The role of repeated sea-level fluctuations in the generation of shrew (Soricidae: *Crocidura*) diversity in the Philippine Archipelago

Jacob A. Esselstyn *, Rafe M. Brown

Department of Ecology and Evolutionary Biology, Biodiversity Research Center, University of Kansas, 1345 Jayhawk Blvd., Lawrence, KS 66045, USA

ABSTRACT

Repeated sea-level fluctuations during and prior to the Pleistocene had a dramatic effect on the distribution of land and connection among islands between the Asian and Australian continents. These cycles of connection and isolation have long been recognized as an important factor determining the distribution and organization of biodiversity in the Philippines. However, surprisingly few studies have tested for predicted patterns of genetic diversity derived from a Pleistocene sea-level model of geography. Here, we examine evidence for fit to such a model in a widely distributed lineage of shrews (*Crocidura*). The topology of relationships among *Crocidura* from the Philippines is concordant with a Pleistocene sea-level model, but (1) AMOVAs reveal that genetic diversity is explained at least as well by modern islands as by Pleistocene islands; (2) Mantel tests reveal a significant influence of isolation by distance; and (3) the degree of genetic divergence between some populations connected by dry land during the last glacial maximum reveals isolation that almost certainly predates the most recent glacial activity. We further employ multiple strategies for inferring time-calibrated phylogenies, but these result in widely varying time estimates for the invasion of SE Asian islands by shrews. Overall, our results suggest Pleistocene sea-level fluctuations have been an important, but not dominant factor shaping shrew diversity.

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1. Introduction

Geographic patterns of variation within lineages reveal basic features of the processes that generate and maintain biodiversity. These patterns, when considered in concert with well-substantiated phylogenetic hypotheses, illuminate evolutionary processes and have important implications for the conservation of biodiversity (Carstens et al., 2004; Evans et al., 2003b; Heaney et al., 2005). Observed patterns may lead to insights regarding the nature (e.g., allopatric vs. sympatric) and tempo of speciation, the temporal and spatial occurrence of barriers to gene flow, the nature of demographic parameters through time, and the appropriate partitioning of diversity into taxonomic units.

The extent of connections among modern islands during Pleistocene (and earlier) sea-level low stands has long been recognized as an important factor in the evolution and assembly of biodiversity in the Philippines and on the Sunda and Sahul shelves (Delacour and Mayr, 1946; Dickerson, 1928; Heaney, 1985; Inger, 1954; Kloss, 1929; Simpson, 1977; Voris, 2000). Deep-water channels generally separate distinctive biological communities, whereas neighboring islands currently separated by shallow water tend to share largely similar biotas (Brown and Diesmos, 2002; Dickerson, 1928; Esselstyn et al., 2004; Heaney, 1986; Heaney

et al., 1998). These shallow-water islands experienced repeated bouts of connection and isolation due to Pleistocene sea-level fluctuations, the magnitude of which ranged from 100 to 140 m below current sea levels (Rohling et al., 1998). During periods of low sea level, five major islands existed in the Philippines; these are referred to as Pleistocene Aggregate Island Complexes (PAICs: Brown and Diesmos, 2002).

The commonly observed pattern of faunal similarity among islands within PAICs and sharp differences between faunas on neighboring complexes (Dickerson, 1928; Heaney, 1986; Heaney et al., 1998) implies that gene flow within PAICs has been common, if intermittent. However, because the role of Pleistocene geography has long been recognized, there is a risk that taxonomic decisions could have been based in part on PAIC geography, and PAIC importance then inferred from taxonomy, thereby resulting in an over-emphasis of the importance of Pleistocene sea-level fluctuations. Thus, there is a need to evaluate the spatial distribution of genetic, morphological, and ecological diversity delimited by criteria independent of PAIC geography. Surprisingly few studies have attempted to do so (though see Brown and Guttman, 2002; Evans et al., 2003a; Heaney et al., 2005; Roberts, 2006a,b), leaving open the question of how pervasive the influence of PAIC geography might have been.

An ideal system for testing for the effects of intermittent land connections on the diversification process would be an organism that: (1) is present on all islands; (2) is short-lived with a rapid rate of substitution (so that genetic signal will be detectable); (3) has a

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* Corresponding author. Fax: +1 785 864 5335.
E-mail address: esselsty@ku.edu (J.A. Esselstyn).

limited ability to cross sea channels; and (4) is commonly collected during biodiversity surveys. Shrews (Soricidae: *Crocidura*) fit this ideal in many respects. They are known from all major islands that have been surveyed for small, non-volant mammals in the Philippines (Esselstyn et al., 2009; Heaney and Ruedi, 1994); they are short-lived, which may result in a rapid rate of molecular evolution; and they have a small body size and high metabolic rate, presumably making them relatively poor over-water colonizers.

Ruedi (1996) used allozyme data to explore diversity in shrews on the Sunda Shelf and the oceanic islands of the Philippines and Sulawesi. He found that isolation by distance failed to explain diversity throughout the region, but that distance explained a significant proportion of variation when the analysis was restricted to the Sunda Shelf. We interpret this to indicate that intermittent, shallow-water barriers isolating islands of the Sunda Shelf have been insignificant in the generation of diversity (relative to distance), whereas, deep-water channels isolating the Philippines and Sulawesi represent significant barriers to dispersal. In contrast, Gorog et al. (2004) considered movements of rodents across lowland areas of Borneo to have been rare during the Pleistocene, perhaps due to aridification and the limited distribution of forests (Bird et al., 2005; Heaney, 1991), implying that land connections on their own, may not be sufficient to provide for dispersal.

Since the publication of Ruedi's (1996) work, the number of *Crocidura* specimens from the region has increased significantly and recent studies (Dubey et al., 2008; Esselstyn et al., 2009) provide phylogenetic context, allowing a test of these patterns within the Philippine archipelago. Nine species of *Crocidura* currently are recognized in the Philippines (Heaney and Ruedi, 1994; Hutterer, 2007). Esselstyn et al. (2009) include seven of these in phylogenetic inferences. Six Philippine species form a well-supported, widespread, monophyletic group (*beatus, grayi, mindorus, negrina, palawanensis,* and *panayensis*). Of the remaining species, two (*batakorum* and *attenuata*) probably represent separate invasions of the archipelago and one (*grandis*) has not been seen since the holotype was collected in 1906 (Esselstyn et al., 2009; Heaney and Ruedi, 1994; Miller, 1910).

If we assume that the history and geography of PAICs was the dominant factor in the evolution of Philippine biodiversity, several predictions may be derived, including: (1) populations on modern islands will be most closely related to adjacent island populations within the PAIC; (2) individual PAICs will hold monophyletic lineages (though gene trees will not always reflect this); (3) degree of genetic divergence between populations on different PAICs will be greater than those between populations residing on the same PAIC; and (4) populations separated by shallow water will have divergence dates associated with the end of the last glacial maximum (LGM).

In this study, we use time-calibrated phylogenetic estimates, analyses of molecular variance (AMOVAs), Mantel tests, and phylogeographic summary statistics to test for an effect of sea-level fluctuations on the generation of genetic diversity within the widespread Philippine clade. In particular, we explore patterns of genetic diversity to address the following questions: (1) Is genetic diversity in shrews partitioned primarily by PAICs and secondarily by islands within these complexes? (2) Do any of the divergences separating populations on neighboring islands within PAICs date to the end of the LGM, when rising sea levels last separated these islands? (3) Do other factors (e.g., isolation by distance and island area) contribute to genetic diversity?

To determine the generality of our conclusions, we make comparisons to a published study of genetic diversity in a small fruit bat endemic to the Philippines, (*Haplonycteris fischeri*; Roberts, 2006b). Given the major differences in natural history between these two lineages (e.g., dispersal capacity, life span, and reproductive rates), any similarities in their patterns of genetic diversity

might indicate pervasive causes. However, we note that *Crocidura* and *Haplonycteris* are not different in all aspects. For instance, both are probably most abundant in mid-elevation forests and moderately tolerant of habitat disturbances (Heaney et al., 1998; Roberts, 2006b). Although we compare patterns within a single named species (*H. fischeri*) to a clade of six named species (*Crocidura*), levels of genetic divergence among island populations within these two groups are similar, suggesting that either different taxonomic standards have been applied to these groups, or the extent of morphological diversification has been greater in *Crocidura*.

Our results show that PAICs explain some genetic variation and hence evolutionary history. However, the proportions explained in *Crocidura* are much less than those noted by Roberts (2006b) in *H. fischeri*. Phylogenetic topology in *Crocidura* fits the PAIC model well, but some divergence dates almost certainly predate the LGM. We further note that the inference of the time of speciation events is dependent on the calibration point used, and choosing among calibration points that produce wildly disparate estimates is difficult. Given our results, it is apparent that Pleistocene sea-level fluctuations are an important factor influencing patterns of variation, but they operated in a context where island area, isolation, and topographic relief, along with features of the organisms themselves, and perhaps other variables, must be taken into account.

2. Materials and methods

We combined mtDNA sequence data from several sources (Bannikova et al., 2006; Brandli et al., 2005; Dubey et al., 2007a,b, 2008; Esselstyn et al., 2009; Ohdachi et al., 2006, 2004; Ruedi et al., 1998) to explore patterns of genetic diversity of shrews within the Philippine Archipelago (see Appendix for details). We follow the taxonomy of Heaney and Ruedi (1994) and Hutterer (2007). Populations were sampled on all major Philippine islands and several small islands; we sampled multiple populations from the large islands of Luzon, Mindanao, and Mindoro (Fig. 1). Based on the results described in Esselstyn et al. (2009), we assign newly discovered populations from Calayan and Samar islands to *C. grayi* and *C. beatus*, respectively.

We simultaneously estimated phylogenetic relationships and divergence dates using sequences of the mitochondrial gene, Cytochrome *B* (CytB). We included all taxa from the species-level alignment of Esselstyn et al. (2009) and added one terminal for all additional species found in Dubey et al.'s (2008) Old World + Asian *Crocidura* clade, including *Diplomesodon*. We included *Suncus murinus* in the analyses to serve as outgroup. These analyses were conducted in BEAST 1.4.8 (Drummond and Rambaut, 2007) using the Yule speciation model and relaxed uncorrelated lognormal clock with sequences partitioned into 1st + 2nd and 3rd codon positions. The GTR + I + Γ model of sequence evolution was chosen using the AIC criterion in MODELTEST (Posada and Crandall, 1998). Parameter estimates were unlinked between the two partitions. Analyses were initiated with an UPGMA starting tree and run for 2×10^7 generations with trees and parameters sampled every 2000 generations. We examined trace files and effective sample sizes of parameters drawn from MCMC chains in Tracer (Rambaut and Drummond, 2007) and compared posterior probabilities of splits between independent runs in AWTY (Nylander et al., 2008) to check for evidence of stationarity and convergence. The first 50% of each run was discarded as burn-in. We applied five calibration strategies to these analyses. All initial calibration strategies relied on normally distributed prior probabilities on the ages of particular nodes in the tree or on the substitution rate. We calibrated analyses with the oldest known fossil *Crocidura* (5.03 My ago: Butler, 1998), secondary calibrations from a recent higher-level phylogenetic analysis (5.75 My ago origin of Old World + Asian *Crocidura*;
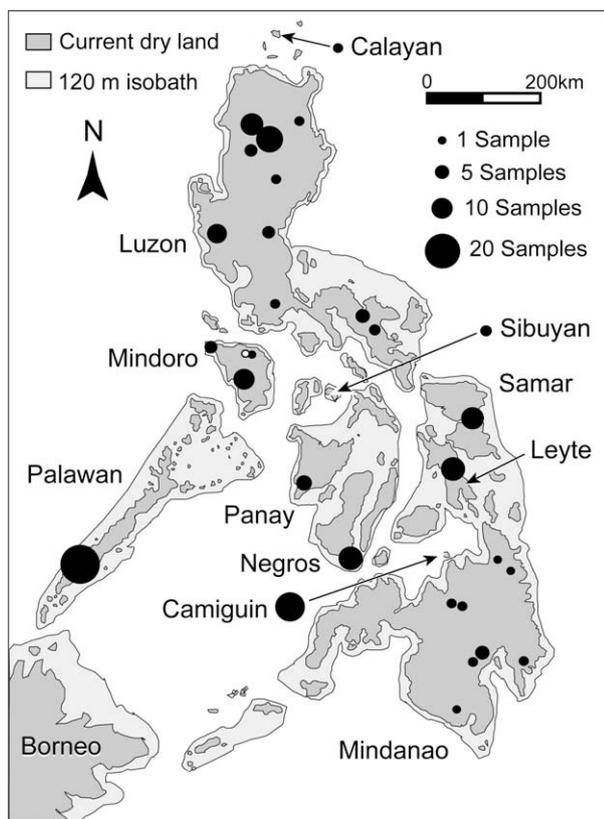
Fig. 1. Distribution of shrew samples from the Philippines. Sample size is indicated by the diameter of the circle. On the island of Mindoro, we sampled two species; *C. mindorus* is noted with a white circle and *C. grayi halconus* with black circles. The modern distribution of land is shown in medium gray with the shorelines during Pleistocene sea-level low stands represented by the 120 m isobath indicated by light gray (after Heaney, 1985).

4.39 My ago origin of a clade found in the Philippines and Sunda Shelf: Fig. 1 of Dubey et al., 2008), and three geological calibrations from the Philippines. The geological calibration points were the uplift of Camiguin Island, occurring primarily around 0.35 My ago (Heaney and Tabaranza, 2006; Sajona et al., 1997), the uplift of the Samar + Leyte region ca. 3 My ago (Sajona et al., 1997), and the collision of the Bicol Peninsula with Luzon Island ca. 3 My ago (Hall, 2002). Each of these ages was used as a calibration point for the most recent common ancestor of the shrew population residing on that block and its sister group. We used 0.5 My as an arbitrarily determined standard deviation for the fossil, secondary, and geological calibrations. We ran additional analyses placing a prior probability on the substitution rate. These relied on the average mammalian rates for synonymous and nonsynonymous substitutions in CytB, determined from Fig. 2 of Pesole et al. (1999). We calculated the proportions of each type of substitution in the *Crocidura* CytB matrix using DnaSP (Rozas and Rozas, 1999) and used these values to calculate a weighted average of the mammalian rates. We then used a normally distributed prior on the per-site substitution rate with a mean of $0.009695\,My^{-1}$ and standard deviation of $0.002\,My^{-1}$. The standard deviation was arbitrarily determined, but our intent was to encompass the range of variation known from mammals (Gissi et al., 2000; Pesole et al., 1999). Two independent runs were completed for each calibration strategy and the final 5000 trees from each run combined to calculate maximum clade credibility trees, posterior probabilities, median node ages, and 95% highest posterior densities of node ages.

A combined analysis that used all of the above calibration strategies was then employed. We changed the oldest fossil *Crocidura*

calibration to a uniform prior with a range of 5–50 My so that it would function as a minimum calibration point. All other priors were used as above. Four runs with these priors were undertaken for 2.5×10^{7} generations. Because of strong conflict among the priors, we were forced to begin each run with a tree that resembled the 'correct' topology. We therefore started each run using the topology with the highest likelihood from the island-age calibrated runs. Again, the first 50% of samples were discarded and the maximum clade credibility tree was computed.

We then posed the question: does the phylogenetic association between island populations conform to the PAIC model more than would be expected by chance? We considered topologies to conform to the PAIC model if all island populations were most closely related to other island populations within their respective PAIC. We excluded the population of *C. beatus* from Camiguin Island because the phylogenetic relations of populations on small, oceanic islands are not informative with regard to the importance of PAICs in structuring genetic diversity. We considered the proportion of possible unrooted trees with 10 terminals (10 modern islands sampled from the Philippines, excluding Camiguin) in which taxa 1, 2, and 3 (Samar, Leyte, and Mindanao) form a monophyletic group, as do taxa 4 and 5 (Negros and Panay). We counted the number of possible trees consistent with this constraint in PAUP 4.0b10 (Swofford, 1999) and divided this by the total number of possible trees with 10 terminals.

We then used a concatenated matrix of 1019 nucleotides of CytB and 1018 nucleotides of ND2 from 173 specimens (Fig. 1), representing six currently recognized species from the Philippines (Appendix). This matrix is complete with no missing characters. We computed several indices of genetic diversity, including the number of haplotypes, nucleotide diversity (π), and uncorrected genetic distance (p), using Arlequin 3.1 (Excoffier et al., 2005).

To determine whether divergences between island populations within PAICs could have originated at the end of the LGM, we calculated the rate of molecular evolution necessary to generate the observed, uncorrected genetic divergence between these populations. This was undertaken for the divergence between the Samar/Leyte clade and the Mindanao populations of *C. beatus*, and between *C. negrina* and *C. panayensis*. We used 10,000 (10 K) years ago as the approximate time when rising waters would have separated these islands (Siddall et al., 2003; Voris, 2000).

Three-way analyses of molecular variance (AMOVAs) were implemented to evaluate the role of Pleistocene sea-level fluctuations in the generation of genetic diversity. AMOVAs were completed with 1000 permutations in Arlequin 3.1 to explore genetic diversity across the entire archipelago with sequences partitioned by current taxonomy, PAICs, and modern islands. We also subjected the *C. grayi* and *C. beatus* complexes to independent AMOVAs, with data partitioned by modern islands and sample sites. Because we observed a large difference between *Crocidura* and *Haplonycteris* in the contribution of PAICs to genetic variation, we wanted to know how much of this difference might be due to disparities in the geographical distribution of samples available for these two lineages. We therefore repeated 10 iterations of the AMOVA of PAICs/modern islands/populations on reduced data sets. These jackknifed data sets were generated by removing randomly selected haplotypes (36–40% of all haplotypes were removed per iteration) and revealed the potential effects of variation in geographic sampling. Because Luzon Island was densely sampled and no other islands within the Luzon PAIC were represented, we analyzed the jackknifed data sets with all Luzon samples excluded.

We then tested for an association between geographic and genetic distances using Mantel tests (Mantel, 1967). Latitude and longitude were taken from museum catalogs, specimen tags, or the field notes of collectors. Specimens sampled within 5 km of each other were considered members of the same population. A matrix

of geographic straight-line distances among populations (including over-water distance when relevant) was generated using ArcGIS tools (Beyer, 2004). Mean among population genetic distances were generated in Arlequin 3.1. Mantel tests were completed in the R package, APE (Paradis et al., 2004; R Development Core Team, 2009), and relied on 5000 permutations to evaluate significance. We applied these methods to (1) all populations of *C. grayi*, (2) *C. grayi* from Luzon Island only, (3) all *C. beatus*, and (4) *C. beatus* from Mindanao Island only.

To test for an effect of island area on diversity, we plotted the per-population nucleotide diversity against the logarithm of island area and fit a least-squares regression to these data. Island areas were garnered from Heaney et al. (2002) and Allen et al. (2006).

3. Results

Phylogenetic analyses resulted in widely varying age estimates for clades of interest. Fossil (Fig. 2) and secondary calibrations (not shown) produced similar results, as did geological (not shown) and substitution rate (Fig. 3) priors. However, the former two produced much younger inferences than the latter two. The analysis combining all calibration points resulted in intermediate age estimates (Fig. 4).

The probability of a phylogeny with randomly determined relationships showing Samar + Leyte + Mindanao and Negros + Panay relationships is 0.001. Thus, the topological relationships show a greater concordance to PAIC geography than would be expected by chance alone (Fig. 5).

One hundred and six haplotypes were identified among 173 mitochondrial sequences for an overall haplotype diversity of 0.6127. Of the 106 haplotypes, 72 were represented by a single individual, 14 by 2 individuals, 18 by 3–4 individuals, and 2 by 7 individuals (Table 1).

If the levels of genetic divergence among islands within PAICs (Fig. 6) are the result of 10 K years of isolation, then rates of substitution necessary to generate the observed divergences between populations on Negros and Panay islands and between Samar/Leyte and Mindanao islands would be 1.39 and 2.82 site^{-1} My^{-1}, respectively. These are two orders of magnitude faster than those typically reported for mammals (Fumagalli et al., 1999; Gissi et al., 2000). Thus, we suggest that a divergence as recent as the end of the last glacial maximum (LGM) is highly unlikely for these populations. If these divergences occurred after the Pliocene–Pleistocene boundary, substitution rates would be approximately $\geqslant 0.0077$ and $\geqslant 0.0157$ site^{-1} My^{-1}, calculations more consistent with what is thought of as typical mammalian rates (Pesole et al., 1999). Thus, it is plausible that these divergences occurred during the early–middle Pleistocene (Figs. 2–4), but extremely unlikely that they occurred after the LGM. However, we note that the populations on Samar and Leyte are genetically indistinguishable and gene flow may have occurred between these two islands during the LGM.

The proportions of genetic variation accounted for by taxonomy, PAICs, and modern islands are similar (48–54%), as are the among-population and within-population comparisons across these three partitioning strategies, at 35–42% and 10–11% (Fig. 7), respectively. In each case, all levels of the three-way AMOVA account for significant proportions of variation ($P < 0.001$).

When samples were analyzed with a PAIC/island/population hierarchy, PAICs explained substantially less genetic diversity in *Crocidura* (29%) than reported for *Haplonycteris* (78%; Roberts, 2006b), with both among-island and within-island comparisons accounting for the difference (Fig. 8). Some of these differences may be due to the extent and distribution of sampling; however, our iterations on reduced *Crocidura* data sets reveal that the pro-

portions estimated are relatively stable, with modern islands retaining greater explanatory power than PAICs (Fig. 9). Removing Luzon from consideration lessens the difference, but modern islands retain slightly more explanatory power (Fig. 9).

The AMOVAs further reveal that among island genetic diversity accounts for a substantially larger proportion of variation in *C. beatus* than in *C. grayi* (Fig. 10). This is due in part to the deep divergence separating populations on Samar and Leyte from those on Mindanao. These islands are separated by very shallow water and probably were connected as recently as 10 K years ago (Siddall et al., 2003; Voris, 2000). In contrast, some islands, which have never been connected to any other, hold populations with only shallow genetic divergences separating them from their presumptive source populations. These include populations of *C. grayi* on Calayan Island and *C. beatus* on Camiguin Island, each with uncorrected genetic divergences ≈ 0.01 (Fig. 6).

Large islands have the potential to provide opportunities for within-island diversification because of the potential effects of isolation by distance, isolated mountain ranges, and elevational habitat gradients (Evans et al., 2003a; Heaney and Rickart 1990; Steppan et al. 2003; Wright 1950). Mantel tests revealed an effect of isolation by distance within the widespread species, *C. grayi* and *C. beatus* (Table 2). All but one test were significant, indicating that isolation by distance has an effect in most cases. The test with the smallest sample size was insignificant, perhaps due to a lack of statistical power. Island area shows a positive, though not statistically significant relationship with mitochondrial diversity in this data set. This is apparent whether nucleotide diversity is considered across an island (Table 1), or at the population level (Fig. 11). Regression of these data failed to reveal significant explanatory power ($R^2 = 0.105$, $P = 0.13$), though the positive trend suggests biological significance.

4. Discussion

Our analyses reveal a complex role for Pleistocene sea-level fluctuations in the diversification of shrews in the Philippines. The topological pattern among island populations is perfectly concordant with PAIC geography (Fig. 5), and the probability of this happening by chance alone is small. However, divergences between some island populations within PAICs almost certainly predate the end of the LGM. These divergences may have occurred earlier in the Pleistocene, perhaps associated with previous fluctuations in sea level. Other island populations within PAICs appear to be more closely related and have yet to achieve reciprocal monophyly (e.g., Samar and Leyte populations of *C. beatus*). Given this variation in pattern, the simple assumption that gene flow occurs wherever and whenever dry land is present is probably incorrect for this system and others (e.g., Gorog et al., 2004; Roberts, 2006b).

The inference of the timing of speciation events is heavily dependent on calibration strategy. Our estimates based on fossil and secondary calibrations broadly overlap, as do those based on the mean mammalian substitution-rate and island-age calibrations. The former two produce very young estimates ($\leqslant 5$ My ago) and the latter two very old estimates ($\leqslant 25$ My ago). There is no overlap in 95% highest posterior densities (HPD) between the young and old estimates (Figs. 2 and 3), making reconciliation of these two sets of dates challenging. However, we consider it likely that the dates inferred from the fossil and secondary calibrations underestimate the true ages. The earliest known fossil *Crocidura* should be considered a minimum calibration point, but we treated it as a mean age for the origin of the genus in our analysis (Fig. 2) because of a lack of potential upper bounds on the age of any node. The secondary calibrations we used also were derived from fossil-calibrated analyses (Dubey et al., 2008) and they produced dates

First Fossil
Crocidura
5.03 mya

Suncus murinus, PH
leucodon
zimmermanni, GR
russula
sicula, MA
obscurior 1, IC
obscurior 3, GU
obscurior 2, IC
batakorum, Palawan, PH
musseri, Sulawesi, ID
sp. 1, Sulawesi, ID
sp. 2, Sulawesi, ID
sp. 3, Sulawesi, ID
lea, Sulawesi, ID
elongata, Sulawesi, ID
rhoditis, Sulawesi, ID
levicula, Sulawesi, ID
sp. 4, IN
Diplomesodon, TU
zarudnyi, IR
shantungensis, TW
mimula, HU
aleksandrisi, LI
sibirica, RU
suaveolens, CH
cf. *tanakae*, VT
*fuliginosa*1, VT
*fuliginosa*2, PM
horsfieldii, TH
watasei, RY
maxi, Sumatra, ID
dsinezumi, JP
kurodai, TW
lasiura, RU
paradoxura, Sumatra, ID
wuchihensis, CH
*attenuata*1, CH
*attenuata*2, CH
lawuana, Java, ID
*beccarii*1, Sumatra, ID
lepidura, Sumatra, ID
*beccarii*2, Sumatra, ID
brunnea, Java, ID
orientalis, Java, ID
foetida, Borneo, MY
nigripes, Sulawesi, ID
malayana, PM
negligens, Tioman, MY
palawanensis, Palawan, PH
*grayi*1, Luzon, PH
*grayi*2, Luzon, PH
*grayi*3, Luzon, PH
*grayi*4, Calayan, PH
grayi halconus, Mindoro, PH
mindorus, Mindoro, PH
mindorus, Sibuyan, PH
negrina, Negros, PH
panayensis, Panay, PH
*beatus*1, Leyte, PH
*beatus*2, Mindanao, PH
*beatus*3, Mindanao, PH
*beatus*4, Mindanao, PH
*beatus*5, Mindanao, PH
*beatus*6, Camiguin, PH

6.0   5.0   4.0   3.0   2.0   1.0   0.0
Millions of Years Ago

Fig. 2. Maximum clade credibility tree from a phylogenetic analysis of *Crocidura*. This tree was calibrated with the oldest known fossil *Crocidura*. Numbers at nodes represent median age estimates in millions of years and medium gray bars represent the 95% highest posterior density of age estimates. Black diamonds indicate node support of ⩾90% posterior probability. The vertical, light-gray bar represents the Pleistocene Epoch. Terminals are labeled with taxonomic names, followed by abbreviated localities (CH, China; GR, Greece; GU, Guinea; HU, Hungary; IC, Ivory Coast; ID, Indonesia; IN, India; IR, Iran; JP, Japan; LI, Libya; MA, Malta; MY, Malaysia; PH, Philippines; PM, Peninsular Malaysia; RU; Russia; RY, Ryukyu islands; TH, Thailand; TW, Taiwan; VT, Vietnam).

similar to, but slightly older than those from our fossil-calibrated analysis. The per-site substitution rate necessary to generate branch lengths in our fossil-calibrated analysis was much faster (mean = 0.044, 95% HPD = 0.033–0.055 site^{-1} My^{-1}) than the mean mammalian rate (0.0097 site^{-1} My^{-1}). Although there is little basis to choose among potential calibration strategies, we are intrigued by the similarity in age estimates derived from the substitution-rate and island-age calibrated trees. These two analyses produced
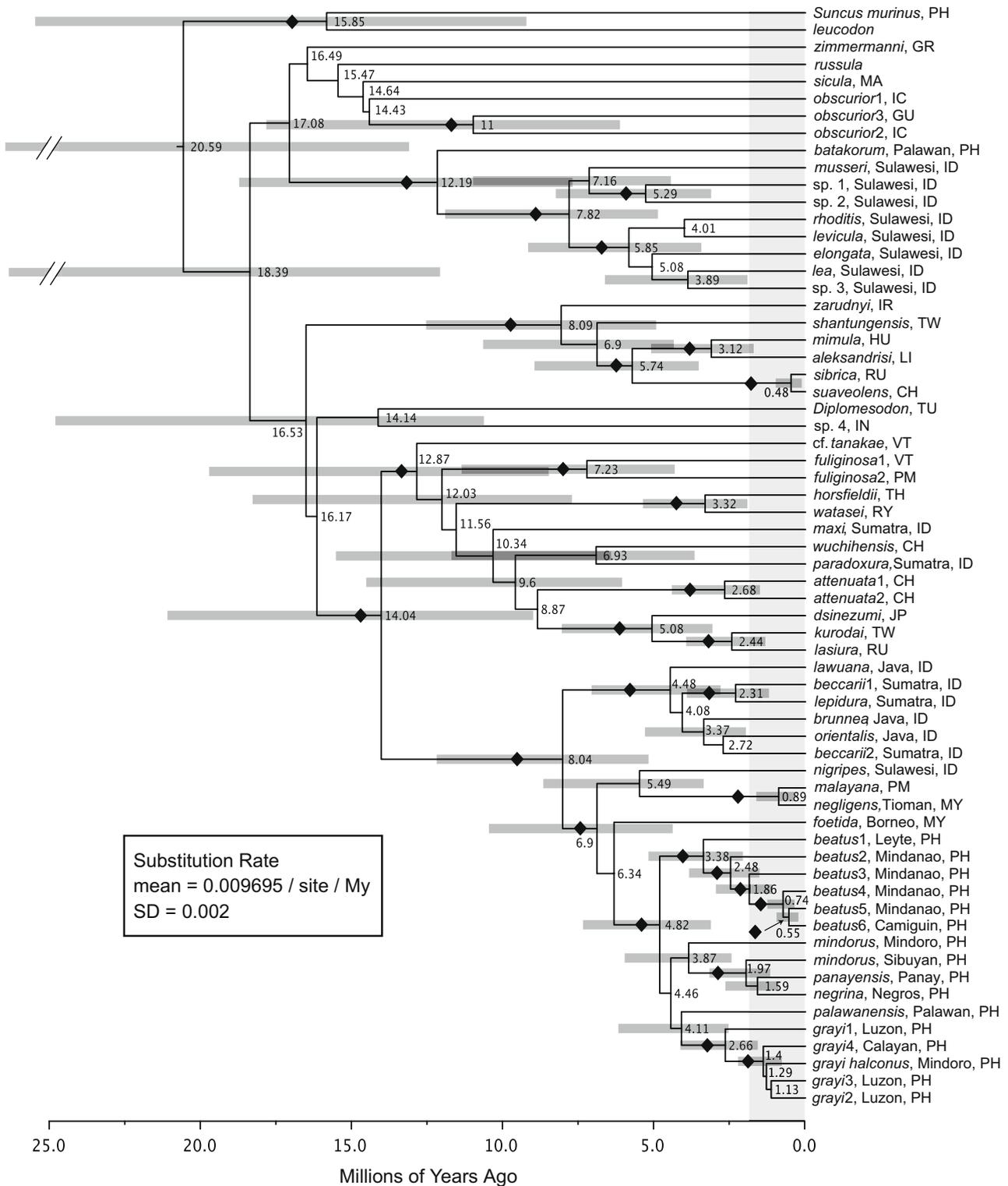
Fig. 3. Maximum clade credibility tree from a phylogenetic analysis of *Crocidura*. This tree was calibrated with a normal prior on the substitution rate (mean = 0.009695, SD = 0.002 site^{-1} My^{-1}). Numbers at nodes represent median age estimates in millions of years and medium gray bars represent the 95% highest posterior density of age estimates. Black diamonds indicate node support of ⩾90% posterior probability. The vertical, light-gray bar represents the Pleistocene Epoch. Terminals are labeled with taxonomic names, followed by abbreviated localities (CH, China; GR, Greece; GU, Guinea; HU, Hungary; IC, Ivory Coast; ID, Indonesia; IN, India; IR, Iran; JP, Japan; LI, Libya; MA, Malta; MY, Malaysia; PH, Philippines; PM, Peninsular Malaysia; RU, Russia; RY, Ryukyu islands; TH, Thailand; TW, Taiwan; VT, Vietnam).

broadly overlapping HPDs of node ages and are derived from unrelated calibration strategies. However, other than the independence of data sources (or lack thereof), there is little information that might be used to choose between the old and young dates. The use of island ages as calibration points on molecular phylogenies

assumes that islands are colonized shortly after they emerge from the sea. Unfortunately, little evidence is available to evaluate this assumption (though see: Brown et al., in press; Steppan et al., 2003). We suggest that an analysis testing for rank-correlations between speciation events and island emergences using numerous
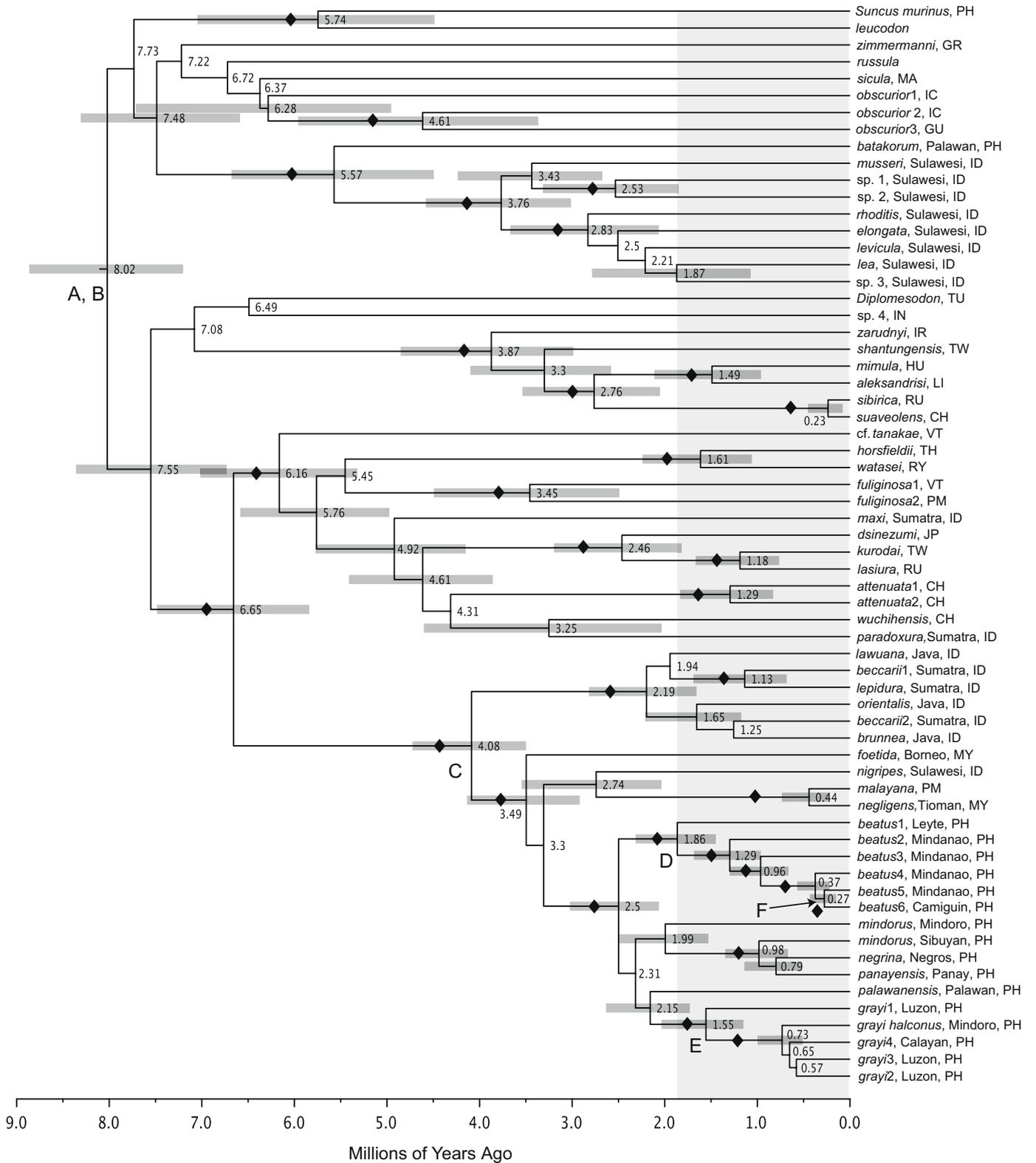
Fig. 4. Maximum clade credibility tree from a phylogenetic analysis of *Crocidura*. This tree was calibrated with a combination of available strategies, indicated by letters at nodes: (A) oldest fossil *Crocidura* (uniform distribution 5–50 My ago); (B and C) secondary calibrations from Dubey et al. (2008: normal distribution with mean = 5.75 My ago, SD = 0.5 My and 4.39 My ago, SD = 0.5 My, respectively); (D and E) geological calibrations from uplift of Leyte and collision of Bicol Peninsula with Luzon (each at mean = 3 My ago, SD = 0.5 My); (F) uplift of Camiguin (0.35 My ago, SD = 0.5 My); and finally with a normal prior on the substitution rate (mean = 0.009695 site^{-1} My^{-1}, SD = 0.002). Numbers at nodes represent median age estimates in millions of years and medium gray bars represent the 95% highest posterior density of age estimates. Black diamonds indicate node support of ⩾90% posterior probability. The vertical, light-gray bar represents the Pleistocene Epoch. Terminals are labeled with taxonomic names, followed by abbreviated localities (CH, China; GR, Greece; GU, Guinea; HU, Hungary; IC, Ivory Coast; ID, Indonesia; IN, India; IR, Iran; JP, Japan; LI, Libya; MA, Malta; MY, Malaysia; PH, Philippines; PM, Peninsular Malaysia; RU, Russia; RY, Ryukyu islands; TH, Thailand; TW, Taiwan; VT, Vietnam).
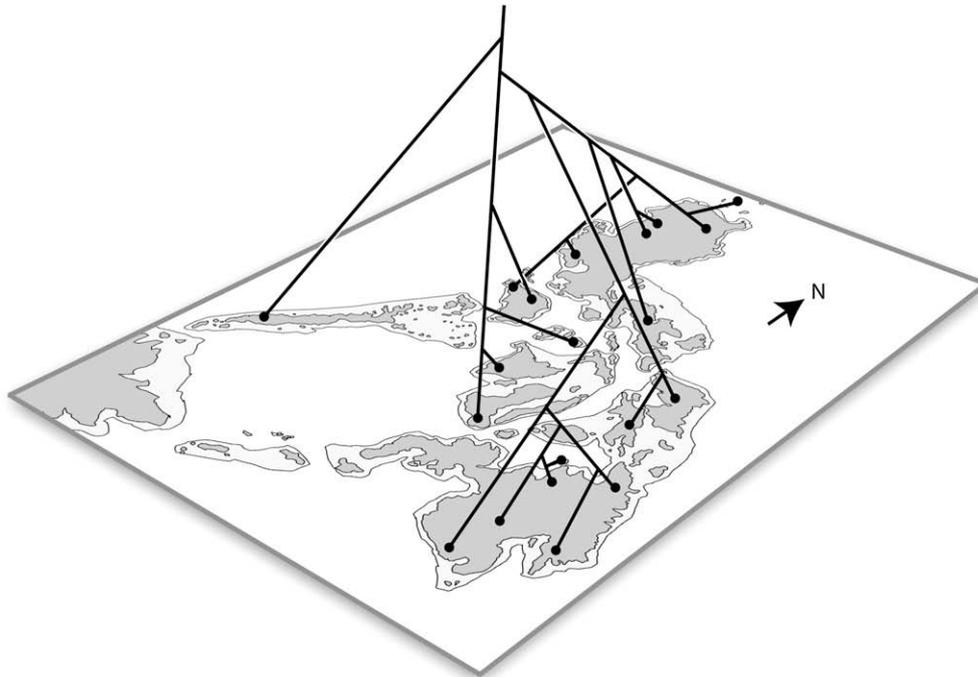
Fig. 5. Our preferred phylogenetic hypothesis for Philippine *Crocidura*, derived from analyses here and in Esselstyn et al. (2009), and mapped on to Pleistocene geography. Modern islands are shown in medium gray, surrounded by the extent of land during Pleistocene sea-level low stands (−120 m) in light gray. Monophyletic lineages tend to be found on Pleistocene islands more often than would be expected by chance alone.

Table 1
Summary of mtDNA sequence diversity in *Crocidura* on Philippine islands.

Species	Island	Area (km^2)	Populations sampled	Haplotype diversity	Nucleotide diversity
C. beatus	Camiguin	265	1	0.5625	0.0020
	Leyte	7213	1	0.4167	0.0074
	Samar	13,429	1	0.7273	0.0097
	Mindanao	96,467	8	0.875	0.0227
C. grayi	Calayan	196	1	1.0	0.0015
	Mindoro	9,735	3	0.6667	0.0054
	Luzon	107,170	10	0.625	0.0279
C. mindorus	Sibuyan	449	1	0.6667	0.0065
C. negrina	Negros	13,670	1	0.4167	0.0051
C. palawanensis	Palawan	11,875	1	0.4348	0.0046
C. panayensis	Panay	12,300	1	0.8333	0.0020

co-distributed lineages might shed light on the validity of this assumption. Our combined analysis, which incorporated all potential calibrations and treated the oldest fossil *Crocidura* as a minimum bound, inferred dates that are intermediate, but closer to the young set of dates derived from the fossil- and secondary-calibrated trees. Clearly, any determination of the number of speciation events that took place during the Pleistocene requires better evidence regarding the validity and variance of available calibrations.

Several very shallow divergences separate populations on islands that have never been connected to another landmass (Calayan, Camiguin, and Mindoro) from their closest relatives on Luzon and Mindanao, perhaps suggesting recent colonization of these islands. Although this is not a prediction that could be derived from a PAIC model, occasional over-water colonization events do not necessarily diminish the importance of PAICs in shaping evolutionary history. Colonization of previously uninhabited islands could reasonably be expected to occur throughout history under a PAIC model. We note that all such island populations (i.e. those that are not part of a PAIC: Calayan, Camiguin, Mindoro, and Sibuyan) are most closely related to populations on large, adjacent islands (Esselstyn et al., 2009), suggesting an element of predictability in
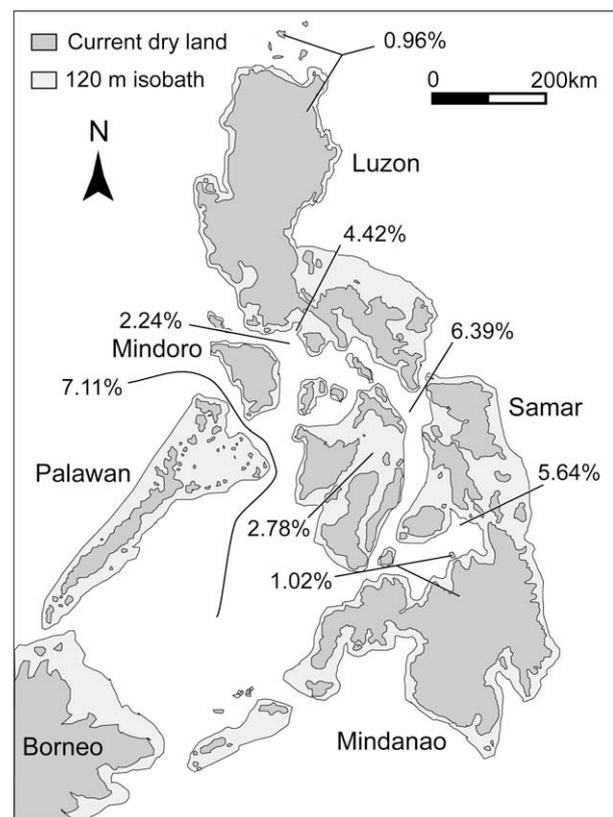
Fig. 6. Percentages of uncorrected divergence observed in mtDNA sequences across several putative barriers to dispersal and corridors for gene flow, as inferred from the Pleistocene distribution of land.

the colonization process. Divergences between these populations and their putative sources range from <0.01 (Calayan) to >0.06 (Sibuyan). Distinguishing between the effects of distance and PAIC
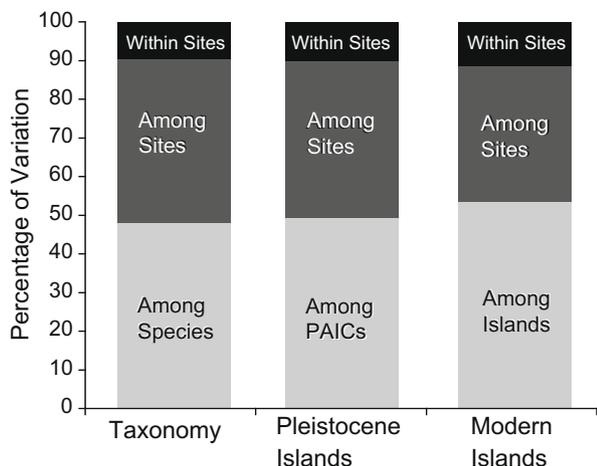
Fig. 7. Results of three-way AMOVAs. Three hierarchies were used to explore the partitioning of genetic diversity in Philippine *Crocidura*. These included partitioning by taxonomy, by Pleistocene Aggregate Island Complex, and by modern islands.
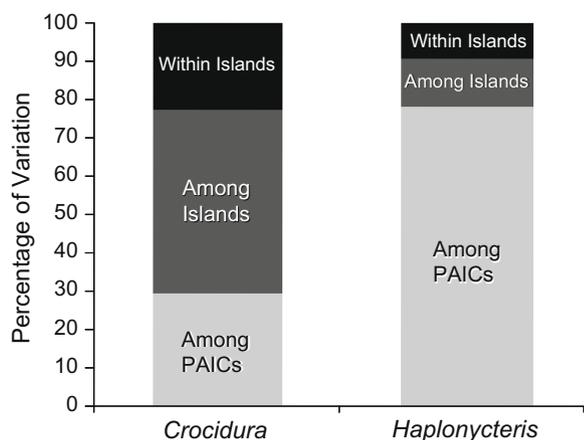
Fig. 8. Results of three-way AMOVAs comparing the role of Pleistocene Aggregate Island Complexes (PAICs) in structuring genetic diversity in Philippine *Crocidura* and *Haplonycteris* (from Roberts, 2006b).

Fig. 9. Results of jackknifing AMOVAs showing the mean percentage of genetic variation explained by Pleistocene Aggregate Island Complexes (PAICs), islands within PAICs, and sample sites within islands when 36–40% of randomly selected haplotypes have been removed (light gray). All samples from Luzon were removed from the same jackknifed data sets (dark gray). Error bars represent ±1 standard deviation.
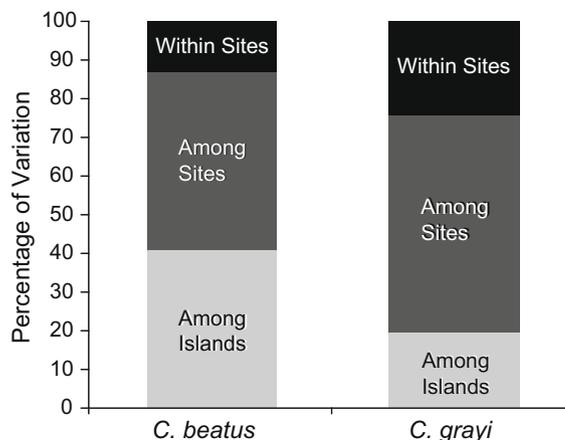
Fig. 10. Results of three-way AMOVAs showing the role of geography in structuring genetic diversity in *Crocidura beatus* from the Mindanao PAIC and *C. grayi* from the Luzon area.

Table 2
Results of Mantel tests of geographic and genetic distances. *P*-values significant at $\alpha \leqslant 0.05$ are noted in bold.

Species	Area	Z-statistic	P-value
C. grayi	Luzon Island only	3.797	**0.0034**
	Luzon, Mindoro, Calayan islands	8.258	**<0.0001**
C. beatus	Mindanao Island only	1.210	0.1270
	Mindanao, Samar, Leyte, Camiguin islands	5.185	**0.0014**

geography will be difficult because islands in close proximity tend to be separated by shallow water.

An effect of geographic distance on genetic diversity also is apparent in the Mantel tests conducted using populations sampled across PAICs and modern islands. The only Mantel test that was not significant was that restricted to Mindanao Island. The number of samples available from the island is limited (Fig. 1) and our failure to reject the null may be due to a lack of statistical power. We note however, that Mindanao Island has a complex geological history that probably includes the accretion of previously isolated islands (Hall, 2002). The geography of these Palaeo islands may have played a role in generating the substantial genetic diversity seen in some lineages (e.g., Jones and Kennedy, 2008; Roberts, 2006b) on modern Mindanao and our failure to find a signature of isolation by distance. Island area is known to have a positive correlation with genetic diversity (Nevo, 1978; Wright, 1931, 1950) and is probably important in shaping patterns of variation in the Philippines, where islands range in area from a few to >100,000 km². As expected, the magnitude and variation of within-population (Fig. 11) and within-island (Table 1) nucleotide diversity rises with increasing island area. The effect is not statistically significant, but the trend suggests biological importance.

AMOVAs revealed a stronger relationship between genetic diversity and modern islands than with PAICs, unlike the pattern

noted by Roberts (2006b) in *Haplonycteris*. The limited explanatory power of PAICs in the *Crocidura* data set appears real, as our jackknifing procedures had no effect on the relative proportions explained by PAICs and modern islands (Fig. 9). This is quite different from the patterns noted by Roberts (2006b) and Heaney et al. (2005) for forest-dependent bats and a rat, where differences among modern islands within PAICs accounted for little genetic diversity. In another study (Roberts, 2006a), three additional lin-
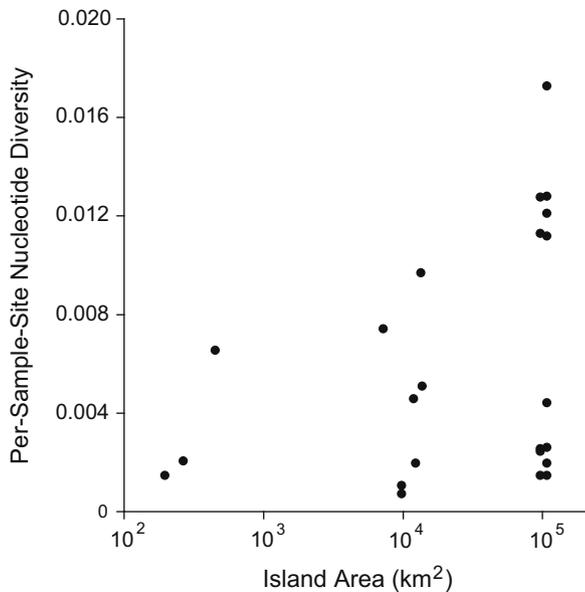
Fig. 11. Semi-logarithmic plot of within-sample-site nucleotide diversity on island area. Sample sites represented by a single sequence (no estimate of nucleotide diversity) have been removed. A least-squares regression was not significant ($R^2 = 0.105$, $P = 0.13$).

eages of bats were found to have little genetic variation explained by PAIC geography. However these lineages are much less genetically diverse than *Haplonycteris* or Philippine *Crocidura*, suggesting that they are either younger or have experienced greater gene flow across the archipelago.

Jones and Kennedy (2008) concluded that PAICs are not important correlates of genetic variation in four lineages of birds. However, we note that the taxa included in their study were represented by relatively few samples and that substantial portions of the archipelago were unsampled. Future efforts at testing PAIC models of diversification will be most powerful if they include much denser geographic sampling than is currently available for any taxon. It is unfortunate that all studies to date (this one included) have suffered from limited sampling across the archipelago. Dense sampling might allow one to isolate the effects of sea-level fluctuations, distance, and island area.

The role of PAICs in structuring shrew diversity is clearly substantial, but not ubiquitous and probably no more important in explaining geographic patterns of genetic diversity than are modern islands. Clearly there is substantial variation among lineages in the degree of fit of genetic diversity to the expectations of PAIC geography, and ecology may play a role in determining these patterns (Heaney et al., 2005). In the future, densely sampled comparative studies of additional lineages of varying ages, ecologies, and dispersal abilities should provide further insights into the pervasiveness of the 'PAIC effect'. Recent developments in the methods of historical demography (e.g., Drummond et al., 2005) and coalescent-based simulations (e.g., Rosenblum et al., 2007) offer much promise for relating current genetic patterns to past geological and climatic processes. Analyses that combine a comparative approach with tests of explicit *a priori* predictions offer the most potential for untangling the web of potential causes of diversification in this dynamic archipelago.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.05.034.

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