Elucidating geological and biological processes underlying the diversification of Sulawesi tarsiers

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Abstract

Because of their exceptionally long independent evolution, a range diminution of their Eocene relatives, and a remarkable subsequent diversification in Southeast Asia, tarsiers are of particular importance to evolutionary primatologists. Little is known, however, on the processes shaping the radiation of these small enigmatic primates—especially on the Indonesian island of Sulawesi, their center of endemism. Geological reconstructions and progress in applying DNA sequence information to divergence dating now provide us with the tools and background to comprehend tarsier dispersal. Here, we describe effects of plate-tectonic movements, Pleistocene sea level changes, and hybridization on the divergence of central Sulawesi tarsiers. We analyzed 12 microsatellites, the cytochrome *b* gene, the hypervariable region I of the mitochondrial control region, and the sex-determining region on the Y-chromosome from 144 specimens captured along a transect crossing a species boundary and a contact zone between 2 microplates. Based on these differentially inherited genetic markers, geographic information, and recordings of vocalizations, we demonstrate that the species boundary coincides with a tectonic suture. We estimate the most recent common ancestor of the 2 taxa to have lived 1.4 Mya, we describe asymmetrical introgressive hybridization, and we give evidence of unbiased dispersal in one species and male-biased dispersal in another species. This study exemplifies that the distribution of tarsier acoustic forms on Sulawesi is consistent with the allocation of genetic variability and that plate-tectonic and glacial events have left traceable marks in the biogeography of this island's unique fauna.

As primatologists are getting close to realizing the diversity of extant primates, the phylogeographic history and underlying processes of several major primate groups are still far

from understood. Among those animals whose phylogenetic uniqueness has sparked high interest and controversial discussion over the past decades are tarsiers. These are small nocturnal faunivores of the Southeast Asian rain forests. Although formerly considered close relatives of lemurs and lorises (1, 2), the perception of tarsiers as a sister group to extant anthropoid primates (3–6) has developed in parallel and is now backed by molecular data, including retropositional evidence (7, 8). The tarsier lineage split from all other extant primates at least 58 Mya (7), and possibly even much earlier (4, 9). Thus, reconstructing the phylogeography of this ancient line might not only help us to understand the current distribution, sociobiology, or conservation status of *Tarsius* but shed light on the evolution of adaptive traits during early stages of primate divergence.

Tarsiid fossils dating from the Eocene to Miocene epochs have been recovered from Asia (10–13) and, if the controversially discussed *Afrotarsius* is included, from Africa (14, 15). There is still no consensus about the affinities of extant tarsiers to the Omomyidae, a family of extinct haplorhine primates with an Eocene distribution throughout most of the northern hemisphere (16). Many phylogenetic reconstructions place omomyids as ancestors to tarsiers, giving rise to only tarsiids (17), to independent tarsiid and anthropoid lineages (16), or to a tarsier-anthropoid clade (5, 6). Recent evidence, however, suggests a sister group relationship between extant haplorhines and omomyids (18).

The once holarctic tarsiiform primates (16) are now represented by a single monophyletic group inhabiting a small fraction of their Eocene range—Southeast Asia, the only region assumed to have retained Eocene-like rain forest habitats through the Neogene (19). All the more intriguing is the unusual recent radiation of *Tarsius* on Sulawesi. This central Indonesian island is the unrivaled hotspot of tarsier diversity, with 7 currently recognized species (20) and several other taxa in line to be named. As the major component of the biodiversity hotspot Wallacea (21), Sulawesi is a melting pot of Asian as well as Australian fauna, and its troubled geological past has promoted an extraordinary 98% of its nonvolant mammals being endemic to the island (22). Sulawesi is a remarkable outdoor laboratory to study the complex processes toward the diversification of *Tarsius* because (*i*) the taxonomic diversity of this genus is by far greatest on this island and (*ii*) tarsiers presumably colonized the Sulawesi region during the Miocene (23), and thus could have been subject to microplate-tectonic movements as well as Pleistocene sea level changes. Long before the latter funneled the radiation of Sulawesi macaques (24–29), the colonization of proto-Sulawesi islands by tarsiers might have shaped the present distribution of these "old endemics" (30). Tarsiers living on this fourth largest of the Indonesian islands are characterized by species-specific duet songs. The differentiation into several vocal-acoustic groups and the preliminary outline of their distribution led to the formulation of a "hybrid biogeographic hypothesis," (31) raising the notion of a causal relationship between plate-tectonic history and the evolution of tarsiers. The exact timing of geological events in this region, however, is still as poorly understood as the distribution of land and sea over evolutionary time scales (32, 33). Thus, it remains difficult to formulate testable hypotheses on consequences of geological action on primate phylogeography. There is one notable exception. At least the northern part of the Palu-Koro fault (Fig. 1*A*), one of the major fissures of mainland Sulawesi, probably remained a water barrier between the eastern and western parts of the island throughout the Neogene (34, 35). The Lariang tarsier (*T. lariang*) supposedly inhabits the northern part of western Sulawesi and borders on its parapatric congener, *T. dentatus*, right along the Palu-Koro

fault (36). Whereas other taxon boundaries among tarsiers (31) coincide with range limits among macaques (24), toads (26), and frogs (27), the geographic subdivision of the central core of Sulawesi is only to be found in *Tarsius*, suggesting the effect of geological action older than, for example, the macaque radiation on the island. In this study, we explore evidence of a fundamental relationship between the geological past and the evolution of a primate group. We also test the influence of natural hybridization on the divergence of 2 tarsier species bordering each other along a major tectonic suture, which, in turn, is not a present geographic barrier.

Results

Geography and Vocalizations.

Zones of contact between *T. dentatus* and *T. lariang* were located near the villages of Winatu [1°33′S, 119°58′E, 850 m above sea level (asl)] and Marena (1°34′S, 120°01′E, 550 m asl; Fig. 1*A*). No present physical barrier to dispersal was identified in this area. Moving along a transect across these zones, the shift from Dian's tarsier-like duet calls to the type of Lariang tarsiers occurs very abruptly (Fig. 1*B*). Thus, it pinpoints the tentative species boundary, clearly evident to the field observer. Except for 3 tarsiers (2 in Winatu, 1 in Marena) whose calls could not be witnessed, all other captured individuals produced the duet song characteristic to the trapping location.

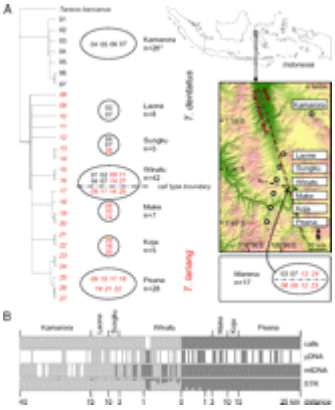

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Fig. 1.

Geographic distribution of genetic and acoustic diversity in central Sulawesi tarsiers. (*A*) Phylogenetic relationships (maximum likelihood) and distribution of 27 HV I haplotypes falling into a *T. dentatus* (*n* = 7) and a *T. lariang* (*n* = 20, red italics) clade. (*B*) Type of vocalization, Y-chromosomal DNA and mtDNA, and score of microsatellite assignment (0–100%) of 127 tarsiers to *T. dentatus* (light gray) or *T. lariang* (dark gray). STR, short tandem repeats.

Individuals were arranged with regard to their distance to the tentative species boundary. White bands denote females (yDNA) or lack of data (calls, mtDNA). We sampled 32 tarsiers from Kamarora (*) yet obtained HV I sequences of only 26 of these.

Sequence Evolution.

FindModel (37) suggested the HKY+G ($\alpha = 0.21$) model to be reflecting Sulawesi tarsier hypervariable region I of the mitochondrial control region (HV I) sequence evolution. We used this model for tree reconstruction and the similarly suitable TN93+G for molecular diversity estimates. Tajima's D ($D = -0.177$, $P = 0.478$ for *T. dentatus*; $D = 0.455$, $P = 0.744$ for *T. lariang*) and Fu's Fs ($Fs = 0.961$, $P = 0.709$ for *T. dentatus*; $Fs = 3.207$, $P = 0.885$ for *T. lariang*) statistics do not point at departure of the tarsier HV I from selective neutrality. The TN93 model was followed in the analysis of cytochrome *b* gene (Cyt *b*) sequence variation among Sulawesi tarsiers, and for divergence dating, the inclusion of the outgroup sequence led us to adopt GTR+G. In light of the homogeneity of sex-determining region on the Y-chromosome (SRY) sequences within study species, only the uncorrected genetic distance between species was calculated.

Intraspecific Genetic Diversity.

Aiming at true species-typical estimates, the computation of molecular diversity statistics [supporting information (SI) Table S1] involved only data obtained from 113 tarsiers whose confirmed vocalizations matched their unambiguous genetic composition (see below). Basic characteristics of the 12 microsatellite markers have been published previously (38). We found the level of their allelic diversity to be similar in both species (Table S1). Of the 99 alleles detected in Dian's tarsiers, 42 are private for this taxon. For Lariang tarsiers, 37 of a total 94 were found to be unique. Mitochondrial HV I pairwise distances among Lariang tarsiers are more than 5-fold higher than among Dian's tarsiers (Table S1). All sampled *T. dentatus* males comprise the same SRY haplotype, and a single haplotype is common to all (male) *T. lariang*. Mantel tests for the association between molecular and Euclidian geographic distances indicated no significant isolation by distance effects within the 2 species (Table S1).

Interspecific Genetic Distances.

Nei's net average distance between Cyt *b* sequences of the 2 species is $D_A = 0.030$, with an intragroup variation of $D_A = 0.003$ ($n = 55$) for Dian's tarsiers and $D_A = 0.004$ ($n = 54$) for Lariang tarsiers. HV I sequences differed by an average of $D_A = 0.388$ between the 2 major clades (Table S1). These highly significant results were corroborated by just as significant genetic distances between the species calculated from the number of different alleles of 12 microsatellite loci (Table S1). Dian's tarsiers' SRY ($n = 30$) differed at 4 loci (2 transitions, 2 transversions) from Lariang tarsiers' SRY ($n = 29$). Based on Cyt *b* genetic distances and a divergence time between Bornean and Sulawesi tarsiers of 11.0 Mya (see *Materials and Methods*), we estimated the split between *T. dentatus* and *T. lariang* to have occurred *ca.* 1.4 Mya (mean = 1.42, SD of mean = 0.01, median = 1.32, 95% confidence interval: 0.56/2.38).

Population Structuring.

The marked structuring of Lariang tarsier mtDNA haplotypes in the sampled populations (F_{ST} = 0.325) is opposed to a high admixture of Dian's tarsier mtDNA (F_{ST} = 0.081). Slightly lower values were obtained when considering only adult individuals (F_{ST} = 0.229 for *T. lariang*, F_{ST} = 0.078 for *T. dentatus*). For microsatellites, R_{ST} values indicate little to moderate genetic differentiation among conspecific populations (Table S1).

Geographic Distribution of Genetic Diversity.

Two mitochondrial haplogroups were found in this study (Fig. 1*A*): a clade of 7 nonidentical HV I haplotypes uniting 58 Dian's tarsiers and 20 haplotypes of a Lariang tarsier variety found among 80 individuals. Close to the species boundary, a total of 20 specimens with *T. lariang* haplotypes were captured in the Dian's tarsier range: 1 at the Sungku study site, 13 in Winatu, and 6 near the village of Marena. Seven Lariang tarsier haplotypes thus introgressed into the Dian's tarsier range (Fig. 1*A*). The frequency of these haplotypes decreased with increasing distance from the species boundary. In Laone, 18 km away, all specimens (n = 8) carried a range-typic *T. dentatus* haplotype. In Sungku, 10 km away, 1 of 5 specimens carried the *T. lariang* type, and in the Winatu region (0–2 km away), 13 of 28 specimens in the Dian's tarsier range were detected with the atypical haplotype (Fig. 1*B*). Mitochondrial gene flow in the opposite direction was not observed. There was also no sign of Y-chromosomal gene introgression. An adult male tarsier and a subadult male tarsier with *T. lariang*-typic SRY were captured together almost 1 km into the Dian's tarsier range. They also comprised *T. lariang*-typic mtDNA and were clearly assigned to Lariang tarsiers according to their microsatellite allelic frequencies. We were unable, however, to record their vocalizations (Fig. 1*B*). A total of 8 male tarsiers comprised *T. dentatus*-typic Y-chromosomes and *T. lariang*-typic mtDNA (see Fig. 1*B* for 7 male tarsiers from Winatu). We found no evidence of the reverse composition.

From an assignment test in *structure* v2.2 (39) based on microsatellite frequencies, it can be inferred that the probable ancestry of individual tarsiers closely corresponds to the vocalization type. For each specimen, the genetic assignment to a particular species matched its call type (Fig. 1*B*). Four individuals were ascribed to a taxon with a probability of 50–60%, with the scores of all other animals being higher. These 4 tarsiers were all captured within 300 m from the species boundary.

Discussion

Population Genetics.

Low fixation indices reflect the absence of geographic pattern in the distribution of microsatellite alleles within both species. mtDNA of Lariang tarsiers, however, shows significantly more geographic structuring than Dian's tarsiers' mtDNA (Fig. 1*A* and Table S1). These different patterns between biparentally and maternally inherited markers lead to the assumption that in Lariang tarsiers, females are the more philopatric sex, whereas migrating males homogenize the gene pool and thus provide for a low F_{ST} value in microsatellites (40). In contrast, there is no indication for sex-biased dispersal in Dian's tarsiers. This interspecific difference could be a

signature of a more recent population expansion of Dian's tarsiers to populate the eastern areas of central Sulawesi. Judging from this species' wide distribution (41), its adaptability to human land use (42), and the presumed displacement of *T. lariang* by *T. dentatus* in parts of its range (see below), Dian's tarsier appears to be a good disperser and probably the more opportunistic of the 2 species.

Pairwise distances among Lariang tarsier HV I sequences and the number of this species' mitochondrial haplotypes are considerably greater than among Dian's tarsiers. The rationale for this remains unknown for now. Spatially elaborated future studies on central Sulawesi tarsiers will help us to understand their population history better, including the potential effects of bottlenecks and rapid expansions.

Hybridization.

Although hybridization often counteracts divergent evolution, variation introduced via introgression can also contribute to adaptation and diversification (43). At least 10% of animal species and most rapidly radiating groups undergo interspecific hybridization (43); thus, observing this phenomenon in the relatively young radiation of Sulawesi tarsiers comes as no surprise. The lack of a physical barrier or a vegetation ecotone between the ranges of Dian's and Lariang tarsiers and the limited degree of gene exchange characterize a hybrid tension zone (44). In light of the presumably long time after secondary contact between the groups, the evident perpetuation of species identities points to continued assortative mating. A well-studied but certainly not exclusive candidate for a mate recognition system among these nocturnal primates is the characteristic duet song (23, 45).

Microsatellite-based estimations of individual ancestry revealed 4 putative F1 hybrids indicating ongoing hybridization. Considering the total number of captures and a sampling design deliberately biased toward individuals from the contact zone, this number is relatively low. Such hybrid zones are highly stable and usually the result of assortative mating (46). Backcrossing occurs into both species' ranges, although to a lesser degree on the *T. lariang* side of the boundary (Fig. 1*B*). Mitochondrial gene flow is strictly unidirectional. There seems to be a pronounced mating bias wherein *T. dentatus* males do not seem to differentiate strictly between conspecific and foreign females. *T. lariang* males, on the other hand, apparently do not successfully mate with *T. dentatus* females. It is yet unclear whether this is attributable to female choice based on the attractiveness of the loud *T. dentatus* male song to females of both species or to another semipermeable pre- or postzygotic barrier. We captured 2 males with *T. lariang*-typic genetic signatures several hundred meters outside of their species' usual range. This observation falls into a radius that tarsiers easily cover within a single night (42, 47). Although these 2 individuals might also have been translocated and released pets, the steep gradients in allele frequencies at the species boundary and the consistent lack of alien genes in the reference populations suggest that accidental introductions do not play an important role in shaping the genetic structure of tarsier populations in a human-altered landscape. We anticipate that future field studies, including radio-tracking of young potential dispersers in the contact zone, will be highly rewarding.

Based on this snapshot picture of tarsier distribution, there is a question that cannot be answered definitively. Is the introgression of Lariang tarsier mtDNA into the Dian's tarsier gene pool attributable to active Lariang female dispersal, or did we register remnants of a Lariang tarsier population displaced by its more opportunistic neighbor in the wake of forest degradation? The majority of mixed genotypes were detected in the vicinity of the species boundary. Furthermore, the introgressed haplotypes are identical or nearly identical to other *T. lariang* types; thus, they are likely to be traces of localized introgression based on present-day hybridization. The backcross found 10 km away from the present boundary, however, could hint at past hybridization and subsequent movement of the hybrid zone (48). Because a recent westward movement of the species boundary is likely (see below), the latter seems sensible. On the other hand, the generally widespread distribution of tarsier mtDNA haplotypes could easily explain the presence of that particular backcross by recent dispersal alone.

Plate Tectonics and Sea Level Changes.

Our research in the area of the Palu-Koro fault revealed a well-maintained boundary between parapatric species of an old endemic genus. The analyses of mtDNA, Y-chromosomal DNA, and microsatellite DNA uniformly indicate a deep divergence between the 2 studied taxa coinciding with different vocalization patterns. Not only does this support the assumption that tarsier calls are species diagnostic (23, 45), but the geographic distribution of this variability, along with results from additional surveys, shows that the boundary between *T. dentatus* and *T. lariang* roughly coincides with the Palu-Koro fault. There is neither a present physical barrier to gene flow nor is this contact zone low-lying, and thus potentially effected by vicariance through glacial sea level changes alone.

Our expectation that this line follows the course of the Palu valley as the topographic landmark for the past geological processes was not entirely met. Dian's tarsiers occurred slightly further west than previously known (36). On the one hand, satellite images and ground surveys showed heavy deforestation in the Winatu area; thus, we might speculate that the widespread Dian's tarsier is better adaptable to human land use than its western neighbor. On the other hand, tension zones can move (44), and in light of secondary contact presumably several hundred thousand years ago (33), the recorded distance of 6 km from the expected location of the species boundary may just be the result of stochastic events rather than directed competitive exclusion. Our surveys of the western coast of central Sulawesi confirmed that Lariang tarsiers inhabit this region and proved the limited extent of Dian's tarsiers' dispersal toward the west.

It has been argued before that Sulawesi's plate-tectonic history might have played a major role in shaping tarsier evolution (31), but the underlying processes leading to dispersal and eventual reproductive isolation remained unknown. Previous studies of other fauna of this island found no phylogeographic breaks in the Palu-Koro area (24, 26–28, 49). The timing of colonization, however, seems crucial to understand Sulawesi's biogeography. A sea lowstand (*ca*. 200 m below present level) most likely influenced macaque distribution in Southeast Asia (not necessarily in Sulawesi) as early as 1.6 Mya (29). This date falls well within the confidence interval of our date estimate for the *T. dentatus*-*T. lariang* split. Thus, a plausible chain of dispersal events could have been as follows. The progenitor of Sulawesi tarsiers diverged from a Sundaland taxon and reached the area of present southern or western Sulawesi at *ca*. 11 Mya,

either via a transitory landbridge (30) or by sea rafting. Subsequently, as the already joined eastern microplates closed in on the western fragment (33), an early-Pleistocene glacial maximum at 1.6 Mya (29) resulted in a land bridge connecting the island precursors and allowing for tarsier dispersal across the Palu-Koro fault and throughout the eastern areas. On inundation of this bridge, the microplates maintained their distance from each other for a sufficient time to permit vicariant divergence between the 2 tarsier taxa. Even without a transient connection between microplate land masses, their potentially increased proximity during ice ages raised the odds of successful overwater dispersal by rafting and subsequent allopatric speciation. Macaques presumably colonized Sulawesi in early-middle Pleistocene (29) and might therefore have "missed" the early chance to colonize eastern central Sulawesi.

This theory, of course, assumes the ancient land masses to be generally hospitable to tarsiers and neglects possible effects of aridification and deforestation at glacial maxima. To date, information on the zoogeographic history of Sulawesi is scarce. The limited fossil record of rain forest fauna makes phylogenetic and demographic reconstructions from molecular data all the more important. At this stage, we do not attempt to decide whether the diversification of central Sulawesi tarsiers is attributable to vicariance or dispersal. The deep divergence, however, exists. Here, we present evidence that plate-tectonic movements as well as Pleistocene sea level changes very likely influenced the radiation of an evolutionary unique primate genus on an island that is now a continuous land mass. We expect that future research resulting in more detailed stratigraphic and palynological information on this region will thoroughly promote our understanding of the historical biogeography of Sulawesi's highly distinctive fauna.

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Materials and Methods

Study Objects.

Dian's tarsiers (*T. dentatus*) and Lariang tarsiers (*T. lariang*) are small (12 cm) nocturnal primate species endemic to Sulawesi, Indonesia. As pointed out by Shekelle and colleagues (50), the widely used name *T. dianae* (51) is likely to be a junior synonym to *T. dentatus* (52). Here, we use the senior denomination. *T. lariang* differs in terms of morphology, acoustics, distribution (36), and genetic characters (37) from Dian's and other tarsiers.

Field Procedures.

Between the years 2001 and 2006, we sampled a total of 144 tarsiers between the villages of Kamarora (01°12′S, 120°08′E) and Peana (01°46′S, 119°55′E) in central Sulawesi (Fig. 1*A*). These 2 sites were the terminal locations of a transect crossing the species boundary between *T. dentatus* and *T. lariang* and were home to species reference populations sampled in this study. Sleeping sites were localized by tracing morning duet songs; trapping involved mist-netting in the vicinity of these sites and also occasional hand-captures. We obtained small ear biopsies (2 × 2 mm) for DNA analyses and stored them in vessels with tissue buffer solution [6 M urea, 10 mM Tris/HCl (pH 8), 10 mM EDTA, 125 mM NaCl, 1% SDS]. The 32 samples from Kamarora were collected in the year 2001 and stored in 70% (vol/vol) ethanol until analyzed 4 years later.

Tarsier morning duet calls were digitally recorded using a Sony MZ-NH900 Hi-MD Walkman connected to a Røde NT3 condenser microphone and visualized using the Syrinx-PC (53) sound analysis software. The calls were classified on the grounds of qualitative traits given in the species descriptions (36, 51). As a working hypothesis, taxonomic affiliation of individuals and a preliminary demarcation of the species boundary followed the tarsiers' acoustic characteristics (31). Field research and shipping of samples (see below) were approved by the Indonesian Institute of Sciences (LIPI) and the Department of Forestry (PHKA) and were conducted abiding by state law and the regulations of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Laboratory Procedures.

We extracted genomic DNA from tarsier ear biopsies using a DNeasy Tissue Kit (Qiagen). Extracted DNA was subjected to whole-genome amplification (WGA) according to the manufacturer's (GE Healthcare) protocol. WGA was necessary to (*i*) comply with Indonesian export regulations and (*ii*) increase the amount of template material. To trace potential sex-biased dispersal in tarsiers, we characterized and amplified by PCR maternally inherited mtDNA markers (Cyt *b* and HV I), paternally inherited Y-chromosomal DNA (SRY), and 12 biparentally inherited microsatellites (38). Primers and PCR conditions are listed in Table S2. PCR products were checked on 1–2% (m/vol) agarose gels and purified using an ExoSAP protocol (54). To confirm the Y-chromosomal origin of the SRY marker, we attempted several amplifications of this gene from female DNA, all with negative results. To minimize the risk of amplifying nuclear mtDNA inserts instead of genuine mtDNA, we performed long-range PCR of 2 fragments (10,100 and 7,600 bp) whose ends overlapped on opposite sides of the mtDNA molecule. Sequences of overlapping parts of the 2 amplificates were identical, suggesting the circular nature, and thus the genuine mitochondrial origin, of the template. Target regions of mtDNA (Cyt *b* and HV I) were amplified from these long-range PCR products and from original WGA products. We sequenced both DNA strands, and we obtained microsatellite data using ABI 377 and ABI 3130*xl* automated sequencers (Applied Biosystems) and standard ABI software. Sequences of the whole data set have been deposited in the GenBank database under accession nos. FJ214312–FJ214337 and FJ614263–614568.

Sequence Data Analyses.

For diversity estimates, we evaluated both mitochondrial markers; for tree reconstruction and population structuring, we analyzed HV I; and for divergence dating, we used the more conservative Cyt *b*. Nucleotide sequences were assembled and aligned in BioEdit 7.0.9.0 (55) with *T. bancanus* (GenBank accession no. AF348159) as an outgroup for tree reconstruction and divergence dating. We used the Web-based program FindModel (37) implementing the Akaike information criterion as the model selection framework to estimate the nucleotide substitution model best describing the evolution of scrutinized sequences. Genetic distances were calculated in MEGA v3.1 (56), and their SE was estimated by bootstrapping (500 iterations). AMOVA computations (Analysis of Molecular Variance, over loci) to test for population structuring were done in Arlequin 3.11 (57) with 20,000 bootstraps for confidence intervals and 10,000 permutations for significance testing. F_{ST} values were estimated in the sense of Weir and Cockerham's θ_w (58) considering (*i*) all age classes or (*ii*) only adults. Mantel tests for isolation-

by-distance using matrices of Euclidean distances and Slatkin's linearized F_{ST} (100,000 permutations) and tests for selective neutrality were also performed in Arlequin (57). Based on HV I sequences, we reconstructed phylogenetic relationships with TREE-PUZZLE 5.2 (59).

We used the BEAST package v1.4.8 (60) for divergence time estimates by Bayesian inference. The analysis comprised the whole suite of different Cyt *b* haplotypes of Dian's and Lariang tarsiers and the sequence of *T. bancanus*. Input files were generated assuming an uncorrelated relaxed log-normal clock for sequence evolution, a GTR+G substitution model, decoupled evolution of codon positions 1 + 2 and 3, and a birth-death model (61) as tree prior. We used default model parameter priors yet calibrated the tree with a basal split between the Western/Philippine tarsier clade and the Sulawesi tarsier group at 11.0 ± 1.0 Mya (95% confidence interval: 9.0/13.0). There is strong evidence for the respective monophyly of both clades (62, 63) yet no reliable information on divergence time. Evidence for the lowest pre-Pleistocene sea level at 11.4–10.5 Mya (33, 64, 65); distinctive sweeps of pollen dispersal across the Makassar Strait at 17, 14, and 9.5 Mya (66); a commencing radiation of *Chitaura* grasshoppers at 14–7 Mya (49); the split between western and Sulawesi squirrels at 11.0 Mya (65); and a previous hypothesis on the separation between eastern and western tarsiers *ca*. 10 Mya (23) give us reason to presume that the calibration date of 11.0 Mya for the deepest split of extant tarsiers is a reasonable choice. After 10% burn-in, 3 runs of Markov Chain Monte Carlo with 20,000,000 cycles each were executed, sampling every 5,000 cycles. Results of the runs were combined in LogCombiner v1.4.8 and displayed in Tracer v1.4 [both from the BEAST package (60)] to check for stationarity and to give mean age estimates and 95% credibility intervals.

Microsatellite Data Analyses.

We computed fixation indices, their significance (10,000 permutations), and 95% confidence intervals (20,000 bootstraps) in Arlequin (57). R_{ST} values and 95% confidence intervals were calculated in RSTcalc v2.2 (67) based on the stepwise mutation model (1,000 permutations, 1,000 bootstraps). In Arlequin (57), we also inferred average pairwise distances as the number of different alleles per locus (10,000 permutations), and we performed Mantel tests for isolation-by-distance (Euclidean distances and Slatkin's linearized F_{ST}; 100,000 permutations).

Based on allelic composition, the tarsiers captured along the transect (excluding Marena) were subjected to a population assignment test in *structure* v2.2 (39) using the following run parameters: 127 individuals, 12 loci, 2 populations assumed, 100,000 burn-in period, 1 million repetitions; and USEPOPINFO turned on (with predefined membership of the reference populations in Kamarora and Peana).

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Footnotes

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- Author contributions: S.M., J.P., and H.Z. designed research; S.M., C.D., and D.P.-F. performed research; S.M., D.P.-F., and H.Z. contributed new reagents/analytical tools; S.M. and C.D. analyzed data; S.M. and H.Z. wrote the paper; and J.P. provided administrative support.
- The authors declare no conflict of interest.
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- Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. FJ214312–FJ214337 and FJ614263–614568).
- This article contains supporting information online at www.pnas.org/cgi/content/full/0900319106/DCSupplemental.

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