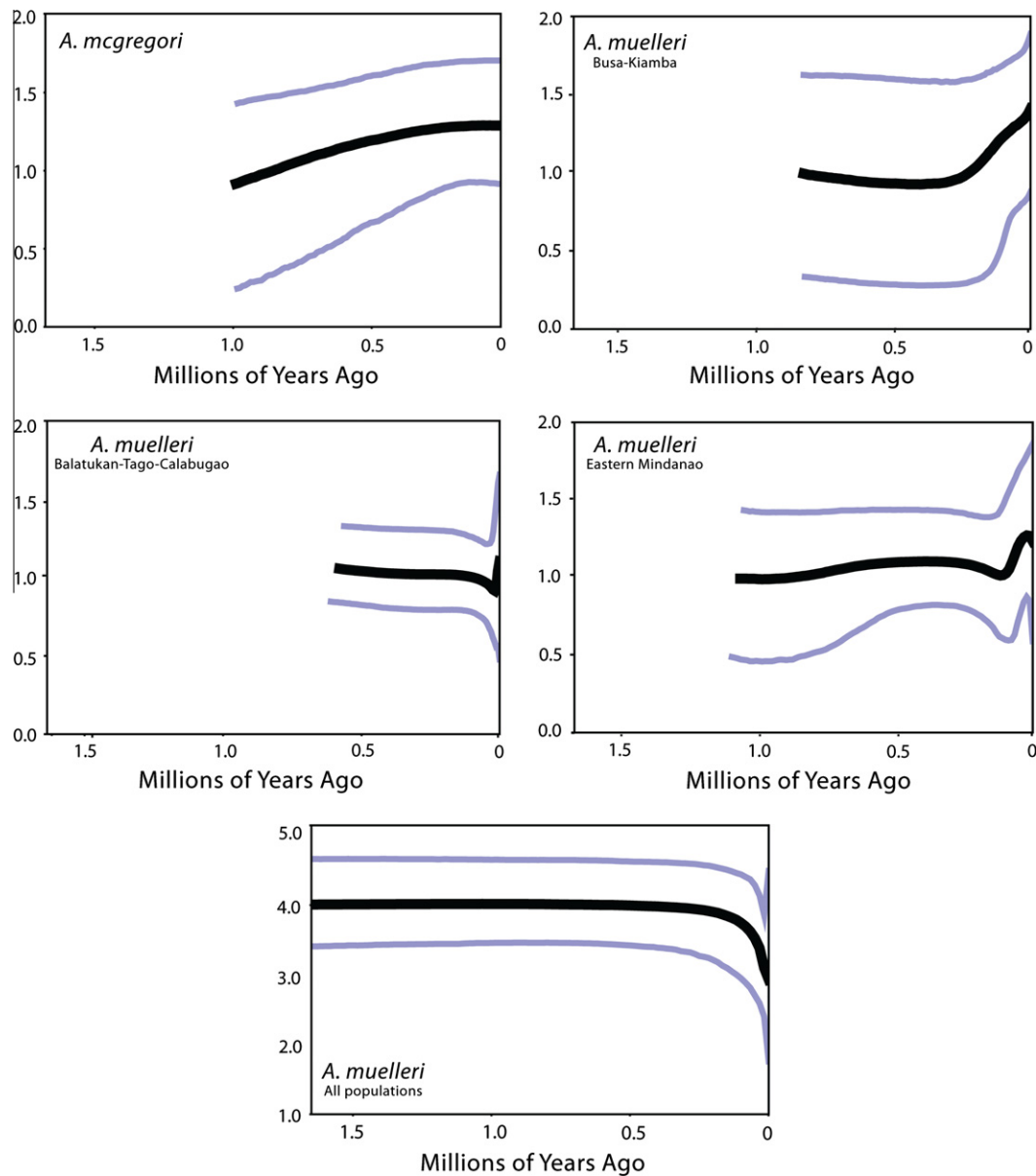


Fig. 5. Bayesian skyline plots for *A. mcgregori*, the three clades of *A. muelleri* from central and eastern Mindanao with sufficient numbers of samples (see Figs. 1 and 2: Kiamba-Busa, Balatukan-Tago-Calabugao, and eastern Mindanao), and for all populations referred to *A. muelleri*. Bold black lines indicate an estimate of median effective population size as a function of time; gray lines indicate the 95% highest posterior density. The horizontal axis has been scaled to show the same time interval both plots, resulting in the truncation of the *A. mcgregori* trace. The vertical axis should be interpreted as the change in effective population size.

status as a distinct species in confirmation of earlier studies (Taylor, 1922c; Inger, 1966).

Our phylogenetic estimates and analyses of sequence divergence suggest two highly divergent haplotype clades (Fig. 3; Table 3), one found at both Murias and Timolan and another at Lake Lanao (Fig. 1). Our preliminary survey of available specimens (Appendix A), plus the types of both *A. mcgregori* and *A. muelleri*, identified no character differences of external morphology that would diagnose these two putative evolutionary lineages as new species (Sanguila, Fuiten, and Brown, unpublished data). At present, due to the potential for species boundaries and relationships to be obscured by mitochondrial lineage sorting and introgression, maintenance of ancestral polymorphisms, and deep coalescent events (Hudson, 1990; Nichols, 2001; Hare, 2001), it is premature to recognize species without diagnostic characters derived from an independent source of data (e.g., adult or larval morphology, bioacoustics, etc.). We refer to these highly divergent genetic lin-

eages as “*Ansonia* sp. 1.” and “*Ansonia* sp. 2” (Fig. 3; Table 3) to emphasize their genetic distinctiveness and to highlight the need for additional study.

Given no strong statistical support for individual demographic, population genetic, or divergent phylogenetic histories in the central and eastern haplotype mtDNA gene lineages, we tentatively identify the widespread, highly structured clade from central and eastern Mindanao as a single species (*A. muelleri*). This arrangement includes five geographically structured haplotype clades, separated from one another by minimal to moderate (1.6–3.8%) sequence divergence (uncorrected *p*-distance; Table 3). Although the exact type locality for *A. muelleri* is not known (the species was described by Boulenger, 1887, from “Mindanao Island”), and the holotype specimen (BM 1947.2.20.57) falls within the range in morphological variation we have observed (Sanguila, Fuiten, and Brown, unpublished data), we follow many other herpetologists in assuming that *A. muelleri* was originally collected in central or eastern

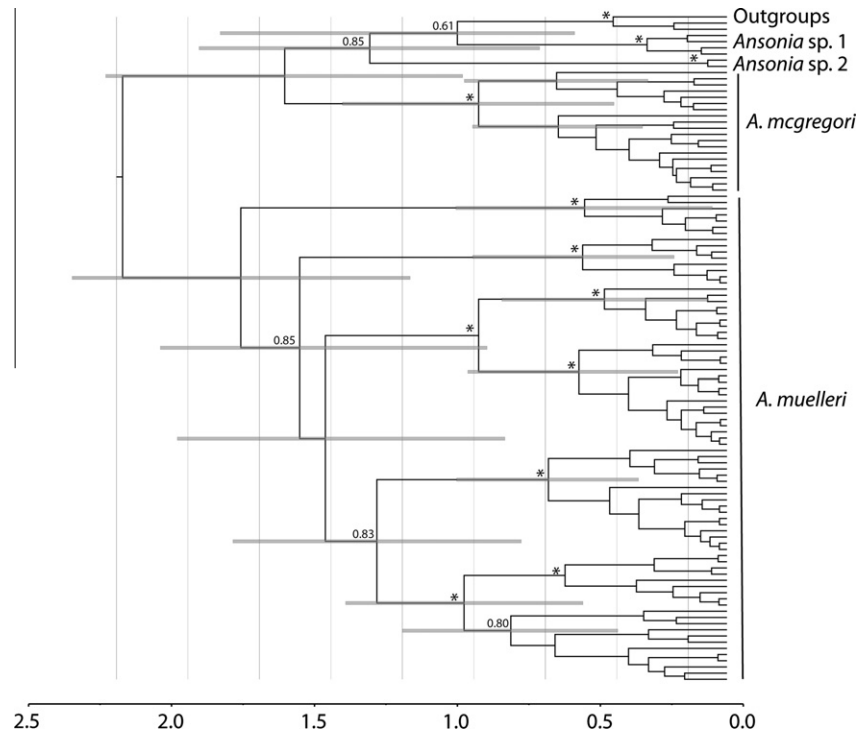


Fig. 6. Bayesian chronogram inferred by using a relaxed molecular clock as implemented in BEAST v1.6.1. Gray nodal bars are highest posterior density intervals, and the time axis is indicated at the bottom (mya). Nodal support values indicate Bayesian posterior probabilities of >0.50 (asterisks = posterior probabilities of >0.99). Note that most major divergences occurred within the last 1.8 my, well within the Pleistocene Epoch.

Mindanao (Inger, 1960, 1965, 1966; Brown and Alcalá, 1970; Alcalá, 1986; Brown and Alcalá, 1989; Brown, 2007). The hypothesis of a highly structured single, widespread species throughout central and western Mindanao, although conservative, presents some obvious and immediate conservation considerations (see below).

4.2. Phylogeography, geographic distribution of genetic variation, and demography

Our widespread geographic sampling across the range of Philippine *Ansonia* represents the first densely sampled phylogeographic study for an endemic Mindanao Island vertebrate. Many previous works have surmised the existence of montane subcenters of biodiversity, or pockets of species endemism on this large, geologically complex amalgamation of paleoislands (Taylor, 1920, 1922a, 1928; Inger, 1954; Leviton, 1963; Kennedy et al., 2000; Brown et al., 2002; Brown and Diesmos, 2009; Heaney et al., 2006a, 2006b, 2010). However, no previous studies have used robust genetic sampling to survey for Areas of Endemism (AOEs; Evans et al., 2003b) across the Mindanao faunal region, nor assessed intraspecific genetic partitioning within a widespread endemic Mindanao species.

Our results unequivocally demonstrate for the first time that widespread endemic Mindanao vertebrates exhibit significant geographical population structure and allopatric genetic AOEs, coincident with all the major upland geological components of the island (Peterson and Heaney, 1993; Heaney et al., 2005, 2006a, 2006b; Esselstyn et al., 2009; Siler et al., 2011). The implications for endemic Mindanao vertebrates known from mid- to high-elevation forested regions of the island may be profound. If many of Mindanao's montane vertebrate species exhibit similar, highly divergent lineages (i.e., close to or bypassing species boundaries), not only have ESUs for conservation gone largely undetected but species-level biodiversity on the whole may be severely underestimated (Brown and Diesmos, 2009).

Our results, together with a new body of well-sampled, geographically robust phylogenetic and phylogeographic studies (e.g., Esselstyn and Brown, 2009; Brown et al., 2010; Linkem et al., 2010a, 2010b; Siler et al., 2010, 2011) suggest that within-island evolutionary diversification may contribute substantially more to Philippine megadiversity than previously recognized (Brown et al., 2008; Brown and Diesmos, 2009; Brown and Stuart, in press). If intra-island landscape processes have contributed substantially to vertebrate diversification in other taxa as they have influenced diversification in *Ansonia*, an expanded characterization of the evolutionary processes of diversification leading to Philippine megadiversity may be required (Brown et al., 2002; Brown and Diesmos, 2002, 2009; Esselstyn et al., 2010).

Our preferred phylogenetic estimate (Fig. 1) suggests a general west-to-east dispersal into central and eastern Mindanao. We have, however, rejected a strict, west-to-east stepping-stone model of dispersion or expansion along the linear Zamboanga Peninsula (Fig. 3a), and several intuitive dispersal-corridor hypotheses are similarly rejected (Fig. 3a–e).

Exploration of the approximate timing of diversification (see below) suggests that *Ansonia* dispersed simultaneously from west-central to east-central, southern, and eastern Mindanao, and that stable populations were established (possibly by constraints on dispersal imposed by larval biology) in the Pleistocene. Given the past several million years of continued uplift of central Mindanao, and numerous oscillations of sea level and terrestrial climate during the Pleistocene (review: Woodruff, 2010), we suspect that today's regionalized, geographically structured *A. muelleri* populations result from Pleistocene habitat fragmentation driven by climate change (i.e., Evans et al., 2003b, 2008). Evidence suggests that interglacial periods were (and are today) characterized by rising sea levels, increased aridification of low-lying valleys and wide inter-montane plains near sea level (Whitmore, 1984, 1987; Lisiecki and Raymo, 2005; Bintanja et al., 2005; Corlett, 2009; Woodruff, 2010; Wurster et al., 2010), suggesting expansion

of environmental barriers to dispersal for anurans with torrent-specialized larvae. A general concordance between the level of divergence between the geographically structured clades of *A. muelleri* (~1.6–3.8%; Table 3), the timing of diversification estimated for nodes within *A. muelleri* (Fig. 6), the approximate age of onset of the decline in effective population size inferred by the Bayesian skyline analysis (Fig. 5), and the known timing of Pleistocene sea-level and climatic oscillations (Lisiecki and Raymo, 2005; Woodruff, 2010) somewhat corroborates our findings and strengthens our confidence in our interpretation.

Coupled with much lower levels of haplotype diversity, polymorphic sites, and nucleotide diversity in *A. mcgregori* as compared to *A. muelleri* (Table 2), plus a significant Fu's F_s in the former but not the latter (Table 4), our results suggest a departure from expectations based on a null model of constant population size for *A. mcgregori*. Additionally, mismatch distributions for this range-restricted species are smooth and unimodal, and the nonsignificant T statistic fails to reject the hypothesis of an expanding population in *A. mcgregori*. On the other hand, the ragged, multimodal mismatch distribution and statistically significant T for *A. muelleri*, suggest a stable, structured population. Finally, Ramos-Onsins and Rozas R_2 statistics were small and significant in both species, suggesting recent demographic expansion in some parts of their ranges. The signal of recent range expansion in *A. mcgregori* may be influenced by the fact that we have collected it in Pasonanca at the tip of the Zamboanga Peninsula, and also at Murias (midway up the peninsula), but not at any intervening or other sites.

4.3. Timing of diversification

Our Bayesian relaxed clock analyses (Fig. 6) yielded broadly overlapping highest posterior density intervals at basal nodes in the tree, suggesting a similar temporal framework (the Pleistocene) for the divergences that produced the major structure, giving rise to the four putative species in our analyses. Our results suggest that species diversity within Mindanao *Ansonia* probably has evolved within the last two million years. Interestingly, inferred divergences within *A. muelleri* are approximately the same or even slightly older than divergences inferred between *A. sp. 1*, *A. sp. 2*, and *A. mcgregori* (~1.6–1.5 mya; Fig. 6), further emphasizing the potential for future taxonomic partitioning of *A. muelleri* and a general Pleistocene framework for diversification of Mindanao *Ansonia*.

We consider Matsui et al.'s (2010) ~20 my estimate of the age of Philippine *Ansonia* suspect (biased towards unrealistically old estimates of clade age) for three main reasons. First, the geological components of Mindanao were widely separated and largely still submerged 20 mya (Hall, 1996, 1998; Yumul et al., 2003, 2004, 2008, 2009), casting doubt on the possibility that vertebrates of low relative dispersal abilities could achieve long-distance dispersal necessary to invade the separate paleoisland precursors of today's Mindanao. Second, the Matsui et al. (2010) analysis inferred a mean *Ansonia*–*Pelophryne* split of 74.9 (95% CI: 56.4–95.2) Mya and an age of 66.4 (49.5–84.0) Mya for the genus *Ansonia*. In well-calibrated analyses involving multiple unlinked loci and multiple nuclear genes, these ages are roughly equivalent to the estimated age of the clade Hyloidea and, as such, are far older than any major divergences within Bufonidae that gave rise to Asian bufonid genera (e.g., ~25–40 mya; Roelants et al., 2007); thus, the Matsui et al. (2010) analysis appears unrealistically biased toward old ages across their tree (possibly an artifact of their external calibration procedure, extreme rate variation across the tree, or a combination of these and other factors; Graur and Martin, 2004). Finally given the shallow divergences detected in Mindanao populations included in Matsui et al.'s (2011) analysis

(2.7–3.0% uncorrected p -distance) and the inference of an ~20 my age of the Mindanao clade, we note that an unrealistically low nucleotide substitution rate (on the order of 0.000000135–0.00000015%; far slower than ever empirically recorded in mitochondrial gene sequences of amphibians) is implied by Matsui et al.'s (2010) result. As a final point, we note that if our approximate divergence dating estimation is reasonably accurate, the timing of diversification inferred here is generally consistent with the Pleistocene sea-level oscillation (and climate-habitat fragmentation) model of diversification previously postulated (Heaney, 1985; Heaney et al., 2005; Esselstyn and Brown, 2009; Linkem et al., 2010a, 2010b; Siler et al., 2010) as a common mechanism that may have promoted vertebrate species diversification in the archipelago (Brown and Diesmos, 2002, 2009).

4.4. Conservation implications

Currently both Philippine species of *Ansonia* are listed in the IUCN Red List of Threatened Species conservation status definitions (IUCN, 2010) as “Vulnerable (B1ab(iii) “populations trend: decreasing”). Given our elucidation of *A. mcgregori* as a potentially range-restricted species, we favor the continuation of this classification. However, we note that no data have ever been presented to suggest that population sizes are declining in *A. mcgregori*.

In contrast our analysis has identified in *A. muelleri* five divergent mitochondrial gene lineages, which may be considered conservation ESUs. To preserve genetic diversity as a much-desirable component of biodiversity (Moritz, 1994; Evans et al., 2003b; Rowley et al., 2009), protection of habitat in all five areas of genetic endemism across the range of *A. muelleri* will be required. We suspect that deep divergences within *A. muelleri* (Fig. 6) may warrant taxonomic partitioning (i.e., additional species recognition) pending the identification of diagnostic characters of morphology and/or bioacoustics.

Two lineages revealed here require immediate taxonomic and conservation status assessments. A highly genetically divergent lineage (“*Ansonia* sp. 1”), forming the sister taxon to all remaining Philippine *Ansonia* was sampled at two mid-peninsular Zamboanga sites: Murias and Timolan. This morphologically indistinguishable lineage was encountered sympatrically and syntopically with *A. mcgregori* at these two sites and may represent a new, undescribed, “cryptic” species. Similarly, two unvouchered genetic samples taken from individuals at the Lake Lanao population (“*Ansonia* sp. 2”) represent a highly divergent lineage placed as the sister taxon to *A. mcgregori* + *A. muelleri*. Our suspicion is that both of these (and, conceivably, other as of yet undiscovered lineages) may represent range-restricted, unrecognized species in need of immediate conservation planning and/or taxonomic recognition. Like the five divergent lineages of *A. muelleri*, these two highly divergent western Mindanao lineages should be treated as ESUs for conservation purposes and should be scrutinized further for diagnostic differences in adult and larval morphology or acoustic characters that may facilitate their recognition as distinct species.

Finally, future phylogeographic and conservation genetic studies of Mindanao's diverse fauna should target genetic sampling from within the AOE's defined by our elucidation of geographically structured genetic variation in *Ansonia*. We would not be surprised if these isolated montane components of Mindanao harbor many additional undiscovered species and undiagnosed ESUs in other vertebrate groups. A multi-taxon comparative approach (e.g., Evans et al., 2003a, 2008; Brown et al., 2010; Setiadi et al., in press) would be a particularly compelling approach for future studies geared towards understanding the origins and maintenance of high endemic species diversity in the southern Philippines.

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Appendix A

Species identity collector number, museum catalog numbers, and localities for all samples included in this study.

Taxon	Field No.	Catalog No.	General Area	Specific collection locality
<i>Ansonia</i> sp. 1	MBS 027	KU 323467	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Pagadian City, Zamboanga del Sur Province
<i>Ansonia</i> sp. 1	MBS 031	KU 323471	West Mindanao	Mt. Timolan, Barangay Limas, Municipality of Guipos, Pagadian City, Zamboanga del Sur Province
<i>Ansonia</i> sp. 1	MBS 030	KU 323470	West Mindanao	Mt. Timolan, Barangay Limas, Municipality of Guipos, Pagadian City, Zamboanga del Sur Province
<i>Ansonia</i> sp. 1	MBS 026	KU 323466	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Pagadian City, Zamboanga del Sur Province
<i>Ansonia</i> sp. 1	MBS 029	KU 323469	West Mindanao	Mt. Timolan, Barangay Limas, Municipality of Guipos, Pagadian City, Zamboanga del Sur Province
<i>Ansonia</i> sp. 2	ACD 3600	No voucher	North (Central) Mindanao	Near Lake Lanao, Marawi City, Lanao del Sur Province
<i>Ansonia</i> sp. 2	ACD 3601	No voucher	North (Central) Mindanao	Near Lake Lanao, Marawi City, Lanao del Sur Province
<i>Ansonia mcgregori</i>	RMB 11079	KU 321113	West Mindanao	Barangay La Paz, Sitio Nancy, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	RMB 11273	KU 321119	West Mindanao	Barangay La Paz, Sitio Nancy, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	RMB 10432	KU 314231	West Mindanao	Barangay Baluno, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	MBS 008	KU 323448	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 005	KU 323445	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 010	KU 323450	West Mindanao	Murias River, Barangay Lourdes, Mt. Murias, Pagadian City, Zamboanga del Sur
<i>Ansonia mcgregori</i>	MBS 012	KU 323452	West Mindanao	Murias River, Barangay Lourdes, Mt. Murias, Pagadian City, Zamboanga del Sur
<i>Ansonia mcgregori</i>	RMB 10395	KU 314194	West Mindanao	Barangay Baluno, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	RMB 10371	KU 314180	West Mindanao	Barangay Baluno, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	RMB 11003	KU 321106	West Mindanao	Barangay Baluno, Municipality of Pasonanca, Zamboanga City
<i>Ansonia mcgregori</i>	MBS 011	KU 323451	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 020	KU 323460	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 009	KU 323451	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 023	KU 323463	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 016	KU 323456	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 007	KU 323447	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 018	KU 323458	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 021	KU 323461	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 019	KU 323459	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia mcgregori</i>	MBS 022	KU 323462	West Mindanao	Mt. Murias, Murias River, Barangay Lourdes, Municipality of Pagadian City, Zamboanga del Sur Province
<i>Ansonia muelleri</i>	MBS 044	KU 323484	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province
<i>Ansonia muelleri</i>	MBS 035	KU 323475	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province
<i>Ansonia muelleri</i>	MBS 041	KU 323481	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province

(continued on next page)

Appendix A (continued)

Taxon	Field No.	Catalog No.	General Area	Specific collection locality
<i>Ansonia muelleri</i>	MBS 043	KU 323483	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province
<i>Ansonia muelleri</i>	ACD 3543	PNM*	North (Central) Mindanao	Mt. Kitanglad, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 036	KU 323476	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province
<i>Ansonia muelleri</i>	MBS 046	KU 323486	North (Central) Mindanao	Near Mt. Gabunan, Sitio Minsaliding, Barangay Rogongon, Municipality of Iligan City, Lanao del Norte Province
<i>Ansonia muelleri</i>	RMB 643	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	RMB 639	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	RMB 640	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	RMB 641	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	RMB 639	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	RMB 642	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	ACD 1617	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	ACD 1640	CMNH*	Apo Massif	Mt. Apo, Barangay Baracatan, Municipality of Toril, Davao del sur Province
<i>Ansonia muelleri</i>	ACD 4265	KU 319745	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4437	KU 319747	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4421	KU 319752	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4284	KU 319755	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4282	KU 319725	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4260	KU 319733	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4264	KU 319762	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4263	KU 319758	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	ACD 4334	KU 319721	North (Central) Mindanao	Mt. Balatukan, Barangay Lunotan, Sitio San Isidro, Municipality of Gingoog City, Misamis Oriental Province
<i>Ansonia muelleri</i>	MBS 064	KU 323504	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 076	KU 323516	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 047	KU 323487	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 075	KU 323515	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 073	KU 323513	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 074	KU 323514	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 066	KU 323506	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 077	KU 323517	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 067	KU 323507	North (Central) Mindanao	Cagang-awan River, Sitio Ananaso, Barangay Dumalaguig, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 052	KU 323492	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 056	KU 323496	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 057	KU 323497	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 051	KU 323491	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 049	KU 323489	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 054	KU 323494	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 050	KU 323490	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 053	KU 323493	North (Central) Mindanao	Yandang, km. 30, Calabugao River, Sitio Nasandigan, Barangay Calabugao, Municipality of Impasugong, Bukidnon Province
<i>Ansonia muelleri</i>	MBS 095	KU 323520	South Mindanao	Nobol River, Sitio Kapalanan, Barangay Gasi, Municipality of Kiamba, Sarangani Province
<i>Ansonia muelleri</i>	MBS 106	KU 323531	South Mindanao	Nobol River, Sitio Kapalanan, Barangay Gasi, Municipality of Kiamba, Sarangani Province
<i>Ansonia muelleri</i>	MBS 100	KU 323525	South Mindanao	Nobol River, Sitio Kapalanan, Barangay Gasi,

Appendix A (continued)

Taxon	Field No.	Catalog No.	General Area	Specific collection locality
<i>Ansonia muelleri</i>	MBS 105	KU 323530	South Mindanao	Municipality of Kiamba, Sarangani Province Nobol River, Sitio Kapalanan, Barangay Gasi,
<i>Ansonia muelleri</i>	MBS 107	KU 323532	South Mindanao	Municipality of Kiamba, Sarangani Province Nobol River, Sitio Kapalanan, Barangay Gasi,
<i>Ansonia muelleri</i>	MBS 098	KU 323523	South Mindanao	Municipality of Kiamba, Sarangani Province Nobol River, Sitio Kapalanan, Barangay Gasi,
<i>Ansonia muelleri</i>	MBS 094	KU 323519	South Mindanao	Municipality of Kiamba, Sarangani Province Nobol River, Sitio Kapalanan, Barangay Gasi,
<i>Ansonia muelleri</i>	PNM/ CMNH 1718	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1475	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1472	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1471	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1643	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1714	CMC 12173	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1644	CMC 12712	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1713	PNM*	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1756	CMC 12174	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	PNM/ CMNH 1757	CMC 12176	South Mindanao	Municipality of Kiamba, Sarangani Province Mt. Busa, Municipality of Kiamba,
<i>Ansonia muelleri</i>	MBS 084	No voucher	East Mindanao	Sarangani Province Bato River, Mt. Talinis – Bato, Barangay Cabugo,
<i>Ansonia muelleri</i>	MBS 088	No voucher	East Mindanao	Municipality of Claver, Surigao del Norte Province Bato River, Mt. Talinis – Bato, Barangay Cabugo,
<i>Ansonia muelleri</i>	MBS 081	No voucher	East Mindanao	Municipality of Claver, Surigao del Norte Province Bato River, Mt. Talinis – Bato, Barangay Cabugo,
<i>Ansonia muelleri</i>	MBS 082	No voucher	East Mindanao	Municipality of Claver, Surigao del Norte Province Bato River, Mt. Talinis – Bato, Barangay Cabugo,
<i>Ansonia muelleri</i>	EMD 308	PNM*	East Mindanao	Municipality of Claver, Surigao del Norte Province Barangay San Antonio, Municipality of Remedios
<i>Ansonia muelleri</i>	MBS 002	KU 323442	East Mindanao	T. Romualdez, Agusan del Norte Province Agay River, Mt. Bato – Batohon (in the Mt. Hilong – Hilong Range), Barangay
<i>Ansonia muelleri</i>	MBS 001	KU 323441	East Mindanao	San Antonio, Municipality of Remedios T. Romualdez, Agusan del Norte Province Agay River, Mt. Bato – Batohon (in the Mt. Hilong – Hilong Range), Barangay
<i>Ansonia muelleri</i>	EMD 301	PNM*	East Mindanao	San Antonio, Municipality of Remedios T. Romualdez, Agusan del Norte Province Agay River, Mt. Bato – Batohon (in the Mt. Hilong – Hilong Range), Barangay
<i>Ansonia muelleri</i>	EMD 302	PNM*	East Mindanao	San Antonio, Municipality of Remedios T. Romualdez, Agusan del Norte Province Agay River, Mt. Bato – Batohon (in the Mt. Hilong – Hilong Range), Barangay
<i>Ansonia muelleri</i>	ACD 3836	KU 319522	East Mindanao	San Antonio, Municipality of Remedios T. Romualdez, Agusan del Norte Province Mt. Magdiwata, Barangay Bayugan 2, Municipality of San Francisco,
<i>Ansonia muelleri</i>	ACD 3871	KU 319527	East Mindanao	Agusan del Sur Province Mt. Magdiwata, Barangay Bayugan 2, Municipality of San Francisco,
<i>Ansonia muelleri</i>	ACD 3958	KU 319522	East Mindanao	Agusan del Sur Province Mt. Magdiwata, Barangay Bayugan 2, Municipality of San Francisco,
<i>Ansonia muelleri</i>	GGT 043	PNM*	East Mindanao	Agusan del Sur Province Purok Kulapi, Barangay Bahi, Municipality of Maragusan,
<i>Ansonia muelleri</i>	GGT 051	PNM*	East Mindanao	Compostela Valley, Davao Province Purok Kulapi, Barangay Bahi, Municipality of Maragusan,
<i>Ansonia muelleri</i>	GGT 065	PNM*	East Mindanao	Compostela Valley, Davao Province Purok Kulapi, Barangay Bahi, Municipality of Maragusan,
<i>Ansonia muelleri</i>	ACD 2631	PNM*	East Mindanao	Compostela Valley, Davao Province Mt. Hamiguitan, Davao Oriental Province
<i>Ansonia muelleri</i>	ACD 2623	PNM*	East Mindanao	Mt. Hamiguitan, Davao Oriental Province Mt. Hamiguitan, Davao Oriental Province
<i>Ansonia muelleri</i>	ACD 2638	PNM*	East Mindanao	Mt. Hamiguitan, Davao Oriental Province Mt. Hamiguitan, Davao Oriental Province
<i>Ansonia muelleri</i>	ACD 2669	PNM*	East Mindanao	Mt. Hamiguitan, Davao Oriental Province Mt. Hamiguitan, Davao Oriental Province
<i>Ansonia muelleri</i>	ACD 2702	PNM*	East Mindanao	Mt. Hamiguitan, Davao Oriental Province Mt. Hamiguitan, Davao Oriental Province
<i>Ansonia muelleri</i>	ACD 2639	KU 326765	East Mindanao	Mt. Hamiguitan, Davao Oriental Province Mt. Hamiguitan, Davao Oriental Province

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