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Complex phylogeographic structuring in a continental small mammal from East Asia, the rice field mouse, *Mus caroli* (Rodentia, Muridae)

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Abstract. We investigated genetic variation in mitochondrial cytochrome *b* within the long-tailed rice field mouse, *Mus caroli* Bonhote, 1902, across its entire geographic range in Southeast and East Asia with a view to: 1) assessing the pattern and causality of phylogeographic structure in a terrestrial small mammal from continental Southeast Asia; and 2) distinguishing genuine insular relics from cases of human-assisted translocation. We identified five main mtDNA lineages which show a similar level of differentiation as the subspecies of *M. musculus* and probably diverged during the Middle Pleistocene. Two of the lineages are restricted to large islands (Taiwan and Java) and their existence is explicable in terms of regional palaeogeographic factors including changes in sea level and climate. The remaining lineages are distributed in different regions on mainland Southeast Asia but vicariant explanations are inappropriate given the relatively short time frame. Dispersal across barriers followed by local differentiation probably explains the observed phylogeographic patterning on the mainland. A close genetic link between Okinawan *M. caroli* and populations in Laos confirms previous suggestions that people carried this species to the Ryukyu Archipelago. However, more intensive regional sampling is needed to identify a precise source area.

Key words: mainland Southeast Asia, mitochondrial cytochrome *b*, *Mus caroli*, Quaternary, Sunda Shelf.

The dramatic climatic perturbations of the late Tertiary and Quaternary period had a profound influence on the genetic structure of many plants and animals. For organisms found in western Europe and North America, the 'genetic imprint' of the Ice Ages is most often related to cyclic retreat of taxa into southern refugia in the wake of expanding continental ice sheets, with reinvasion of northern areas following glacial retreat (e.g. Hewitt 1996, 1999, 2000; Riddle 1996). In contrast, genetic patterns in parts of the world that remained ice-free are frequently more complex and there is debate over the general relevance of refugial models for these areas (e.g. Patton and Smith 1992; Patton et al. 1994; Joseph et al. 1995; da Silva and Patton 1998). Nevertheless, organisms in these regions commonly show phylogeographic structure of latest Tertiary and Quaternary age, as documented for sub-Saharan Africa (e.g. Arctander et al. 1999), the Andean mountains (e.g. Patton and Smith 1992) and Amazonian basin (e.g. da Silva and Patton 1998) of South America, the Sunda Shelf region of Southeast Asia (e.g. Gorog et al. 2004), the Philippine Archipelago (e.g. Steppan et al. 2003; Heaney et al. 2005), and tropical northeastern Australia (e.g. Schneider et al. 1998).

Two factors are commonly identified as major 'drivers' of micro-evolutionary differentiation in all of these

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regions, namely: 1) changes in geography related to sealevel fluctuations, including the submergence and emergence of both extensive continental shelves and land bridges—thereby producing cycles of isolation and recombination between populations (e.g. Steppan et al. 2003; Gorog et al. 2004); and 2) changes in the distribution of vegetation types related to global climatic shifts (e.g. Comes and Kadereit 1998), with associated changes in spatial distribution and abundance of dependent animal species (e.g. Joseph et al. 1995; Zamudio and Greene 1997; Brunhoff et al. 2003).

Mainland Southeast Asia is a region of exceptional biological diversity and complexity (Gaston et al. 1995; Myers et al. 2000; Sodhi et al. 2004), yet comparatively little is yet known regarding the scale or biotic impact of late Tertiary and Quaternary climatic changes in this region (Hope et al. 2004). In addition, very few studies have investigated phylogeographic patterning within the exceptionally rich small mammal fauna of mainland Southeast Asia. Notable exceptions include the studies of Ruedi et al. (1996) on soricid insectivores, Ruedi and Fumagalli (1996) on erinaceid insectivores, Mercer and Roth (2003) on squirrels, and of Ruedas and Kirsch (1997), Terashima et al. (2003) and Gorog et al. (2004) on murine rodents.

The long-tailed rice field mouse, *Mus caroli* Bonhote, 1902, is a good candidate for phylogeographic analysis. It is widely distributed across Southeast and East Asia, from Myanmar and Yunnan (southern China) in the west, to Taiwan and the Ryukyu Archipelago in the east, and there are widely disjunct populations on the Indonesian islands of Sumatra, Java, Madura and Flores in the southeast (Marshall 1977; Corbet and Hill 1992; Aplin et al. 2004). The Indonesian populations are generally treated as recent introductions (Musser 1981; Musser and Newcomb 1983), as are those in the Ryukyu Archipelago (Motokawa 2000; Motokawa et al. 2003). Finally, an earlier pilot study of genetic variation in *M. caroli* revealed surprisingly high levels of regional differentiation in mtDNA (Terashima et al. 2003), and highlighted the potential of this species for more intensive phylogeographic studies. This paper reports our analysis of mitochondrial cytochrome *b* (cyt *b*) gene sequence variation in a sample of 41 individuals drawn from across almost the entire reported geographic range of *M. caroli*.

Materials and methods

Population sampling

Mus caroli is generally found in rice field habitats across its range (Fig. 1a) and little is known of its ecology outside of this anthropogenic habitat (Marshall 1977; Aplin et al. 2004). As a rule the species is quite

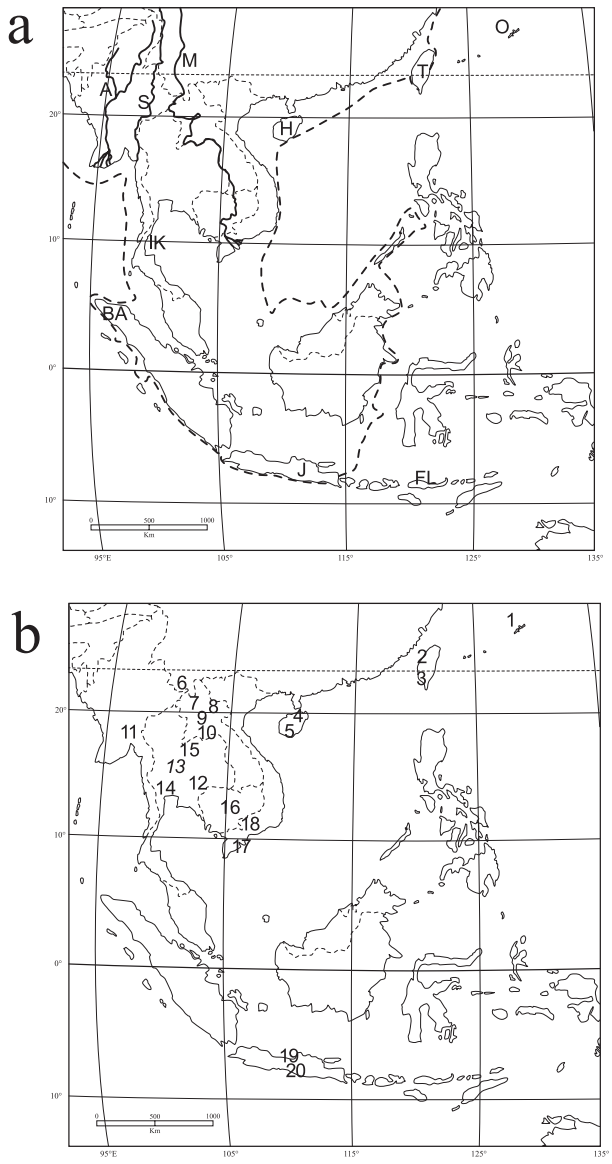

Fig. 1. Maps showing the geographic context and collection localities of samples used in this study. Key for Fig. 1a: Solid pale lines are country boundaries; solid dark lines are major river systems; dashed line is approximate –120 m contour showing extent of aerial exposure of Sunda Shelf during glacial maxima (after Voris 2000). A = Ayerawaddy River, BA = Banda Aceh, Sumatra Island; FL = Flores Island, H = Hainan Island, IK = Isthmus of Kra, J = Java Island, M = Mekong River, O = Okinawa Island, S = Salween River, T = Taiwan Island. Key for Fig. 1b: numbers (1–20) are collecting localities as listed in Table 1. The exact location of Locality 13 is unknown.

difficult to trap, although it can be locally abundant, judging from numbers of active burrows. In better surveyed parts of its range, such as in Cambodia (A. Frost, University of Queensland, unpublished), the distribution of *M. caroli* is very patchy compared with that of *M. cervicolor* Hodgson, 1845, a more ubiquitous rice field mouse of similar size and habits. Most of the material included in this study was obtained opportunistically during the course of general surveys of rice field pest rodent communities or during collection of rodents for disease studies; additional samples were obtained from rodent researchers in Indonesia, Taiwan and Japan.

The 41 individuals of *M. caroli* used in this study come from 20 localities, with sample sizes (N) ranging from one to seven per locality (Table 1, Fig. 1b). For most localities where N > 1, mice were collected on dif-

Table 1. List of samples used in this study. Figure references correspond to the localities in Fig. 1.

Country	Fig ref.	Locality (region or closest city)	Specimen code	Code	Accession no.
Japan	1	Shuri, Okinawa	HS598	Okinawa-1	AB033698[a]
			HS2766	Okinawa-2	AB109792[b]
			HS2784	Okinawa-3	AB213488
			HS2785	Okinawa-4	AB213489
			HS2786	Okinawa-5	AB213490
			HS2787	Okinawa-6	AB213491
			HS2789	Okinawa-7	AB213492
Taiwan	2	Taichung	HS1526	Taiwan-1	AB109793[b]
	3	Zhanghua	HS3144	Taiwan-2	AB213493
			HS3145	Taiwan-3	AB213494
			HS3146	Taiwan-4	AB213495
			HS3147	Taiwan-5	AB213496
			HS3148	Taiwan-6	AB213497
China	4	Haikou, Hainan	MG619	Hainan-1	AB109794[b]
	5	Sanya, Hainan	MG630	Hainan-2	AB109795[b]
	6	Menghai, Yunnan	MG600	Yunnan-1	AB109796[b]
			MG602	Yunnan-2	AB109797[b]
Laos	7	Luang Namtha Province	HS3241	Laos-1	AB213498
			HS3242	Laos-2	AB213499
			HS3243	Laos-3	AB213500
	8	Hatsua, Luang Prabang Province	HS2951	Laos-4	AB213501
	9	Houay Khot, Luang Prabang Province	CM28366/HS2958	Laos-5	AB213502
	10	Vientiane Municipality	HS2995	Laos-6	AB213503
Myanmar	11	Hmawbi, Yangon Division	HS3248	Myanmar-1	AB213504
			HS3249	Myanmar-2	AB213505
Thailand	12	Nakhon Ratchasima	R1245/HS3289	Thailand-1	AB213506
	13	Thailand	—	Thailand-2	AY057812[c]
	14	Kanchanaburi	R1755/HS3293	Thailand-3	AB253437
	15	Loei	HS3579	Thailand-4	AB253438
Cambodia	16	Samroang, Kampong Cham Province	HS2963	Cambodia-1	AB213507
			HS3001	Cambodia-2	AB213508
Vietnam	17	Tuyen Nah	HS2048	Vietnam-1[d]	—
	18	Cu Chi District, Ho Chi Minh City Province	HS2961	Vietnam-2	AB213509
			HS2996	Vietnam-3	AB213510
			HS2997	Vietnam-4	AB213511
Indonesia	19	Yogyakarta, Java	MZB24350/HS2474	Java-1	AB109798[b]
			MZB24351/HS2475	Java-2	AB109799[b]
	20	Bantul, Java	MZB24352/HS2476	Java-3	AB109800[b]
			MZB24353/HS2477	Java-4	AB109801[b]
			MZB24354/HS2478	Java-5	AB109802[b]
			MZB24355/HS2479	Java-6	AB109803[b]

[a]Suzuki et al. (2004), [b]Terashima et al. (2003), [c]Lundrigan et al. (2002), [d]Chinen et al. (unpublished).

ferent occasions and in fields separated by distances of several hundreds of meters to kilometers. Accordingly, these samples are unlikely to include close familial relatives and locality samples with low haplotype diversity are assumed to be representative of regional populations. Voucher specimens from Cambodia, Myanmar and Vietnam are lodged in the Australian National Wildlife Collection, Canberra (Table 1; CM prefix); those from Java are lodged in the Museum Zoologicum Bogoriense, Indonesia (MZB prefix); those from Thailand are held at the Research Center for Emerging Viral Diseases, Salaya, Thailand (R prefix). Other samples were not vouchered. The DNA samples, with serial number HS, are preserved in the Laboratory of Ecology and Genetics, Graduate School of Environmental Earth Science, Hokkaido University.

Cyt *b* gene sequences for fifteen previously sequenced individuals of *M. caroli* and single individuals of *M. cookii* Ryley, 1914 and *M. cervicolor* were obtained from the nucleotide databases DDBJ, EMBL and GenBank (Table 1). *Mus cookii* and *M. cervicolor* were used as outgroups for *M. caroli*; the three taxa together comprise the *M. cervicolor* Species Group of the subgenus *Mus* Linnaeus, 1766, as defined by Suzuki et al. (2004). Within this group, *M. cervicolor* and *M. cookii* probably form a sibling clade to the exclusion of *M. caroli* (Suzuki et al. 2004).

PCR amplification and DNA sequencing

We determined complete sequences for the mitochondrial cyt *b* gene (1140 bp), using previously described methods (Suzuki et al. 1997, 2000). The amplification reactions were carried out for thirty five cycles, each consisting of 30 sec at 96°C for denaturation, 30 sec at 50°C for annealing and 30 sec at 60°C for extension. The reaction mixtures (20 μl) contained 2.5 mM $MgCl_2$ (1.25 mM for second PCR). Both DNA strands of the product of the second PCR were directly sequenced using a Dye Terminator Cycle Sequencing Kit (ABI) and an automated sequencer (model 3100, ABI). The nucleotide sequences are deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession numbers: AB213488-AB253438 (Table 1).

Phylogenetic and sequence analyses

Thirty-four distinct haplotypes were recovered from the thirty-nine individuals of *M. caroli*. A total of 151 segregating sites (13%) were found and 113 of these were informative for parsimony analyses.

Phylogenetic trees based on cyt *b* sequences were constructed by using four tree building methods with contrasting optimality criteria: 1) the Neighbour-joining (NJ) method of Saitou and Nei (1987); 2) a Maximum parsimony (MP) method; 3) a Maximum likelihood (ML) method; and 4) Bayesian inference (BI). Neighbour-joining (NJ), Maximum parsimony (MP) and Maximum likelihood (ML) analyses were performed in PAUP* 4.0b10 (Swofford 2003). Bayesian Inference (BI) was implemented in MrBayes v.3.1 (Ronquist and Huelsenbeck 2003).

The best-fit model of sequence evolution in the datasets for the NJ, ML and BI analyses was chosen on the basis of hierarchical likelihood ratio tests, as implemented in PAUP* 4.0b10 and Modeltest v3.06 (Posada and Crandall 1998). We employed a four-parameter Tamura-Nei likelihood model (Tamura and Nei 1993) with correction for variation in rates by site and for invariant sites (TrN + G + I) selected by Modeltest v3.06.

For MP analyses, 100 heuristic searches were conducted with the tree-bisection reconnection (TBR) option, in which the input order of taxa is randomized. Consensus trees were constructed using the strict consensus method. ML model parameters were estimated from a heuristic search with PAUP* 4.0b10 (Swofford 2003). Bootstrap analysis was carried out 1000, 1000, and 100 in the NJ, MP, and ML analyses, respectively.

Posterior probabilities for the Bayesian Inference analysis were determined by running one cold and three heated chains for 20,000,000 generations. To ensure trees were sampled after convergence, we discarded the first 100,000 generations as burn-in. Topology and model parameters were sampled every 100th generation and assessed as the proportion of the trees sampled after burn-in in which that particular topology was observed. Posterior probabilities for nodes were based on the remaining 1,000 topologies.

We used Tamura-Nei likelihood model distances (*d*) for estimates of nucleotide diversity (π) within various geographic samples and for estimates of total and net sequence divergence (*Dxy* and *Da*) between identified clades within *M. caroli* (Nei 1987). Each statistic was computed with its standard error (*SE*) based on 1000 replicates using MEGA 3.0 (Kumar et al. 2004). Confidence intervals (95%) for estimates of net sequence divergence were calculated as ±1.96 *SE*.

Shortest air distance between each pair of collecting localities, excluding Locality 13 where the exact location

is unknown (Fig. 1b), was measured from regional maps with equal area projections. These data were used to test the null hypothesis that genetic divergence between populations is a simple product of isolation by distance. The relationship was tested by inspection of bivariate plots and by matrix comparisons using Mantel tests implemented in GENALEX 6 (Peakall and Smouse 2006). The use of air distance separations for the island populations on Taiwan and Java are justified by the presence of former land-bridge connections between each island and the mainland and the conclusion (see below) that each of these islands population has a long history of occupancy.

Estimation of divergence times between lineages

The estimation of lineage divergence times from molecular data rests on two primary assumptions, namely 1) that substitutions are accumulating at a relatively even rate across all lineages; and 2) that the rate of molecular evolution is known with some degree of accuracy. The first of these assumptions was tested for the *M. caroli* dataset using the branch-length test as implemented in LINTREE (Takezaki et al. 1995). The second assumption is usually defended by the application of a general evolutionary rate derived from calibration against a fossil record. In the case of murine rodents, the common practice is to calibrate the rate of molecular evolution by using the genetic divergence between *Mus* and *Rattus* Fischer, 1803 and assuming a divergence time of 12–10 million years ago as indicated by palaeontological evidence (see Michaux et al. 2002 and references therein; also Suzuki et al. 2003). However, the validity of this practice is challenged by mounting evidence that the rate of molecular evolution is time dependent even within a single gene lineage, perhaps due to a combination of purifying selection and the presence of mutational hotspots (Howell et al. 1996; Lambert et al. 2002; Ho et al. 2005). Consequently, it may be invalid to extrapolate molecular rates of change across different evolutionary timescales and estimates of recent divergence times based on long-term values (such as that derived from the *Mus-Rattus* divergence) may seriously overestimate the true divergence times. In the absence of any independent estimate of the short term rate of molecular change in *Mus*, we used the conventional long-term evolutionary rate for all substitutions in the cyt *b* gene (0.024 substitutions per lineage per million years; Suzuki et al. 2003) but regard the estimates as maximum divergence times for the primary mtDNA lineages within *M. caroli*.

Results

Thirty-four distinct haplotypes were recovered from the thirty-nine individuals of *M. caroli*. A total of 151 segregating sites (13%) were found and 113 of these were informative for parsimony analyses. The phylogenetic analysis produced essentially identical topologies under NJ, MP, ML and BI methods. The NJ tree is illustrated in Fig. 2, annotated with bootstrap values for NJ, MP and ML methods and with posterior probabilities for BI; the three strict consensus trees featured all of the same illustrated clades.

The trees show strong phylogeographic structure. A total of eleven well-supported terminal clades (i.e. with PP > 0.95 and BS values generally >90%; labeled A to K on Fig. 2) are identified. In all but two cases, discrete geographic neighborhoods yielded representatives of single haplotype groups, as follows: Okinawa Island (A); Thailand (C); central Cambodia (E); Java Island (F); southern China and northern Laos (G); Hainan Island (H); Myanmar (J); and Taiwan (K). The two exceptions are the Mekong Delta region of southern Vietnam (D and I) and southern Laos (B and G). Conversely, only one of the haplotype groups (G) was detected in more than one geographic region.

Bayesian posterior probabilities (PP) of >0.9 were obtained for two larger clades (identified as Lineages I-II on Fig. 2). Lineage I includes haplotype groups A + B + C + D + E + F (PP = 0.96); and Lineage II includes groups G + H + I (PP = 0.91). Bootstrap support is relatively low for each of these lineages but the fact that they also represent different geographic neighbourhoods lends credence to the groupings. Leaving aside the remote Okinawan population (A), Lineage I is effectively confined to lower elevation regions in the southern part of mainland Southeast Asia, while Lineage II is largely confined to northern regions, including the elevated plateaux of northern Laos and China.

Three populations from peripheral parts of the range of *M. caroli* appear to be more isolated phyletically. Haplotype group F from Java Island shows a weak association with Lineage I (PP = 0.59), whereas haplotype groups J from Myanmar and K from Taiwan show no preferential association with any other group. Including these isolated groups, we thus distinguish a total of five principal mtDNA lineages within *M. caroli*, each of which has a discrete geographic focus, as follows: I (southern mainland), II (northern mainland), III (Myanmar) and IV (Taiwan).

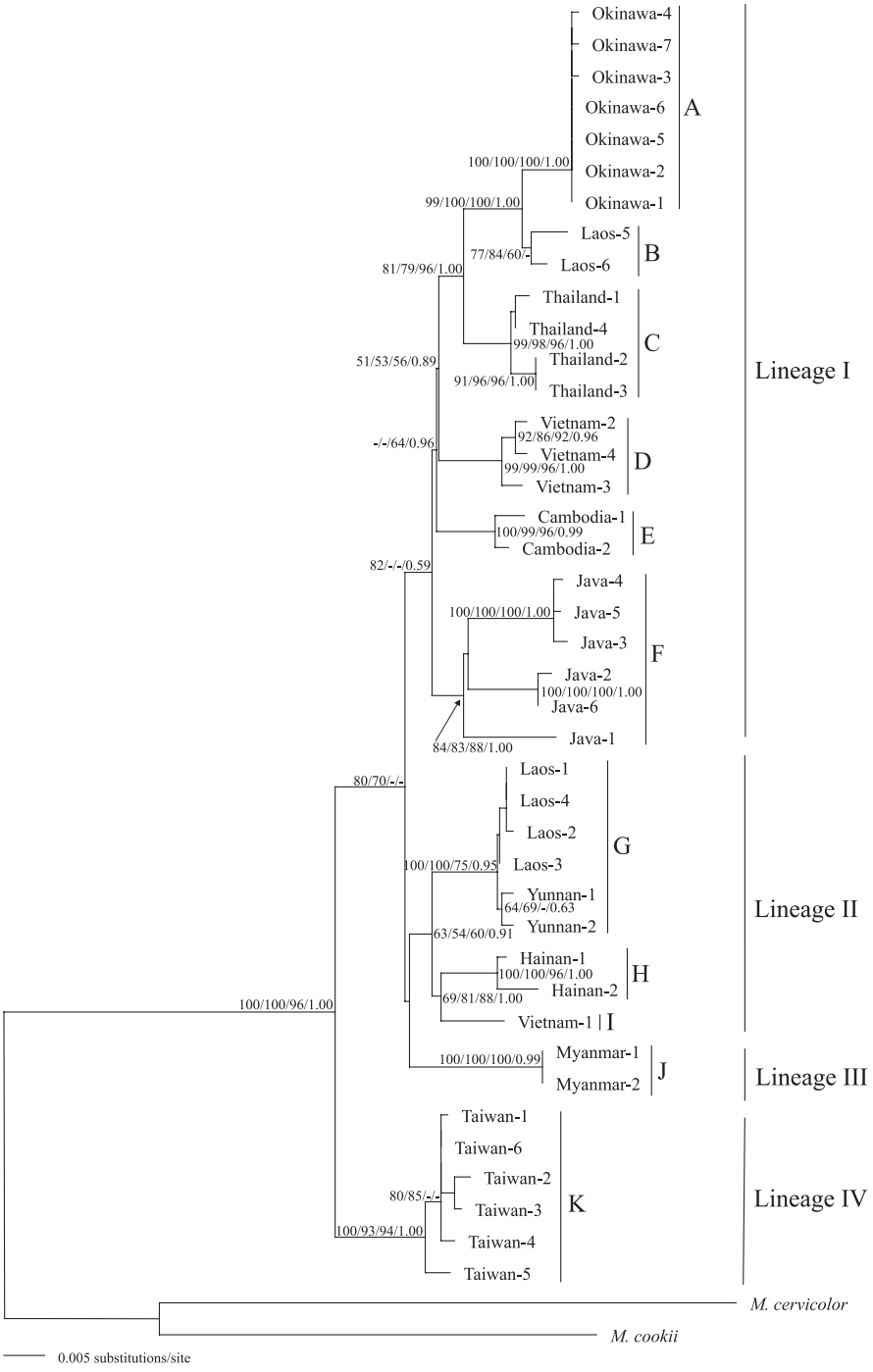

Fig. 2. Neighbour-joining (NJ) tree constructed for cytochrome *b* gene sequences of *Mus caroli*, using *M. cervicolor* and *M. cookii* as outgroup taxa to root the tree. Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian inference (BI) analyses produced an essentially identical topology. Bootstrap analysis was carried out 1000, 1000, and 100 in the NJ, MP, and ML analyses, respectively. Bootstrap values and posterior probabilities for the BI analysis are shown at each node, where values exceed 50% for the NJ, MP and ML and 0.5 for the BI (NJ/MP/ML/BI).

Nucleotide diversity was 2.85% across the entire species and ranged from 0.00 to 2.31% within each of the four lineages (Table 2). Total (raw) divergences (*Dxy*) between the lineages were estimated at 2.76–4.06%, while net divergences (*Da*) ranged from 0.95% to 3.83% (Table 2). Nucleotide diversities (π) differed markedly between the three better-sampled insular populations. The seven individuals from Okinawa shared a near iden-

Table 2. Diversity estimates for the four major cytochrome *b* lineages in *Mus caroli*. Numbers along the diagonal are nucleotide diversities, π (in bold). Below the diagonal is the total, raw DNA divergence (*Dxy*) and above the diagonal is the net divergence (*Da*). Standard errors based on 1000 bootstrap replicates are given in parentheses. All estimates are expressed as percentages.

Lineage	Representative localities	n	I	II	III	IV
I	Okinawa, Java	24	**2.31 (0.28)**	0.95 (0.18)	2.10 (0.41)	2.70 (0.45)
II	Yunnan, Hainan	9	2.76 (0.32)	**1.31 (0.22)**	2.15 (0.43)	2.67 (0.47)
III	Myanmar	2	3.25 (0.47)	2.81 (0.58)	**0.00 (0.00)**	3.83 (0.60)
IV	Taiwan	6	4.06 (0.50)	3.54 (0.48)	4.00 (0.32)	**0.42 (0.12)**

Note: All estimates were calculated with Tamura-Nei model.

Table 3. Mantel tests for correlations between the genetic distances and geographical distances of all pairs of individuals in populations. Each name of populations and lineages is identical to the name used in Fig. 2.

Population (n)	*r*	*P*-value
All individuals from all lineages (40)	0.417	<0.001
Excluding Okinawa (A) population (33)	0.606	<0.001
All individuals from Lineage I (23)	0.739	<0.001
Excluding Okinawa (A) population (16)	0.755	<0.001
All individuals from Lineage II (9)	0.888	0.007
Excluding Vietnam-1 (I) (8)	0.959	0.015

tical sequence (π = 0.08 ± 0.04%), whereas the six individuals from Java showed remarkably diverse sequences (π = 1.65 ± 0.26%). The six individuals from Taiwan showed an intermediate level of sequence diversity (π = 0.42 ± 0.12%).

Mantel tests revealed a positive correlation between genetic and geographic distances (Table 3), both for the entire taxon sample and for each of Lineages I and II. However, the correlation coefficients are considerably higher for each of Lineage I ($r = 0.739$, $P < 0.001$) and Lineage II ($r = 0.888$, $P = 0.007$) than for the entire sample ($r = 0.417$, $P < 0.001$). Excluding the Okinawa population (suspected of being a long-range introduction —see below) further increased the correlation for the entire taxon sample and for Lineage I. Similarly, exluding one Vietnamese individual suspected of being translocated (Vietnam-1) produced a more robust correlation ($r = 0.959$) for Lineage II, although the null hypothesis was not rejected at the 0.01 level of significance ($P = 0.015$). Overall, the results indicate that isolation by distance accounts from much of the diversity within the individual lineages, but is less effective an explanation for the total pattern of genetic diversity. This latter point is given further emphasis by the fact that several geographically proximate populations are strongly divergent genetically (e.g. southern and northern Laos, western Thailand and Myanmar).

The branch length test as implemented in LINTREE indicated that cyt *b* has evolved at a relatively constant rate in all lineages ($P < 0.01$). This issue was further explored using a relative rate test (two-cluster test) as implemented in PHYLTEST (Kumar 1996); this test also failed to reject the null hypothesis of rate constancy at a 5% significance level.

Estimates of divergence times between each of the five primary mtDNA lineages ranged from 0.58–0.83 million years ago (mya) based on *Dxy* and 0.21–0.52 mya based on *Da* (Table 4). As noted earlier, these values probably overestimate the true lineage divergence times, albeit to an unknown degree. Based on these values and considerations, it thus seems likely that all of the contemporary mtDNA diversity within *M. caroli* has developed with the time frame of the Middle to Late Pleistocene (Webb and Bartlein 1992).

Table 4. Estimates of divergence times (mya, million years ago) between each of the four primary lineages based on the total raw DNA divergence (*Dxy*) and the net divergence (*Da*). Standard errors (*SE*) are shown in parentheses.

Lineage	*Dxy* (*SE*)	mya	*Da* (*SE*)	mya
Lineage IV vs. Lineage I, II & III	0.040 (0.0047)	0.83	0.025 (0.0043)	0.52
Lineage III vs. Lineage I & II	0.032 (0.0044)	0.66	0.019 (0.0039)	0.40
Lineage II vs. Lineage I	0.028 (0.0032)	0.58	0.010 (0.0018)	0.21

Note: All estimates were calculated with Tamura-Nei model.

Discussion

Our study considerably extends the previous account of mtDNA diversity in *M. caroli* by Terashima et al. (2003). In particular, our analysis has benefited from the inclusion of much new material from Laos, Thailand and Cambodia, and from the discovery and sampling of the genetically distinct population in Myanmar. Even so, the present dataset supports only tentative conclusions regarding the biogeographic history of *M. caroli*, due to two main limiting factors. The first of these is the geographically sparse sampling and small population samples, reflecting both the relative difficulty of capturing *M. caroli* and its huge geographic range. Indeed, the fact that 34 discrete haplotypes were obtained from 39 mice suggests that a significant proportion of the total haplotype diversity within *M. caroli* remains undiscovered. The second stems from our reliance on mtDNA which presents serious limitations, both for phylogenetic inference and for demographic analyses (Ballard and Whitlock 2004). Despite these limitations, we suggest that the mtDNA results show sufficiently strong phylogeographic structure to support speculation on a number of points of taxconomic and bioeographic interest.

Taxonomic issues

Our expanded mtDNA dataset confirms the essential unity of *M. caroli* as delimited by Musser and Carleton (2005). The type locality of *M. caroli* Bonhote is Okinawajima in the Ryukyu Archipelago and our results confirm the close relationship between this population and those referred to this species by Marshall (1977), Corbet and Hill (1992) and others from mainland Southeast Asia. It also confirms the presence of this taxon on each of Taiwan and Java islands, and thus the referral to *M. caroli* of *Mus formosanus* Kuroda, 1925 (type locality: Taihoku, Taiwan) and of *Mus musculus ouwensi* Kloss, 1921 (type locality: Probolinggo, Java). However, we note that Corbet and Hill (1992) questioned the allocation of *ouwensi* to *caroli* and we encourage re-examination of the type material.

Further consideration is also required for two names synonymised under *M. caroli* by Kaneko and Maeda (2002) and subsequently by Musser and Carleton (2005). These are *Mus boninensis* Kuroda, 1930 from the Bonin or Ogasawara Islands (southeast of Honshu) and *Mus kurilensis* Kuroda, 1924 from Shimoshire (Simashur) in the Central Kuril Islands, northeast of Hokkaido. In each case, these localities would represent significant range extensions for *M. caroli* and we anticipate that both names are based on examples of *M. musculus*.

The question of whether subspecies might be meaningfully delimited within *M. caroli* cannot be decided from the mtDNA evidence alone. However, we note that the level of divergence among the five identified mtDNA lineages within *M. caroli* is approximately equal to that reported among the subspecies of *M. musculus* (Terashima et al. 2006). When this fact is combined with the observation that each of the identified mtDNA lineages within *M. caroli* occupies largely or wholly discrete geographic ranges, and with the mounting evidence for regional differentiation in both external proportions and cranial morphometrics (Macholán 2001; Motokawa et al. 2003), we anticipate that subspecies will one day be recognised. However, identification of appropriate units will require incorporation of evidence from nuclear genes, as well as from morphology.

Biogeography of island populations

Our results substantially clarify the status of three insular populations of *M. caroli*. The population on Okinawajima in the Ryukyu Archipelago is important for several reasons. As indicated above, it is the type locality of *M. caroli* and it is also the most northerly and isolated population of the species. Terashima et al. (2003) were uncertain as to the status of this population which they found to be highly divergent in its mtDNA from all mainland populations. Our broader sampling demonstrates a close relationship between the Okinawan haplotype group and populations of Lineage I on mainland Southeast Asia, and a more distant relationship to the geographically proximate Taiwanese Lineage IV. Within Lineage I, the Okinawan haplotype group is closest to haplotype group B from localities in Vientiane and Luang Prabang Province of Laos. These new findings convince us that the Okinawan population of *M. caroli* was introduced from a source on mainland Southeast Asia but the lack of an exact haplotype match means that we are still unable to pinpoint a precise place of origin.

Studies of other Okinawan mammals suggest that some exotic species arrived during prehistoric times (i.e. a few thousands of years ago), while others arrived historically (i.e. within the last few hundreds of years). The white-toothed shrews (Motokawa et al. 2000) and Polynesian rats (*Rattus exulans* Peale, 1848; Motokawa et al. 2001), both found in the Central Ryukyu Islands, are thought to be historical introductions. In contrast, house mice (*M. musculus*) are usually regarded as a pre-

historic introduction to the Ryukyu and Japanese Islands, with their arrival linked to episodes of prehistoric human migration (Moriwaki et al. 1994). The presence of some haplotype diversity within the Okinawan population of *M. caroli* favours a more ancient arrival but this requires confirmation through archaeological discovery.

The Taiwanese population yielded six different haplotypes that together comprise one of the primary mtDNA lineages within *M. caroli*. This finding confirms the status of *M. caroli* as a member of the original fauna of Taiwan and invites new questions regarding the historical circumstances behind isolation of the Taiwanese population. Geological evidence dates the initial emergence of Taiwan from below sea level to the period 4–5 mya and the attainment of its current configuration and topography around 2 mya (Hsu 1990). Despite its isolation, Taiwan is a continental island with water depths across the Strait of Taiwan mostly less than 100 m deep. Intermittent land bridge connection with the adjacent Chinese mainland thus existed through the late Pliocene and Quaternary periods, most recently during the Last Glacial Maximum (LGM) between 30–16,000 BP (Lambeck and Chappell 2001). This cyclic pattern of interchange and local differentiation is reflected in the complex nature of the Taiwanese biota. For example, among lacertid lizards of the genus *Takydromus* Daudin, 1802, four different invading lineages are identified, with estimated dispersal times ranging from 1.0–0.24 mya (Lin et al. 2002). For *M. caroli*, the dispersal event probably occurred within the interval 0.83–0.21 mya, the maximum age being the estimated time of divergence of the Taiwanese haplotype lineage from all mainland lineages, and the minimum age being the estimated time of divergence among the various endemic Taiwanese haplotypes.

Three islands in Indonesia support populations of *M. caroli*—Sumatra, Java and Flores. Musser and Newcomb (1983) regarded all of these as likely historical introductions but Terashima et al. (2003) found several unique haplotypes in the Javan population and concluded that it was probably indigenous. Our broader geographic sampling confirms the isolated status of the Javan haplotypes but also suggests a possible link between the Javan haplotype group and populations of Lineage I, a group that has its primary distribution in the lower Mekong Basin of mainland Southeast Asia.

A phyletic connection between Javan and Lower Mekong Basin populations of *M. caroli* is consistent with the known palaeogeography and environmental history of this region. Java is located on the southeastern margin of the Sunda Shelf, a broad continental platform that connects the Thai-Malay Peninsula and the southern margin of Indochina with the major islands of Sumatra, Borneo and Java (Verstappen 1975; Tjia 1980). Through the Quaternary period, this platform was periodically exposed to create broad land bridges between many or all of the major islands, including Java (Voris, 2000). The nature of vegetation cover on the exposed shelf is debated but several authors have postulated a glacial 'dry corridor' running behind the rain shadow of the Thai-Malay, Sumatran and Javan 'ranges' (Morley and Flenley 1987; Heaney 1991), while others have posited even more widespread deforestation (Brandon-Jones 1996). Palynological studies confirm the presence of open communities including grasslands on the exposed shelf (Caratini and Tissot 1999; Kershaw et al. 2001; Hope et al. 2004) and the contraction of closed forests, even in the most humid areas (Stuijts 1993; van der Kaars and Dam 1995; Taylor et al. 2001; van der Kaars et al. 2001). Meijaard (2003) recently attempted to delimit the location of open communities by examining the fauna of small satellite islands to deduce the vegetation type present on the adjacent major landmass just prior to post-glacial sea level rise. Interestingly enough, his analysis identifies two likely regions of open vegetation in the vicinity of Java, one in the probable rain-shadow to the northeast, and the other in the low-lying area between the ranges of South Sumatra and West Java. Other areas of open vegetation are indicated around Borneo and on the east side of the Thai-Malay 'range'.

The distinctiveness and diversity of the Javan haplotype group marks *M. caroli* as a long-term component of the Sundaic fauna. However, the same genetic attributes suggest that Sundaic populations of *M. caroli* were highly discontinuous, even under optimum conditions, thereby promoting interpopulational divergence through genetic drift or local selection. In the case of Java, high local diversity of haplotypes might be due to a large population size and long term stability. However, an alternative scenario is that the Javan populations were formerly fragmented but that forest removal to create lowland rice fields has created new opportunities for gene flow across the island. It would be of great interest to now examine the mtDNA of *M. caroli* populations on Sumatra and Penang, Malaysia (Langham and Lam 1977), both on the 'Sunda Shelf', and the one on Flores, which lies off the shelf. We predict that the two former populations are relictual and will yield new haplotype

lineages, while that on Flores is a product of human translocation and will prove to be genetically identical or very close to one of the Javan lineages.

A final dimension to the Javan *M. caroli* story is provided by a fossil *Mus* close in morphology to *M. caroli* from the Early Pleistocene Kali Glagah Formation of Central Java (van der Meulen and Musser 1999). Dentally, *M. caroli* is one of the more distinctive members of this genus (Aplin, unpublished data) and the presence of this morphology on Java at such an early time raises the possibility that *M. caroli* is of Sundaic origin. At any rate, it suggests that the species has a longer history than would be inferred from the genetic structure of its extant populations. This is consistent with the long basal branch that separates *M. caroli* from each of its closest living relatives, *M. cookii* and *M. cervicolor*, with the interspecific time of divergence estimated from nuclear gene sequences at 1.4–2.4 mya (Suzuki et al. 2004)

Biogeography of the mainland populations

The three mtDNA lineages were identified on mainland Southeast Asia occupy largely discrete geographic areas, with distributions divided between Myanmar in the west, the lower catchment of the Mekong River in the south, and the upland plateau of Laos and southern China, together with Hainan Island, in the north. As reported here, LINTREE estimates suggest that these lineages diverged during the Middle Pleistocene. This is much younger than the age of the various major topographic features that appear to subdivide the current mtDNA diversity within *M. caroli* on mainland Southeast Asia. These features, which include the elevated Shan Plateau of eastern Myanmar, the Bilauktaung Range that runs down the narrow neck of the Thai-Malay Peninsula as far as the Isthmus of Kra (Woodruff 2003), and the eastern foothills of the Tibetan plateau in northern Laos and southern China, were all emplaced well prior to the start of the Quaternary period (Hutchinson 1989; Brookfield 1998; Woodruff 2003). Accordingly, any biogeographic explanation of lineage differentiation within mainland *M. caroli* must be framed in terms of essentially modern topography.

The Myanmar population may be geographically isolated from those to the east and north. Known localities for *M. caroli* in Myanmar cluster around two regions of lowland rice field habitat, one at Hmawbi in Yangon Division and the other near Mawlamyine in Mon State, and the species was not found in agricultural habitats on the Shan Plateau. We suspect that *M. caroli* is restricted in Myanmar to the lower central basin, an area of low basal elevation (<100 m above seal level) and limited topographic relief that is enclosed on both sides by north-south trending ranges. On the eastern side, the probable barriers are the Shan Plateau and the Bilauktaung Range. We further suspect that one or more chance crossings of this barrier during the Middle Pleistocene led to the establishment of the Myanmar population and that a subsequent cessation of gene flow or its low frequency ensured the persistence of this distinct mtDNA lineage.

The 'southern' Lineage I is considerably more widespread on the mainland, with populations spread throughout the lower catchment of the Mekong River. This huge drainage basin includes parts of Laos, most of the eastern half of Thailand, all of Cambodia, and the southeastern corner of Vietnam. Evidence for the long-term presence of *M. caroli* in this areas comes from the Khao Samngam fossil locality in Thailand which Chaimanee (1998) assigned to the Early Pleistocene due to the presence of several archaic murines.

Mus caroli is closely associated with rice-field habitats throughout the lower Mekong catchment (Aplin et al. 2004). For this reason, Aplin and Singleton (2003) included it within a 'rice paddy' rodent guild that they saw as benefiting from the spread of rice cultivation in Southeast Asia during prehistoric to contemporary times. Under this model, *M. caroli* might be expected to show low levels of genetic diversity over large areas of the lower Mekong Basin, with a confused admixture of haplotype lineages. As reported here, the reality is strikingly different. Populations of *M. caroli* in the Mekong Basin actually show locally diverse and regionally differentiated haplotypes. Indeed, the level of genetic differentiation between regional populations (*Da* 0.014–0.024) suggests divergence times of tens or even hundreds of thousands of years, far beyond the time range for agricultural activity or any recorded anthropogenic disruption of forest cover (Penny 2001; Hope et al. 2004). The presence of such deep genetic structuring suggests that the original distribution of *M. caroli* in these areas was extensive but fragmented—perhaps linked to a particular habitat type such as semi-permanent wetlands or natural grassland communities. Following widespread forest clearance for wetland agriculture, numerous local populations of *M. caroli* may have spread out to colonize the newly created landscape, thereby preserving much of the original genetic structure.

Most populations of the 'northern' Lineage II come from upland regions where elevation is >300 m above

sea level. However, within these regions, populations of *M. caroli* are typically restricted to areas of low local relief, e.g. in narrow, valley floor or swampy habitats on elevated plateaux. The specific habitat of *M. caroli* on Hainan Island, where both upland and lowland habitats are found, is not reported. Despite the inherent patchiness of such habitats, we note that populations of this lineage from widely separated localities (e.g. Luang Prabang in Laos and Kunming in China) can be closely related. Further sampling is needed to test the generality of this observation. At any rate, the Hainan population is more distinct.

The causal basis of the separation into 'southern' and 'northern' lineages is not immediately obvious. No major barrier separates these populations and there is no obvious ecological or physiological difference between them. One possible explanation is that the pattern reflects two distinct phases in the history of *M. caroli*, perhaps under contrasting climatic regimes. The first phase would involve range expansion through long distance dispersal, leading to a distribution similar to that observed at the present time but perhaps extending further north on the mainland to the latitude of Taiwan. The second phase would involve a contraction of range into local refugia perhaps involving an increased dependence on wetland or grassland habitats as alluded to above. If this second phase were maintained over a sufficiently long period with the likelihood of gene flow determined largely by geographic isolation, the expected outcome would be a fragmented genetic landscape combined with a weak pattern of isolation by distance. Under this model, the upland-lowland division might simply be coincidental rather than causal. At any rate, further speculation is unwarranted in the absence of a nuclear gene dataset to complement the mtDNA presented here.

Geographic overlap between the 'southern' and 'northern'; lineages was detected in two widely separated areas—at an upland locality in mid-northern Laos and a lowland locality in southern Vietnam. In both cases, we suspect that this overlap is the product of recent road transport of mice between formerly more isolated regions.

Phylogeographic structure of M. caroli in the context of Southeast Asian mammal biogeography

Biogeographic studies of the Southeast Asian region remain in their infancy compared with most other parts of the world. This is particularly so for mainland Southeast Asia, which has attracted much less interest than the classic biogeographic arenas of the Indonesian and Philippine archipelagos (Heaney 1986; Steppan et al. 2003; Heaney et al. 2005; Jansa et al. 2006).

In one of the earliest attempts to identify zoogeographical 'realms', Wallace (1876) distinguished between the Indo-Chinese and Indo-Malayan realms, with the boundary drawn at the northern root of the Malayan Peninsula. Later adaptations, such as that of Lekagul and McNeely (1977) distinguished the same regions but moved the boundary south to the Isthmus of Kra; their Indochinese sub-region extended to southern China and Taiwan in the north and east and to the central basin of Myanmar in the west. Although this primary division was maintained by Corbet and Hill (1992), they further subdivided each of the two sub-regions into six 'divisions'. Within the Indochinese sub-region, their schema distinguishes the Taiwanese mammal fauna from that of the adjacent Chinese mainland, and separates a southern Chinese Division from an Indochinese Division.

Since the mainland range of *M. caroli* falls entirely within the Indochinese Division of Corbet and Hill (1992), their classification is insensitive to phylogeographic structure within this species. However, a survey of geographic ranges within other genera of terrestrial mammals reveals numerous similarities with the distributions of major lineages within *M. caroli*, thereby raising the possibility of a commonality of causal factors. To cite but a few examples: the shrew *Crocidura horsfieldi* (Tomes, 1856) shows a comparable range disjunction between southern Chinese and Taiwanese populations; among the Leaf Monkeys, *Semnopithecus cristatus* (Raffles, 1821) has a distribution very like that of the 'southern' lineage of *M. caroli*, while *S. phrayei* (Blyth, 1847) covers the combined ranges of the 'northern' and Myanmar lineages of *M. caroli*; the sciurid squirrel *Callosciurus finlaysoni* (Horsfield, 1823) is endemic to the lower Mekong Basin area but with an isolated and distinctive population in the central basin of Myanmar [*Callosciurus f. ferrugineus* (Cuvier, 1829)]; and lastly, the murid rodent *Rattus argentiventer* Robinson and Kloss, 1916 is present in the lower Mekong Basin through to the Sundaic islands (also in northern Vietnam, but probably as a recent introduction; Suzuki and Aplin, unpublished). The phylogeographic structure of each of these taxa could be usefully compared with that of *M. caroli* in the search for more general patterns and causes.

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