Carving out turf in a biodiversity hotspot: multiple, previously unrecognized shrew species co-occur on Java Island, Indonesia

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Abstract

In theory, competition among species in a shared habitat results in niche separation. In the case of small recondite mammals such as shrews, little is known about their autecologies, leaving open questions regarding the degree to which closely related species co-occur and how or whether ecological niches are partitioned. The extent to which species are able to coexist may depend on the degree to which they exploit different features of their habitat, which may in turn influence our ability to recognize them as species. We explored these issues in a biodiversity hotspot, by surveying shrew (genus *Crocidura*) diversity on the Indonesian island of Java. We sequenced portions of nine unlinked genes in 100–117 specimens of Javan shrews and incorporated homologous data from most known *Crocidura* species from other parts of island South-East Asia. Current taxonomy recognizes four *Crocidura* species on Java, including two endemics. However, our phylogenetic, population genetic and species delimitation analyses identify five species on the island, and all are endemic to Java. While the individual ranges of these species may not overlap in their entirety, we found up to four species living syntopically and all five species co-occurring on one mountain. Differences in species' body size, use of above ground-level habitats by one species and habitat partitioning along ecological gradients may have facilitated species diversification and coexistence.

Keywords: *Crocidura*, cryptic diversity, diversification, ecological niche, Soricomorpha, species delimitation

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Introduction

Accurate quantification of species diversity is critical to our understanding of fundamental, ecological and evolutionary phenomena (Fujita *et al.* 2012). For example, studies of the relationship between the geography of islands and species diversity (e.g. Heaney 1986) may be severely biased if the diversity of some islands (or clades) is underestimated (e.g. Heaney *et al.* 2009, 2011;

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Balete *et al.* 2012). This problem is especially common in the tropics and among young and morphologically conservative clades, in which diversity is often underestimated because of the existence of cryptic species (Bickford *et al.* 2007; Scheffers *et al.* 2012). Recently diverged species possess ancestral polymorphisms in their DNA and are more likely to experience hybridization, further challenging species delimitation efforts (Grant *et al.* 2008; Esselstyn *et al.* 2012b; Fujita *et al.* 2012). These obstacles are pervasive in poorly characterized groups such as beetles and molluscs (e.g. Puillandre *et al.* 2012), but they also remain a substantial challenge in relatively well-characterized vertebrate lineages, including mammals (Helgen 2005; Musser *et al.* 2006; Reeder

et al. 2007; Heaney *et al.* 2009, 2011; Esselstyn *et al.* 2012a,b).

There are few terrestrial ecosystems where the need for accurate quantification of species diversity is more pressing than in South-East Asia. The region has extremely high levels of both diversity and endemism and is experiencing one of the world's highest rapid rates of tropical deforestation (Sodhi *et al.* 2004). Moreover, South-East Asian islands serve as natural laboratories for understanding modes of diversification and community assembly in the context of dynamic geological and climatic processes (Heaney 2001; Brown & Diesmos 2009; Esselstyn *et al.* 2011; Lohman *et al.* 2011). A variety of mechanisms of speciation may operate in the region, including allopatric diversification due to vicariance or interisland dispersal and parapatric speciation along ecological gradients (Heaney & Rickart 1990; Brown & Diesmos 2009; Lohman *et al.* 2011). Among vicariant scenarios, sea level fluctuations, savannah barriers to forest-dependent organisms and geological uplift have all been considered potential mechanisms of speciation (Gorog *et al.* 2004; Heaney *et al.* 2005; Esselstyn *et al.* 2009). Speciation events may also be generated through the accumulation of Bateson–Dobzhansky–Muller incompatibilities, which could be catalysed through isolation-by-distance (Ruedi 1996) or divergent selection along a cline (Agrawal *et al.* 2011).

Mechanisms of speciation are relevant to our ability to quantify diversity. Closely related species that diverge as a consequence of adaptation may have an enhanced capacity to co-occur through niche partitioning because they will tend to have ecologically important differences (Schluter 2000; Ackerly *et al.* 2006). These species may also be easier to identify than allopatric or nonadaptively splitting species if their adaptations are conspicuous to humans. Nevertheless, some lineages may have diversified through adaptive processes but still be difficult to identify because of a lack of connection between their external morphology and their adaptations (e.g. Rowe *et al.* 2011).

The Indonesian island of Java, the focal habitat of this study, is large (128 300 km^2) and densely populated (~141 million people) and has served a long-term role as a centre of trade. As a result, the island's fauna is well-characterized relative to other parts of South-East Asia. Most of the known endemic terrestrial mammals were described during the 19th and early 20th centuries (e.g. Lesson 1840; Peters 1870; Sody 1931; Bartels 1937). Currently, 29% of the terrestrial, nonvolant mammal fauna of Java is considered endemic to the island (Okie & Brown 2009). Biogeographically, Java is one of three major islands (with Sumatra and Borneo) on a shallow continental region known as the Sunda Shelf. Through several Pleistocene periods of low sea level, and as

recently as 10 kya, Java had a broad dry land connection to Sumatra, Borneo and the Asian mainland (Rohling *et al.* 1998; Voris 2000). In the light of this intermittent connectivity, one might expect many widespread species to occur across the large islands of the Sunda Shelf and the mainland, with only minimal genetic divergences between island populations (Okie & Brown 2009). However, this assumption has often been rejected by phylogeographical and faunal similarity studies that incorporate phylogenetic information (Campbell *et al.* 2004; Gorog *et al.* 2004; Janečka *et al.* 2008; Esselstyn *et al.* 2010; Oliveros & Moyle 2010; Wilting *et al.* 2012), suggesting that levels of endemism, although substantial, are still underestimated on the Sunda Shelf. Intriguingly, these studies highlight the question of why Java should have endemic species in the first place.

Geographically, Java has a relatively linear shape that runs nearly parallel to latitude (Fig. 1). The western and central parts of the island are wetter than the eastern side, resulting in a transition from moist tropical forests in the west to more seasonal deciduous forests in the east (Whitten *et al.* 1996; van der Kaars *et al.* 2010; van Welzen & Raes 2011). In addition, Java contains a string of volcanoes (Fig. 1) that reach as high as 3676 m and generate strong gradients of temperature and precipitation (van Welzen & Raes 2011). The island thus contains sharp ecological gradients, and associated natural selection could potentially drive both diversification and extinction.

Taxonomic context and goals

Shrews are small insectivorous mammals found on all continents except Australia and Antarctica. Diversity in the Old World is highly variable, with some tropical African communities containing as many as 17 species (Lunde 2007). In Indonesia, the major islands of the Sunda Shelf are thought to contain relatively few species. Most Indonesian shrews are in the Afro-Asian genus *Crocidura*, the focal taxa of this study, but a few indigenous representatives of other genera are present (e.g. *Suncus* on Borneo and *Chimarrogale* on Sumatra). Shrews in the genus *Crocidura* provide an interesting test case for the expected patterns of endemism on the Sunda Shelf (Ruedi 1996; Esselstyn *et al.* 2009) because they are widespread and ubiquitous members of small mammal communities. Currently, only two species of shrew (*Crocidura maxi* and *Crocidura monticola*) are believed to occur naturally on multiple islands on the Sunda Shelf, while all others are endemic to a single island (Kitchener *et al.* 1994; Ruedi 1995; Omar *et al.* 2013). However, previous investigations suggest that the taxonomy of these shrews is not yet well resolved
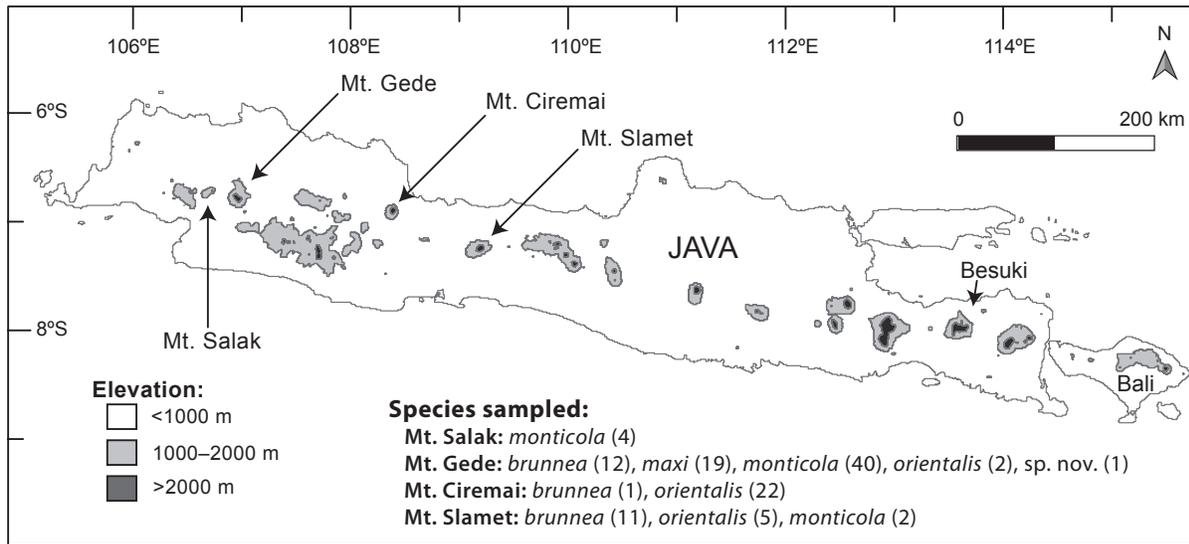
Fig. 1 Map of Java Island, showing high-elevation areas, sample localities and other localities discussed in the text. For each mountain sampled, we list the species and, in parentheses, sample sizes of *Crocidura* that we sequenced.

(Abramov *et al.* 2008; Esselstyn *et al.* 2009; Esselstyn & Oliveros 2010).

Javan *Crocidura* have been examined morphologically over the last three decades in a series of papers that recognized different sets of species (Davison *et al.* 1982; Jenkins 1982; Ruedi 1995; Omar *et al.* 2013), but no investigations of genetic diversity have focused on Javan shrews. All four of the putative Javan species that have been recognized during this time can be placed into either a 'small' (3–7 g; *C. maxi* and *C. monticola*) or 'large' (10–18 g; *Crocidura brunnea* and *Crocidura orientalis*) body size category. In the small category, Jenkins (1982) and Omar *et al.* (2013) recognized both *C. maxi* from eastern Java (type locality east Besuki, East Java) and *C. monticola* primarily from western Java (type locality Mt. Lawu, Central Java), citing several diagnostic morphological characters. However, Ruedi (1995) considered the characters referenced by Jenkins (1982) to be inconsistent and treated all small Javan shrews as *C. monticola*. Small Bornean and Malay Peninsula shrews have also been referred to *C. monticola* (Davison *et al.* 1982; Jenkins 1982; Ruedi 1995; Omar *et al.* 2013), and Omar *et al.* (2013) assessed a Sumatran specimen as either *C. monticola* or *Crocidura neglecta* (Esselstyn *et al.* 2009 mistakenly referred the same specimen to *C. maxi*); other populations have been assigned to *C. maxi*, including small shrews from Bali, the Lesser Sunda Islands and Aru Island (Kitchener *et al.* 1994). Hence, the *maxi-monticola* complex is currently considered to be widespread from the Malay Peninsula to the major islands of the Sunda Shelf (Java, Sumatra and Borneo), the Lesser Sunda Islands and eastern Indonesia (Aru Island).

The larger shrews of Java were treated as members of the widespread species *Crocidura fuliginosa* (Jenkins 1982) until Ruedi (1995) broke up this complex and recognized two species: the low-elevation *C. brunnea* (type locality probably West Java) and the high-elevation *C. orientalis* (type locality Mt. Gede, West Java). Ruedi (1995) considered each to be endemic to Java, and within each of these species, he recognized a western (*C. b. brunnea* and *C. o. orientalis*) and an eastern (*Crocidura brunnea pudjonica* and *Crocidura orientalis lawuana*) subspecies. In addition, Jenkins (1982) and Ruedi (1995) mentioned the possible presence on Java of an undescribed, long-tailed species that superficially resembles *Crocidura paradoxura* from Sumatra.

In this study, we characterize the level of species diversity and plausible mechanisms of diversification of shrews on Java. Specifically, we use genetic data to test whether shrews from Java contain one or two species each within the *maxi-monticola* and *brunnea-orientalis* complexes and whether other undocumented shrew species occur on the island. In so doing, we illuminate the fine geographical scale of endemism in these small cryptic mammals.

Methods

Data collection

We analysed genetic samples from 119 shrews (100–117 individuals per gene fragment) from three mountains in West Java (Mts. Salak, Gede and Ciremai) and one mountain in Central Java (Mt. Slamet; Fig. 1;

Appendix S1, Supporting information). Our Javan material is taken from highland areas because the lowlands are almost exclusively anthropogenic habitats—very little information is available on how tolerant of habitat disturbance species of *Crocidura* are on Java. We also include putative *Crocidura maxi* from Bali, the Lesser Sunda Islands and Aru Island, putative *Crocidura monticola* from Borneo and the Malay Peninsula, and 1–2 samples per species from ~22 other species from the Philippines, Indonesia, Malaysia, Taiwan, China, Cambodia and Vietnam. All Javan specimens used are preserved as vouchers and deposited at the Museum Zoologicum Bogoriense, Bogor, Indonesia, Field Museum of Natural History, Chicago, USA, or Royal Ontario Museum, Toronto, Canada (Appendix S1, Supporting information). Samples from other areas are also preserved as voucher specimens and held in a variety of museums (Appendix S1, Supporting information). All species identifications were made using meristic and mensural morphological characters following Jenkins (1982) and Ruedi (1995). We gathered measurements of length of head and body from the field notes of collectors and summarized them as means and ratios of means between members of each species pair.

We sequenced portions of one mitochondrial protein-coding gene (cytochrome *b*: *Cytb*) and eight nuclear loci [seven exons: apolipoprotein B (*ApoB*), brain-derived neurotrophic factor (*BDNF*), breast cancer susceptibility 1 (*BRCA*), growth hormone receptor exon 10 (*GHR*), prostaglandin E4 receptor (*PTGER4*), recombination activating protein 1 (*RAG1*) and von Willebrand factor exon 28 (*vWF*) and one intron: mast cell growth factor (*MCGF*)] in Javan shrews and closely related species (Appendix S1, Supporting information). We focused our sequencing efforts on exons to avoid laboratory problems associated with length heterogeneity, which is commonly encountered while sequencing introns. Initial phylogenetic analyses based on mitochondrial sequences showed populations of putative *C. maxi* from Bali, the Lesser Sunda Islands and Aru to be very distant relatives of the *maxi-monticola* complex, and they were not included in subsequent nuclear DNA sequencing efforts.

General methods of DNA extraction, amplification and sequencing follow Esselstyn *et al.* (2008, 2009). Polymerase chain reaction protocols for previously used loci (*Cytb*, *MCGF* and *ApoB*) are described in Esselstyn *et al.* (2009); new protocols and primers are detailed in Table S1 (Supporting information). DNA sequences were edited and aligned using GENEIOUS 5. Alignments were inspected visually and were unambiguous. To estimate phylogenetic relationships among the Javan species of *Crocidura*, we added new sequences to the *Cytb* alignment of Esselstyn & Oliveros (2010) and

supplemented these with data from Bornean and Malay Peninsula populations of putative *C. monticola* (Omar *et al.* 2013), resulting in an alignment that includes most of the known species from the Sunda Shelf, Sulawesi, the Lesser Sunda Islands and the Philippines, plus several species from Indochina. We included one *Suncus murinus* as an outgroup. We trimmed this alignment to a length of 1110 nucleotides to minimize missing data and eliminated duplicate representatives of individual species that were included by Esselstyn & Oliveros (2010). The final alignment contained 18 putative *C. maxi* from Mt. Gede; 44 putative *C. monticola* from Mts. Gede, Salak and Slamet; 17 putative *Crocidura brunnea* from Mts. Gede, Ciremai and Slamet; 22 putative *Crocidura orientalis* from Mts. Gede, Ciremai and Slamet; and one specimen of the undescribed long-tailed shrew mentioned by previous authors (Jenkins 1982; Ruedi 1995) from Mt. Gede (Fig. 1). Before analysing these mitochondrial DNA (mtDNA) sequences, we reduced the matrix of 146 individuals to the set of unique sequences, resulting in a final alignment of 66 haplotypes.

Phylogenetic context of Javan shrews

Phylogenetic analyses were conducted using Bayesian and likelihood methods of gene tree estimation and Bayesian species tree inference. First, we identified an appropriate model of sequence evolution for each locus using JMODELTEST 0.1 (Posada 2008). Models were fit to a fixed BIONJ-JC phylogeny and compared using the Bayesian information criterion. The best-fit models (Table S2, Supporting information) were then used for phylogenetic inference in BEAST 1.6.2 (Drummond & Rambaut 2007) and GARLI 2.0 (Zwickl 2006).

Our mtDNA alignment was analysed with four independent MCMC runs of 3×10^7 generations, with parameters logged every 3000 generations. Prior settings included a relaxed log-normal clock with an exponentially distributed standard deviation for the substitution rate parameter ($\bar{x} = 1, \sigma = 0.5$) and a coalescent constant-population-size model of tree shape.

We estimated individual gene trees for nuclear sequences in a likelihood framework using GARLI 2.0 (Zwickl 2006). These analyses used alignments of all phased alleles from all available sequences from Java. We completed 10 replicated searches for the maximum-likelihood solution, followed by 100 bootstrap replicates, for each locus. Bootstrap scores were summarized on the maximum-likelihood tree using the SumTrees script of DENDROPY (Sukumaran & Holder 2010).

We conducted species tree analyses of the eight nuclear loci to infer relationships among the species we delimit herein through other analyses. Species tree

inference consisted of four runs of 2×10^8 generations, with parameters logged every 4000 generations. We employed a relaxed log-normal clock scaled to a rate of 1.0 for *ApoB*. Relaxed clock priors on the substitution rate were exponentially distributed with a mean of 1. We used a Yule speciation prior on tree shape and the piecewise linear and constant root population size model. Analyses were initiated with a random starting tree for each locus.

We sought evidence of convergence in all Bayesian analyses by examining the trends, distributions and effective sample sizes (ESS) of parameters in TRACER 1.5 (Rambaut & Drummond 2007). We summarized the posterior distributions of trees as a maximum clade credibility tree, with node ages presented as medians, using LOG COMBINER 1.6.2 and TREE ANNOTATOR 1.6.2.

Population structure

Our phylogenetic estimates based on mitochondrial and nuclear sequences, described below, suggest that specimens of small Javanese shrews (*C. maxi* and *C. monticola*) form a clade and that large Javanese shrews (*C. brunnea* and *C. orientalis*) are closely related and may represent a clade. However, the two species pairs are not closely related to each other. We therefore performed separate population genetic analyses on each species pairs. We inferred alleles from degenerate sequences for each individual in all nuclear loci using PHASE 2.1 (Stephens & Donnelly 2003). Phased alleles from all individuals of each pair were then analysed in STRUCTURE 2.3.4 (Pritchard *et al.* 2000) to determine whether individuals could be grouped into distinct populations consistent with our morphological identifications and mitochondrial relationships. We excluded sequences of mtDNA to avoid circularity. Structure assumes an a priori number of populations, but makes no assumptions about assignment of individuals to populations. All analyses were conducted using the admixture model with allele frequencies correlated among populations (Pritchard *et al.* 2000). We ran STRUCTURE analyses for 10^5 generations after an initial burn-in of 10^4 generations. Each species pair was analysed with an expected number of populations (*K*) of 1–10 (although we expected a maximum of four populations per species because the samples were taken from up to four mountains), with 10 replicates per *K*. We then determined the preferred value of *K* following Evanno *et al.* (2005) and using STRUCTURE HARVESTER 0.6.93 (Earl & vonHoldt 2012).

To further visualize population genetic structure, we reduced each locus-specific alignment of phased alleles to the set of unique alleles. We then employed the NeighborNet algorithm (Bryant & Moulton 2004) in the

program SPLITSTREE 4.10 (Huson & Bryant 2006) to generate independent phylogenetic networks of alleles for each locus. To assess the support for inferred splits in the network, a bootstrap analysis was conducted with 1000 replicates. We also plotted pie charts to visualize the relative frequencies of each allele in each population.

Multilocus species delimitation

We conducted multilocus species delimitation analyses using the program BPP 2.1 (Rannala & Yang 2003; Yang & Rannala 2010). This method estimates the number of species in a sample using a prespecified guide tree. Assumptions include (i) the species tree is either equivalent to, or can be obtained by collapsing nodes in, the guide tree, (ii) species are panmictic and reproductively isolated from each other and (iii) population size is constant across speciation events. Gamma priors are assigned to the population size (θ) and age of the root in the species tree (τ; Yang & Rannala 2010). We analysed each species pair separately using a sister relationship between the pair members as the guide tree. For the diminutive shrews, the guide tree explicitly follows our estimated mtDNA gene tree and species tree. However, our phylogenetic analyses were more ambiguous for the larger shrews. *Crocidura brunnea* and *C. orientalis* formed a well-supported clade with *Crocidura lepidura* from Sumatra, but relationships among these three were not well supported by either data set. In addition, *C. brunnea* and *C. orientalis* were not monophyletic in the mtDNA gene tree. Thus, it is unclear whether our guide tree reflects the true relationship between *C. brunnea* and *C. orientalis*. Nevertheless, because we assigned individuals to species based on qualitative morphological comparisons, rather than mtDNA clustering, the delimitation analyses should provide a meaningful test of whether these two morphologically distinguishable entities have distinct nuclear DNA sequences. Because BPP analyses assume panmixia, we included only samples of *C. maxi* and *C. monticola* from Mt. Gede, where they occurred in syntopy. For *C. brunnea* and *C. orientalis*, we conducted independent analyses using only samples from West Java, only samples from Central Java and all samples pooled.

Each data set, consisting of phased alleles for all eight nuclear loci (mtDNA excluded), was analysed using both rjMCMC algorithms provided in the program. Each algorithm was run with both the default [*G*(2, 1000)] and more diffuse [*G*(0.01, 5)] priors on θ and τ. These two prior distributions have the same mean (0.002), but the latter has much greater variance (0.002) than the default (2×10^{-6}). We confirmed that

adequate state swaps were occurring in each analysis according to the BPP documentation (Yang & Rannala 2010). All BPP analyses were run twice, and results were compared for consistency among runs.

Estimating divergence times

We estimated divergence times for each species pair using the isolation model (no migration) in MIMAR (Becquet & Przeworski 2007). MIMAR uses summary statistics to estimate demographic parameters, such as divergence time. The summary statistics are the number of derived mutations fixed in one population (s_f), the number that are polymorphic in both populations (s_s) and the number that are polymorphic in the first population, but not the second (s_1), and vice versa (s_2). Our MIMAR analyses used only synonymous sites from nuclear loci.

We estimated the polarity of polymorphisms by inferring ancestral DNA sequences for each locus in each species pair using the BASEML program of PAML 4.4 (Yang 2007). We chose outgroups based on the relationships in our estimated mtDNA gene tree; for the *maxi-monticola* complex, the outgroup was *Crocidura tanakae*, and for the *brunnea-orientalis* complex, we used a new species from Java, discussed below, as the outgroup. We estimated relative rates of mutation by calculating mutation rate scalars using the Jukes–Cantor synonymous distance from an ingroup specimen to the outgroup using DnaSP 5 (Librado & Rozas 2009). We estimated two time-calibrated nuclear mutation rates as the mean across loci of the Jukes–Cantor synonymous distance to an outgroup species of soricine shrew (*Sorex* or *Blarinella*), divided by twice the age of (i) the oldest known fossil crocidurine shrew (13 Ma; Storch *et al.* 1998) and (ii) the origin of crocidurines (34 Ma; Douady & Douzery 2003) based on external calibrations. These estimates are based on only five of the eight nuclear loci due to a lack of homologous data in soricine shrews for three loci (*RAG1*, *MCGF* and *PTGER4*). We assumed a generation time of 1 year, which approximates an estimate derived from a temperate species of *Crocidura* (Jeanmaire-Besançon 1988).

We also attempted to fit the data to the isolation/migration model in MIMAR. However, these analyses failed to converge on the posterior distributions for the time of divergence and ancestral polymorphism (Θ_A) parameters. For the isolation model, we performed goodness-of-fit tests using the median parameter estimates as a fixed value for simulations, as described by Becquet & Przeworski (2007).

To provide a coarse comparison with MIMAR estimates of divergence times, we also calculated Jukes–Cantor distances between *Cytb* sequences of *C. maxi* and *C. monticola* and between *C. brunnea* and *C. orientalis* in DNASP 5 (Librado & Rozas 2009) and compared these

with published estimates of mutation rates in shrews and their relatives (Nabholz *et al.* 2008).

Results

Morphological identifications and geographical distributions

We assigned recently collected specimens of shrews from Java to the species *Crocidura brunnea*, *Crocidura maxi*, *Crocidura monticola*, *Crocidura orientalis* and an undescribed species using the characters and definitions in Jenkins (1982) and Ruedi (1995; Fig. 1; Appendix S1, Supporting information).

On Mt. Gede, where we recorded all five species, we found *C. brunnea* and *C. monticola* at three sites from 1000 to 1400 m and *C. maxi*, *C. monticola*, *C. orientalis* and the new species at ~1600 m. On Mt. Slamet, we found only *C. brunnea* at sites at 700–1000 m and *C. monticola* and *C. orientalis* at sites around 2000 m. On Mt. Ciremai, we found *C. orientalis* at sites between 1500 and 1800 m and one specimen of *C. brunnea* at 1650 m. On Mt. Salak, we collected only *C. monticola* at ~1400 m. At this time, it is unclear the degree to which the variation in diversity among sites and mountains is the result of differences in trapping effort or differences in species distributions and abundances.
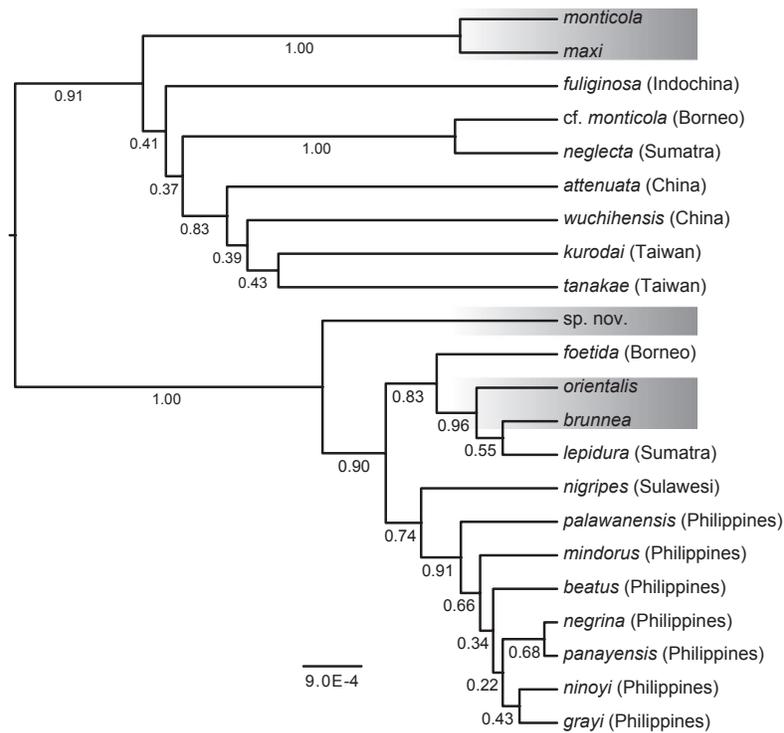
Means of head and body length in mm were as follows: 69.0 (*C. maxi*; $n = 6$), 60.22 (*C. monticola*; $n = 9$), 87.82 (*C. brunnea*; $n = 11$), 84.43 (*C. orientalis*; $n = 7$) and 77.0 (*Crocidura* sp. nov.; $n = 1$). Body size ratios (larger species mean/smaller species mean) were 1.15 (*maxi-monticola*) and 1.04 (*brunnea-orientalis*).

Phylogenetic context of Javan shrews

All four BEAST runs for both gene tree and species tree analyses appeared to converge within the first million generations. We conservatively discarded the first 50% of each run, leaving 2×10^4 (mtDNA) and 2.5×10^4 (species) trees in the posterior distributions. ESSs for all parameters exceeded 1800 in the gene tree analysis and 500 in the species tree analysis.

Mitochondrial and species tree phylogenetic estimates strongly support *C. maxi* and *C. monticola* as sister species and demonstrate that both lineages are Javan endemics (Figs 2 and 3). The lineage of putative *C. maxi* from Bali, the Lesser Sunda Islands and Aru Island is most closely related to the clade of shrews from Sulawesi and *Crocidura batakorum* from the Philippines. Putative specimens of *C. monticola* from Borneo and the Malay Peninsula are closely related to *C. neglecta* from Sumatra (Figs 2 and 3). This Sumatra–Borneo–Malay clade is more closely related to taxa from Indochina

Fig. 2 Phylogenetic relationships estimated from sequences of mitochondrial DNA. Branches are labelled with Bayesian posterior probabilities (when ≥0.5) and horizontal bars indicate the 95% highest posterior densities of node ages. Terminals are labelled with taxonomic identities, geographical localities noted parenthetically and museum catalogue or GenBank accession numbers. Javan lineages are highlighted in grey.

than to *C. maxi* or *C. monticola* from Java (the type local-ity). Their taxonomic association with the small Javan shrews is clearly due to their similarities in body size, but does not reflect their phylogenetic relationships.

Crocidura brunnea and *C. orientalis* formed a clade in the mitochondrial gene tree, but support for the node uniting them was not strong (posterior probabil-ity = 0.69; Fig. 2). However, this pair of species did not

Fig. 3 Species tree estimate for a clade of South-East Asian shrews. Nodes are labelled with posterior probabilities. Terminals are labelled with the specific epithet, and region of origin is noted parenthetically. Javan species are highlighted with a grey background.

form a clade in the species tree analysis, where *C. brunnea* was sister to *Crocidura lepidura*, but with little support (posterior probability = 0.55; Fig. 3). Surprisingly, all specimens from Mt. Slamet identified morphologically as *C. orientalis* had mitochondrial sequences more closely related to those from *C. brunnea* than to mtDNA from other populations of *C. orientalis* (Fig. 2). These analyses also placed an undescribed species from Java in a clade containing species from the Philippines, Borneo and Sulawesi (mtDNA gene tree; Fig. 2) or the Philippines, Borneo, Sulawesi, Sumatra and Java (species phylogeny; Fig. 3). Individual nuclear gene trees of all Javan samples also show close relationships between *C. maxi* and *C. monticola* and between *C. brunnea* and *C. orientalis* (Fig. S1, Supporting information). In addition, they support the distinction of the undescribed species from all known Javan species (Fig. S1, Supporting information).

Population structure

Analyses of eight nuclear loci for signals of population structure provided strong support for the genetic distinctiveness of *C. maxi* and *C. monticola* (Fig. 4). Likelihoods of models in STRUCTURE analyses with $K = 4$ were highest, but the $K = 2$ model was favoured by the *ad hoc* ΔK test (Fig. 4). Likelihoods of the model with a single population were far worse than any other models

(Fig. 4). In all cases with $K \geq 2$, specimens identified morphologically as *C. maxi* and *C. monticola* were clearly separated (Fig. S2, Supporting information).

Crocidura brunnea and *C. orientalis* were also readily distinguished by STRUCTURE (Figs 4 and S2, Supporting information). The $K = 3$ model had the highest likelihood and was favoured by the *ad hoc* ΔK test. It distinguished *C. brunnea* and western and central populations of *C. orientalis* (Fig. 4). *Crocidura brunnea* was genetically homogeneous across all three mountains for all values of K (Fig. S2, Supporting information). One specimen from Mt. Slamet (Central Java) identified morphologically as *C. orientalis* (MZB 32146) appears to be a hybrid between *C. orientalis* and *C. brunnea* based on shared alleles at *BDNF*, *BRCA1* and *RAG1* (Figs 4, 5, S1 and S2, Supporting information).

Gene networks and allelic distributions

Individual loci varied widely in their allelic diversity in our sample of Javan shrews (Figs 5, S1 and S3, Supporting information). For instance, the locus *vWF* contained 39 alleles, while *BDNF* and *PTGER4* each had only 13 alleles. In all loci except *BDNF*, allelic networks showed relatively close affinities between *C. maxi* and *C. monticola* and between *C. brunnea* and *C. orientalis*, with longer branches separating the two species pairs (Fig. 5). Although the majority of alleles were restricted
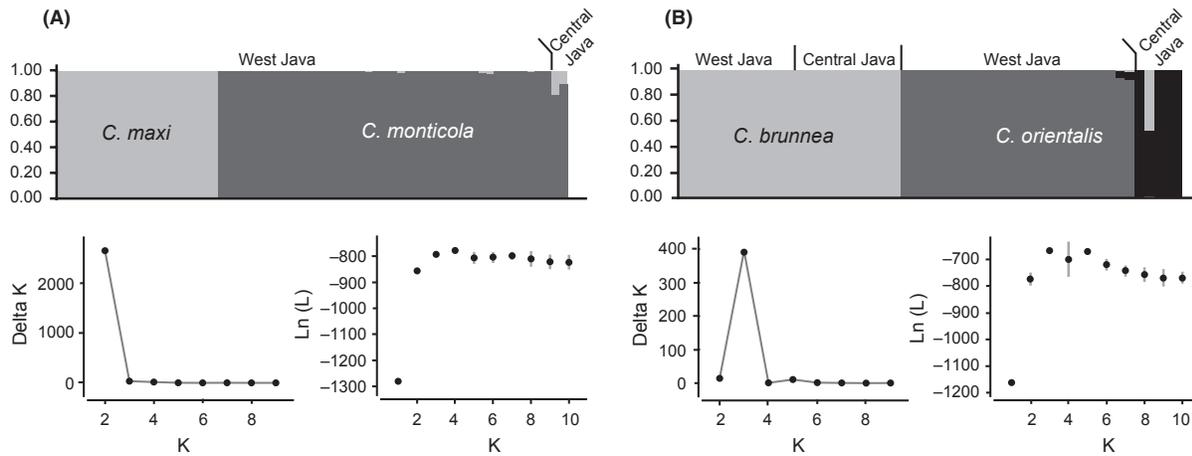
Fig. 4 Results from STRUCTURE analyses of eight nuclear loci from *Crocidura maxi* and *Crocidura monticola* (A) and *Crocidura brunnea* and *Crocidura orientalis* (B). Only the favoured models, as chosen by the *ad hoc* ΔK test (Evanno *et al.* 2005), are shown. Lower plots show bivariate plots of ΔK and the likelihood score against K for each data set. Results for K = 2–6 are presented in Fig. S2 (Supporting information).

to one species, all loci except *vWF* had at least one allele shared between species (Fig. 5). However, no alleles of any locus were shared between the *maxi-monticola* and *brunnea-orientalis* complexes. The new species was homozygous at all loci with unique alleles at six loci. It shared alleles with *C. brunnea* at *MCGF* and *PTGER4* (Figs 5 and S3, Supporting information).

Multilocus species delimitation

Species delimitation analyses conducted in BPP provided strong support for the recognition of *C. maxi*, *C. monticola*, *C. brunnea* and *C. orientalis* as separate species. All analyses, irrespective of the rjMCMC algorithm or prior probabilities used, supported the two species model for each species pair with a posterior probability of 1.0 (Table S3, Supporting information). For the small shrews, our choice of priors had little effect on the results, with broad overlap between the 95% HPDs for all parameters. Estimated divergence times ranged from 0.0004 to 0.0018 expected substitutions per site (Table S3, Supporting information).

For *C. brunnea* and *C. orientalis*, estimated divergence times (τ) and population sizes (θ) were dependent on the localities included. As expected, estimates of θ were higher when all samples were analysed than when West and Central Javan samples were analysed separately (Table S3, Supporting information). Estimated divergence times were more recent when Central Javan samples were included (0.0003–0.0013 substitutions per site) than when they were excluded (0.0008–0.0027; Table S3, Supporting information). These results are consistent with the possibility of occasional gene flow

occurring between *C. brunnea* and *C. orientalis* in Central Java, as implied by our mitochondrial phylogeny (Fig. 2) and STRUCTURE analyses of nuclear DNA (Figs 4 and S2, Supporting information).

Estimating divergence times

Median estimated divergence times derived from the no-migration model of MIMAR were 177 557 (95% HPD: 60 693–391 468; 13 Ma calibration) or 515 467 (175 821–1 138 360; 34 Ma calibration) generations ago for *C. maxi* and *C. monticola* and 167 515 (79 362–315 968; 13 Ma calibration) or 489 603 (235 620–910 913; 34 Ma calibration) generations ago between *C. brunnea* and *C. orientalis*. For the *maxi-monticola* MIMAR analysis, goodness-of-fit tests indicated a good fit for all of the segregating site summary statistics, F_{ST}, Tajima's D and nucleotide diversity (π) for each population (Fig. S4, Supporting information). However, for the *brunnea-orientalis* MIMAR analysis, a poor fit was suggested for π and Tajima's D for *C. brunnea*, in that the observed values were much smaller than the simulated values (Fig. S4, Supporting information). One possible explanation is that *C. brunnea* experienced recent population expansion and therefore has an excess of rare variants (and consequently a negative Tajima's D). In any case, it is not clear whether this violation of model assumptions would affect the estimates of divergence time. Strasburg & Rieseberg (2010), for example, found that population growth did not affect divergence time estimates in an IMa2 analysis, which deploys a similar model to MIMAR.

By way of comparison, Jukes–Cantor distances in mtDNA sequences using West Javan specimens were
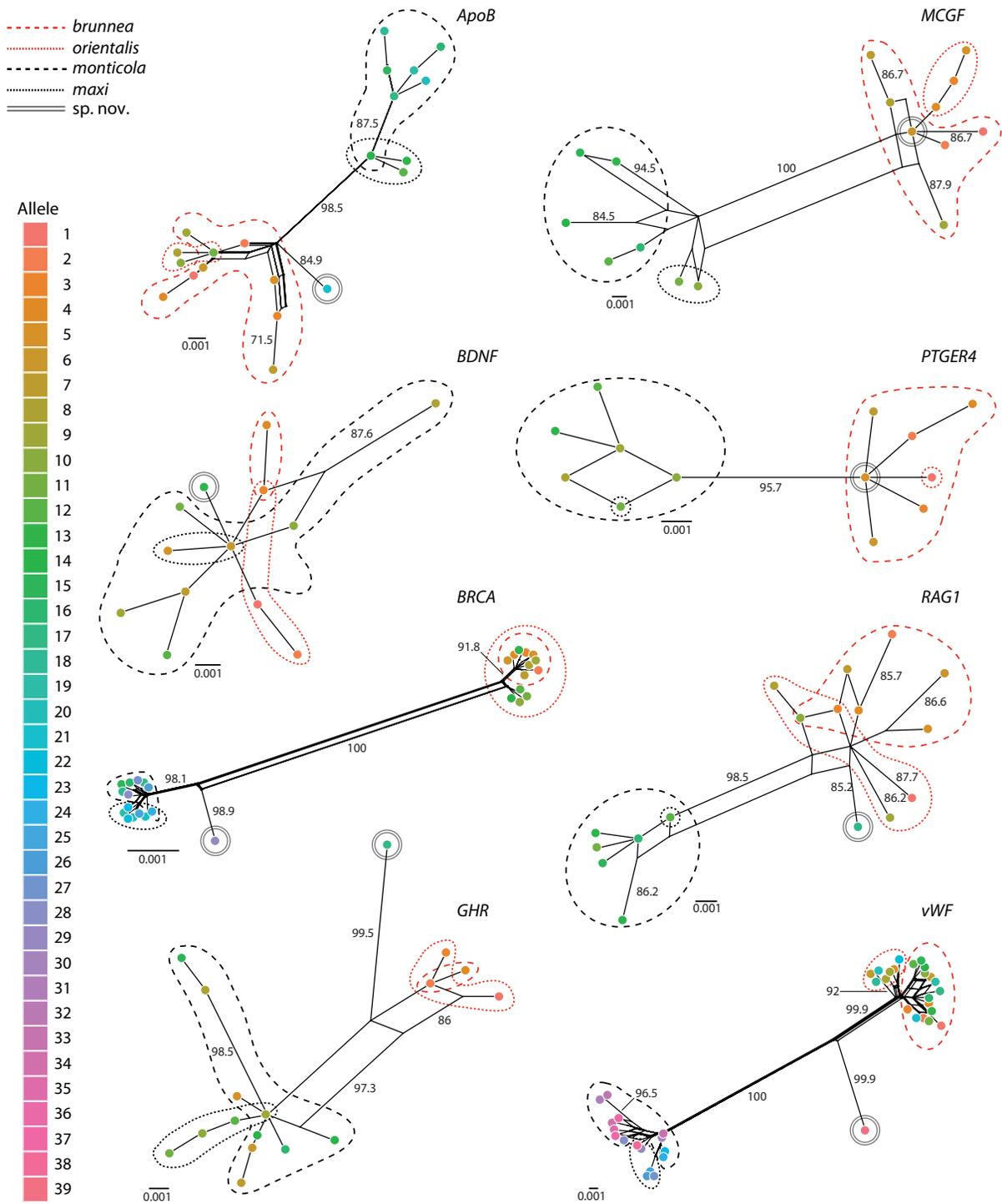
Fig. 5 Gene networks of all alleles sampled from eight nuclear loci from Javanese *Crocidura*. Alleles are colour-coded and named 1 through *n*, where *n* is the number of alleles. Solid and broken lines around nodes indicate the species each allele is found in. Colour codes are identical to those in the allele frequency plots of Fig. S3 (Supporting information).

0.0388 (all positions), 0.0983 (3rd codon positions) and 0.1651 (silent sites) between *C. maxi* and *C. monticola* and 0.0542 (all positions), 0.1561 (3rd codon positions) and 0.2387 (silent sites) between *C. brunnea* and *C. orientalis*. Using a previously published 3rd codon position mutation rate for eulipotyphlans (shrews, moles and hedgehogs) of 0.05/site/Ma (Nabholz *et al.* 2008), we estimate crude (see Galtier *et al.* 2009) divergence times of 0.9 (*maxi-monticola*) and 1.5 Ma (*brunnea-orientalis*).

Discussion

Accurate quantification of species diversity is pivotal to our understanding of speciation and adaptation. When cryptic species go unrecognized, our comprehension of how closely related species fill the environment is biased. Here, we resolve long-standing issues in the taxonomy of Indonesian shrews, providing an empirical example of how similarities in body size in a morphologically conservative group can lead to the confounding of endemic species in purportedly widespread taxa. Our results provide strong support for the recognition of *Crocidura brunnea*, *Crocidura maxi*, *Crocidura monticola*, *Crocidura orientalis* and an undescribed species. All five species are probably endemic to Java, and surprisingly, at least four of them occur syntopically in one area, with all five co-occurring on a single mountain (Mt. Gede).

Our finding of 100% endemism among Javan *Crocidura* contrasts with the a priori expectation that species in continental island groups should be widespread with only minimal genetic differences between populations isolated most obviously by ephemeral sea barriers. This suggests that (i) some barrier(s) has isolated Java consistently from neighbouring islands, enabling the evolution of endemic species, (ii) ecological adaptation occurs on a small enough geographical scale that species are not shared between large islands or (iii) extinctions have occurred in such a way that species were once widespread but now remain on only one island. If eco-geographical barriers are responsible for the localized nature of endemism on the Sunda Shelf, then the intermittent sea barriers may have been supplemented by dry savannah habitats and together restricted the dispersal of forest-dependent organisms over evolutionary timescales (Bird *et al.* 2005; Wurster *et al.* 2010). If ecological adaptations have occurred on small geographical scales, then species should be confined to a particular habitat type, rather than a particular island. Unfortunately, the geographical distributions of mammals within the major islands of the Sunda Shelf are, at this time, too poorly documented to be able to test this hypothesis. However, van Welzen & Raes (2011) show such a pattern among the plants of Java, with West

Javan plant communities being most similar to Sumatran and Malay Peninsula plant communities and those in the east of Java being most similar to Lesser Sunda and Wallacean plant communities. Extinction has almost certainly played a role in generating localized endemism on the Sunda Shelf (Heaney 1986; Okie & Brown 2009). Nevertheless, our identification of at least one speciation event probably having occurred within Java raises questions about the assumption that all or most species were widespread on the shelf in the past. This assumption is built into estimates of insular extinction rates (Heaney 1986; Okie & Brown 2009). Our results are not unique in the inference of localized endemism, but rather contribute to an emerging multi-taxon picture of an unexpectedly partitioned biodiversity, with substantial faunal differences between Java, Sumatra, Borneo and other islands that were connected (or nearly so) during the Pleistocene (Gorog *et al.* 2004; Janečka *et al.* 2008; Blackburn *et al.* 2010; Esselstyn *et al.* 2010; Oliveros & Moyle 2010; Wilting *et al.* 2012).

Timing of shrew diversification on Java

Our analyses suggest that the divergence dates between the members of each Javan species pair are quite recent, perhaps within the last million years. The MIMAR estimates of divergence times based on nuclear loci had medians of ≤515 K generations ago and upper limits on 95% HPDs ≤1.1 million generations ago. These divergence times are of comparable magnitude but younger than the crude estimates derived from mtDNA using a molecular clock (0.9 and 1.5 Ma). At least a portion of the differences between these dates (assuming one generation approximates 1 year based on a temperate species of *Crocidura*; Jeanmaire-Besançon 1988) can be attributed to the coalescent (gene divergences frequently precede species divergences; Edwards & Beerli 2000). Perhaps more importantly, the fossil calibration we used (13 Ma) probably underestimates the divergence time between soricine and crocidurine shrews, which would bias our nuclear divergence estimates towards younger than actual dates. Nevertheless, the slower mutation rate derived from the older calibration (34 Ma; Douady & Douzery 2003) still yielded divergence estimates mostly within the last million years. The divergence time for *C. brunnea* and *C. orientalis* inferred by MIMAR analyses may also be biased towards recent times by the gene flow that apparently occurred between these species in Central Java. Given these possibilities, we suspect the actual species divergences are somewhat older than our 13 Ma calibrated estimates. Overall, however, the speciation events that produced the shrews endemic to Java appear to have occurred relatively recently, probably during the Pleistocene, and

perhaps contemporaneously. This recent time frame helps explain some of the confusion in the taxonomy of these shrews—there has been little time for readily observable morphological differences to evolve and reproductive isolation may not yet be complete in one species pair.

Tempo and mechanisms of speciation

Theory of diversification (Schluter 2000) and numerous empirical studies [see meta-analyses of McPeek (2008) and Phillimore & Price (2008)] suggest that diversification occurs until ecological opportunities are exhausted. Thus, the observation of relatively recent speciation events that generated Javan endemic shrews is consistent with the hypothesis that South-East Asian *Crocidura* is an immature radiation that is still diversifying (Esselstyn *et al.* 2009). The process of diversification in insular terrestrial faunas is often linked with dispersal ability relative to isolation (MacArthur & Wilson 1967). Shrews are clearly able to disperse across marine barriers because they occur on islands that have never had a mainland connection (e.g. the Philippine and Lesser Sunda islands; Heaney & Ruedi 1994; Kitchener *et al.* 1994; Esselstyn & Oliveros 2010). However, interisland colonization is not the only factor that generates species. We find evidence for at least one speciation event having occurred within Java, and within-island diversification has also taken place on Sulawesi (Ruedi *et al.* 1998; Esselstyn *et al.* 2009). Similar surveys are needed to determine the extent of within-island diversification among the shrews of Sumatra and Borneo. Although it is clear that within-island speciation happens, the mechanisms that promote it remain difficult to decipher. However, given Java's strong ecological gradients associated with its east–west moisture gradient and replicated elevational clines, there exists a plausible mechanism of differential adaptation and speciation. Ruedi (1995) recognized eastern and western subspecies of both *C. brunnea* and *C. orientalis*, the ranges of which correspond approximately to wet and dry areas of Java. Similarly, the geographical distribution of specimens identified by Jenkins (1982) as *C. maxi* and *C. monticola* implies that *C. maxi* is more common in East Java, while *C. monticola* is more prevalent in West Java. Although we lack samples from East Java, we found *C. monticola* at most of the sites we sampled, but *C. maxi* at only one midelevation site in West Java. If *C. maxi* and *C. monticola* are indeed mostly isolated along Java's east–west axis, and both *C. brunnea* and *C. orientalis* contain distinct eastern and western lineages, this is consistent with Java's climate gradient playing a role in either generating species or providing

existing species with sufficient environmental variation to maintain ecological separation.

Niche partitioning by Javan shrews

Adaptation to ecological opportunity may facilitate diversification and/or coexistence (Rundell & Price 2009; Losos 2010). We documented all five Javan species of shrew living on Mt. Gede. Among these taxa, four species are terrestrial, while one (the undescribed species) has the morphology (long tail, slender body) of a scansorial (climbs on low trees and shrubs) species. The four terrestrial taxa include two small and two relatively large species. Previous research has shown differences in body size among co-occurring shrews to be associated with differences in foraging mode and dietary composition (e.g. Churchfield *et al.* 1999; Brannon 2000). Thus, differences in body size potentially contribute to Javan species' capacity to coexist (e.g. Churchfield *et al.* 1999; Brannon 2000; Esselstyn *et al.* 2011). Partial vertical stratification, presumably achieved via the presence of the undescribed scansorial species, may also contribute to ecological differentiation (Leite *et al.* 1996).

At the most diverse site (1600 m on Mt. Gede) we surveyed, we found two small (*C. maxi* and *C. monticola*), one large (*C. orientalis*) and one scansorial (*Crocidura* sp. nov.) species occurring within the reach of a single trap line (~100 m long). Among the eight sites we sampled, we found members of different species pairs (i.e. in distinct body size categories) co-occurring at five sites, and members of the same species pair co-occurring at two sites. Although the Javan species that have similar body sizes are able to coexist in at least two locations, this does not necessarily suggest that multiple closely related species living in syntopy is the norm on Java. Rather, members of the same species pair were found to co-occur only at midelevation (1600–1700 m) sites. We found *C. brunnea* (<1650 m) and *C. orientalis* (>1500 m) occurring in syntopy at one midelevation (1650 m) site on Mt. Ciremai, which is consistent with Ruedi's (1995) suggestion that these species are elevationally separated. Species restricted to high-elevation habitats should have more isolated populations, leading to the evolution of distinct lineages on each mountaintop. This prediction is consistent with our finding of geographical structure in the high-elevation *C. orientalis*, but not in the low-elevation *C. brunnea* (Figs 4 and S2, Supporting information). However, N_e estimates from MIMAR and BPP were higher for the low-elevation species than for the high-elevation species, suggesting that although *C. orientalis* may be more fragmented (which could elevate N_e estimates), *C. brunnea* is more abundant, possibly because of more extensive habitat. For

C. maxi and *C. monticola*, we only have conclusive evidence of them occurring in syntopy on Mt. Gede at 1600 m. We did not find *C. maxi* on any of the other mountains we surveyed and they may be more common in dryer habitats in East Java (Jenkins 1982).

Hutchinson & MacArthur (1959) theorized that body size differences in animals of ~1.3-fold should be adequate to allow closely related species to coexist at high abundances. Consistent with this theory, Brannon (2000) found that body length differences of ~1.2 were associated with differential microhabitat use by two syntopic shrews in the Appalachian Mountains. For the Javanese shrews, the ratio of body sizes is only 1.15 for *C. maxi* to *C. monticola* and 1.04 for *C. brunnea* to *C. orientalis*, both well below Hutchinson and MacArthur's threshold, which may explain the limited evidence for co-occurrence within body size categories. While body size differences may contribute to community structure, we note that species of *Crocidura* in Africa have a much greater range of body size, with some species exceeding 100 g—the largest species in South-East Asia are around 20 g. Greater body size diversity coupled with a longer history of diversification (Dubey *et al.* 2008) may, in part, explain why African shrew communities are so much more diverse than South-East Asian shrew communities.

Conclusions

Javan shrews are more diverse, with a higher degree and finer scale of endemism, than previously appreciated. Similarities in body size and a conservative morphology led to the confounding of distantly related species into widespread taxa. At least two Javan species are sister taxa, suggesting a role for within-island speciation in the generation of the modern Javan shrew fauna. The inferred speciation events that produced four of Java's shrews probably occurred during the Pleistocene, and one pair of species probably experienced gene flow after divergence. Up to four species of shrew occur syntopically on Java. Differences in body size and the use of vertical habitats may enhance local community diversity. The 100% endemism in Javan *Crocidura* suggests that biodiversity on the Sunda Shelf is more highly fragmented and localized than is generally appreciated, perhaps implying that ecological adaptation occurs on a small scale relative to island area.

Acknowledgements

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Data accessibility

DNA Sequences: GenBank Accession nos KF282769–KF283985.

DNA Alignments: Dryad Accession doi: 10.5061/dryad.6dn5p.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 List of the museum voucher and GenBank accession numbers for all specimens used in this study.

Fig. S1 Maximum-likelihood gene tree estimates of phased alleles from all available Javan *Crocidura* for (A) *ApoB*, (B) *BDNF*, (C) *BRCA*, (D) *GHR*, (E) *MCGF*, (F) *PTGER4*, (G) *RAG1*, and (H) *vWF*. Numbers at nodes represent per cent bootstrap support.

Fig. S2 Results from Structure analyses of eight nuclear loci from *Crocidura maxi* and *Crocidura monticola* (A) and *Crocidura brunnea* and *Crocidura orientalis* (B) with the number of populations (*K*) set from 2 to 6.

Fig. S3 Pie charts showing per-population allele frequencies for Javan *Crocidura*.

Fig. S4 Results of goodness-of-fit tests for MIMAR analyses of (A) *Crocidura maxi* and *Crocidura monticola* with fast substitution rate, (B) *Crocidura brunnea* and *Crocidura orientalis* with a fast substitution rate, (C) *Crocidura maxi* and *C. monticola* with slow substitution rate, and (D) *C. brunnea* and *C. orientalis* with a slow substitution rate. Full height vertical bars show observed values.

Table S1 Primers used in this study, with associated annealing temperatures.

Table S2 Best-fit models of sequence evolution, as determined using the Bayesian information criterion in jModelTest v 0.1.1.

Table S3 Results of Bayesian species delimitation analyses using BPP (Yang & Rannala 2010).