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A multi-locus phylogeny of Nectogalini shrews and influences of the paleoclimate on speciation and evolution

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ABSTRACT

Nectogaline shrews are a major component of the small mammalian fauna of Europe and Asia, and are notable for their diverse ecology, including utilization of aquatic habitats. So far, molecular phylogenetic analyses including nectogaline species have been unable to infer a well-resolved, well-supported phylogeny, thus limiting the power of comparative evolutionary and ecological analyses of the group. Here, we employ Bayesian phylogenetic analyses of eight mitochondrial and three nuclear genes to infer the phylogenetic relationships of nectogaline shrews. We subsequently use this phylogeny to assess the genetic diversity within the genus *Episoriculus*, and determine whether adaptation to aquatic habitats evolved independently multiple times. Moreover, we both analyze the fossil record and employ Bayesian relaxed clock divergence dating analyses of DNA to assess the impact of historical global climate change on the biogeography of Nectogalini. We infer strong support for the polyphyly of the genus *Episoriculus*. We also find strong evidence that the ability to heavily utilize aquatic habitats evolved independently in both *Neomys* and *Chimarrogale* + *Nectogale* lineages. Our Bayesian molecular divergence analysis suggests that the early history of Nectogalini is characterized by a rapid radiation at the Miocene/Pliocene boundary, thus potentially explaining the lack of resolution at the base of the tree. Finally, we find evidence that nectogalines once inhabited northern latitudes, but the global cooling and desiccating events at the Miocene/Pliocene and Pliocene/Pleistocene boundaries and Pleistocene glaciation resulted in the migration of most Nectogalini lineages to their present day southern distribution.

1. Introduction

In terms of species diversity, shrews (Soricidae) constitute the fourth largest mammalian family (376 species; Wilson and Reeder, 2005), and are among the most successful clades of extant mammals. They are widely distributed in Europe, Asia, Africa, and from North America to northern South America, and adapted to varied habitats from tropical forest to arctic tundra, and from marshy or semi-aquatic regions to arid areas (Nowak, 1999). Shrews have evolved distinct behavioral and morphological adaptations to these ecologically diverse conditions by utilizing one of six feeding and foraging categories: terrestrial, semi-aquatic, semifossorial, scansorial psammophilic or anthropophilic (Hutterer, 1985). Previous phylogenetic and taxonomic research has divided shrews into three subfamilies (Soricinae, Crocidurinae and Myosoricinae; Hutterer, 2005) whose phylogenetic interrelationships are well-resolved (Dubey et al., 2007; Ohdachi et al., 2006). Of the three subfamilies, Soricinae is notable for its remarkable morphological diversity in contemporaneous genera (Repenning, 1967); this is especially evident in the six genera and 23 described species that comprise the tribe Nectogalini.

Nectogaline shrews are remarkable in that they possess morphological adaptations to utilize four of the six soricid foraging and feeding categories (Hutterer, 1985): *Chimarrogale*, *Nectogale*, and *Neomys* are semi-aquatic; *Soriculus nigrescens* is semifossorial; *Chodsigoa sodalis* and *Episoriculus macrurus* are scansorial; and all other species are terrestrial. The tribe's diversity is even more impressive in light of its relatively young age (several million years; Dubey et al., 2007; Reumer, 1998). Therefore, Nectogalini is a promising model for research into mammalian morphological, anatomical and physiological evolution and ecological adaptation. Unfortunately, progress into this research is hampered by the lack of a robust phylogenetic framework.

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Ohdachi et al.'s (2006) phylogenetic analysis of the family Soricidae using the mitochondrial cytochrome *b* (cyt-*b*) gene included 13 species representing all six nectogaline genera. The results supported the monophyly of Nectogalini, but a majority of the relationships among the genera were not well-resolved (Fig. 1a). A subsequent analysis by Dubey et al. (2007) included two mitochondrial genes and two nuclear genes, and represented eight species of four nectogaline genera. This study was able to determine two inter-generic relationships with statistical support: *Neomys* was the sister lineage to the remaining three sampled genera, and *Episoriculus fumidus* and *Chodsigoa* form a well-supported clade exclusive of other genera. However, the sampling of nectogaline taxa was small (eight species; Fig. 1b). Thus, the evolutionary history of this clade remains somewhat ambiguous.

The ambiguous and even conflicting phylogenetic relationships inferred by these previous studies underscore the need for more extensive analysis of the evolutionary relationships of nectogaline shrews. In order to better resolve nectogaline phylogeny, we conducted analyses of a large DNA data set, including eight mitochondrial and three nuclear loci, and extensive taxon sampling. More specifically, we evaluated three major evolutionary questions. (1) What are the phylogenetic relationships among the six genera of Nectogalini? (2) Is the genus *Episoriculus* monophyletic? (3) Do the three genera of water shrews (*Chimarrogale*, *Nectogale* and *Neomys*) form a clade? The last question is particularly interesting because non-monophyly would suggest that their semi-aquatic ("aquatic" hereafter) lifestyle, already relatively rare within shrews (present in only two other North American shrew species, *Sorex bendirii* and *S. palustris*), evolved independently.

Furthermore, we used Bayesian relaxed molecular clock phylogenetic methods (Drummond et al., 2006; Drummond and Rambaut, 2007) and paleoclimatic and fossil data to analyze the correlation between evolutionary history of Asian Nectogalini and the paleoclimate. Reumer (1989) concluded that temperature and humidity are the two most crucial factors influencing shrew distribution, abundance and/or diversity. Although *Neomys* species are widely distributed in the Palearctic region, and *Chimarrogale* species are widely distributed in East and Southeast Asia (including Taiwan, Japan and Indonesia), their distribution is centered in the cool and humid highland and mountains in East Himalaya–Hengduan Mountains regions for Asian groups (Hutterer, 2005). Available evidence suggests that the evolutionary history of Soricidae in Europe is greatly influenced by climate change; cooling and desiccating events caused shrews to retreat into more southern latitudes, whereas warming events were responsible for the fast speciation (Reumer, 1984, 1989). Three specific paleoclimatic events played important roles in the evolutionary history of shrews in Europe: the cooling and desiccating events around the Miocene/Pliocene (M/P) boundary and the Pliocene/Pleistocene (P/P) boundary, as well as the Pleistocene glaciation and subsequent warming in Holocene (Cosson et al., 2005; Dubey et al., 2006; Reumer, 1989; Vogel et al., 2003). Thus, we also address whether the distribution and evolution of nectogaline shrews, especially the Asian groups, were similarly influenced by past climate change.

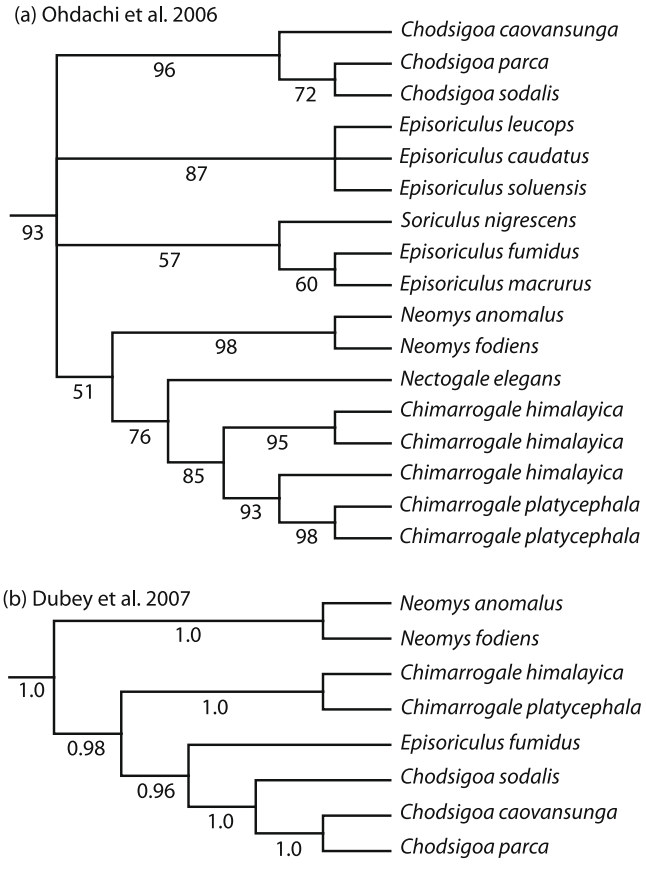

Fig. 1. Maximum likelihood tree of tribe Nectogalini based on (a) 1140 bp mitochondrial cyt-*b* gene sequences (Ohdachi et al., 2006) and (b) 3314 bp nuclear and mitochondrial gene sequences (Dubey et al., 2007). Whole numbers represent bootstrap proportions and decimal numbers represent Bayesian posterior probabilities. Clades with less than 50% or 0.50 clade support are collapsed. For clarity, the Dubey et al. (2007) tree was pruned to include only one representative per species.

2. Materials and methods

2.1. Taxon sampling

Our taxon sampling included 46 samples including representatives of *Crocidura* (Crocidurinae), *Anourosorex* (Soricinae), *Blarinella* (Soricinae) and *Sorex* (Soricinae) as outgroups. We sampled all six described nectogaline genera including one (of six described) species of *Chimarrogale*, three (of eight) *Chodsigoa*, one (of three) *Neomys*, all four *Episoriculus* species and the two monotypic genera *Nectogale* and *Soriculus*. Additional sample information is provided in the Table 1.

2.2. DNA extraction, PCR, cloning and sequencing

All samples were frozen in ethanol at −70° before DNA extraction. Whole genomic DNA was extracted by the phenol/proteinase K/sodium dodecyl sulphate method (Sambrook et al., 1989) or using the DNeasy Tissue kit (Qiagen) from either liver or muscle tissues. Three nuclear (ApoB [615 bp], BRCA1 [792 bp], and RAG2 [675 bp]) and eight mitochondrial (12S rRNA [972 bp], 16S rRNA [1575 bp], cyt-*b* [1140 bp], ND2 [1041 bp], and partial COI [591 bp], ND4 [627 bp], ND5 [1146 bp], and ATP6 [603 bp]) gene regions were amplified with rtaq DNA Polymerase (Takara, Dalian, China) using primers provided in Table 2.

PCR conditions were variable using different primers and different taxa. Annealing temperature varied from 47 to 60 °C and PCR cycles from 29 to 35 cycles. All PCR products were purified using UNIQ-10 spin column DNA gel extraction kit (Shengong, Shanghai, China). Most purified products were directly sequenced, but a few products that could not be sequenced easily were cloned into a T–A cloning site of pMD19-T vector (TaKaRa, Dalian, China), and sequenced with BcaBESTTM sequencing primers RV-M and M13–47 primers. Sequencing was conducted using the BigDye Terminator Cycle kit v3.1 on an ABI 3730xl sequencer.

Table 1
Samples and sequences used in this study.

Genus	Species	Collection code	Collecting site	*abbreviation*	cytb	coI	atp6	nd2	nd4	nd5	12s	16s	Brca1	ApoB	Rag2
Anourosorex	*Squamipes*	18959	China, Yunnan	*Ansquam1*	GU981256	GU981210	GU981135	GU981302	GU981348	GU981394	GU981014	GU981066			
Anourosorex	*Squamipes*	16164	China, Yunnan	*Ansquam7*									GU981181	GU981106	GU981440
Anourosorex	*yamashinai*	astw.1	China, Taiwan	*Anyamas0*									DQ630266*	DQ630185*	
Anourosorex	*Yamashinai*	THUB-S-00008	China, Yunnan	*Anyamas2*	GU981257	GU981211	GU981136	GU981303	GU981349	GU981395	GU981015	GU981060	GU981182	GU981107	
Blarina	*brevicauda*	BLB.I	USA, Michigan	*Blabrev2*									DQ630276*	DQ630196*	
Blarinella	*griselda*	BLG	Vietnam, Ha Tinh	*Blagris0*									DQ630268*	DQ630187*	
Blarinella	*griselda*	19677	China, Yunnan	*Blagris1*	GU981258	GU981212	GU981137	GU981304	GU981350	GU981396	GU981016	GU981067	GU981183	GU981108	GU981441
Blarinella	*griselda*	19702	China, Yunnan	*Blagris2*	GU981259	GU981213	GU981138	GU981305	GU981351	GU981397	GU981017	GU981068	GU981184	GU981109	GU981442
Chodsigoa	*hypsibia*	16076	China, Shaanxi	*Chhypsi1*	GU981260	GU981214	GU981139	GU981306	GU981352	GU981398	GU981018	GU981069	GU981185	GU981111	GU981443
Chodsigoa	*hypsibia*	16054	China, Shaanxi	*Chhypsi2*	GU981261	GU981215	GU981140	GU981307	GU981353	GU981399	GU981019	GU981070			
Chodsigoa	*hypsibia*	16077	China, Shaanxi	*Chhypsi3*	GU981262	GU981216	GU981141	GU981308	GU981354	GU981400	GU981020	GU981071	GU981186	GU981111	GU981444
Chimarrogale	*himalayica*	18962	China, Yunnan	*Chimhim1*	GU981263	GU981217	GU981142	GU981309	GU981355	GU981401	GU981021	GU981061			
Chimarrogale	*himalayica*	19703	China, Yunnan	*Chimhim2*	GU981264	GU981218	GU981143	GU981310	GU981356	GU981402	GU981022	GU981062	GU981187	GU981112	GU981445
Chimarrogale	*platycephala*	3.3.15.1	China, Taiwan	*Chimpla2*									DQ630249*	DQ630166*	
Chodsigoa	*parca*	19704	China, Yunnan	*Chparca1*	GU981265	GU981219	GU981144	GU981311	GU981357	GU981403	GU981023	GU981063			
Chodsigoa	*parca*	19705	China, Yunnan	*Chparca2*	GU981266	GU981220	GU981145	GU981312	GU981358	GU981404	GU981024	GU981072	GU981188	GU981113	GU981446
Chodsigoa	*parca*	19706	China, Yunnan	*Chparca4*	GU981267	GU981221	GU981146	GU981313	GU981359	GU981405	GU981025	GU981073	GU981189	GU981114	GU981447
Chodsigoa	*parca*	19443	China, Yunnan	*Chparca8*	GU981268	GU981222	GU981147	GU981314	GU981360	GU981406	GU981026	GU981074	GU981190	GU981115	GU981448
Chodsigoa	*sodalis*	SIS.2	China, Taiwan	*Chsodal0*									DQ630274*	DQ630194*	
Chodsigoa	*sodalis*	THUB-S-00002	China, Taiwan	*Chsodal1*	GU981269	GU981223	GU981148	GU981315	GU981361	GU981407	GU981027	GU981075			
Chodsigoa	*sodalis*	THUB-S-00007	China, Taiwan	*Chsodal2*	GU981270	GU981224	GU981149	GU981316	GU981362	GU981408	GU981028	GU981076	GU981191	GU981116	GU981449
Crocidura	*fuliginosa*	19701	China, Yunnan	*Crofuli1*	GU981271	GU981225	GU981150	GU981317	GU981363	GU981409	GU981029	GU981077	GU981192	GU981117	GU981450
Crocidura	*malayana*	IZEA3550	Malaysia,Ulu Gombak	*Crymala1*									DQ630211*	DQ630124*	
Cryptotis	*magna*	X4	Mexico, Oaxaca	*Crypmag1*									DQ630267*	DQ630186*	
Episoriculus	*caudatus*	19716	China, Yunnan	Epicau08	GU981272	GU981226	GU981151	GU981318	GU981364	GU981410	GU981030	GU981078	GU981193	GU981118	GU981451
Episoriculus	*caudatus*	19717	China, Yunnan	Epicau11	GU981273	GU981227	GU981152	GU981319	GU981365	GU981411	GU981031	GU981079			
Episoriculus	*caudatus*	19718	China, Yunnan	Epicau12	GU981274	GU981228	GU981153	GU981320	GU981366	GU981412	GU981032	GU981080	GU981194	GU981119	GU981452
Episoriculus	*caudatus*	18946	China, Yunnan	Epicau13	GU981275	GU981229	GU981154	GU981321	GU981367	GU981413	GU981033	GU981081			
Episoriculus	*caudatus*	19719	China, Yunnan	Epicau18	GU981276	GU981230	GU981155	GU981322	GU981368	GU981414	GU981034	GU981082	GU981195	GU981120	GU981453
Episoriculus	*caudatus*	19435	China, Yunnan	Epicau19	GU981277	GU981231	GU981156	GU981323	GU981369	GU981415	GU981035	GU981083			
Episoriculus	*fumidus*	SIF.2	China, Taiwan	Epifumi0									DQ630273*	DQ630193*	
Episoriculus	*fumidus*	THUB-S-00005	China, Taiwan	Epifumi1	GU981278	GU981232	GU981157	GU981324	GU981370	GU981416	GU981036	GU981084	GU981196	GU981121	GU981454
Episoriculus	*fumidus*	THUB-S-00009	China, Taiwan	Epifumi2	GU981279	GU981233	GU981158	GU981325	GU981371	GU981417	GU981037	GU981064			
Episoriculus	*fumidus*	THUB-S-00004	China, Taiwan	Epifumi6	GU981280	GU981234	GU981159	GU981326	GU981372	GU981418	GU981038	GU981085			
Episoriculus	*leucops*	19720	China, Yunnan	Epileu01	GU981281	GU981235	GU981160	GU981327	GU981373	GU981419	GU981039	GU981086	GU981197	GU981122	GU981455
Episoriculus	*leucops*	19721	China, Yunnan	Epileu02	GU981282	GU981236	GU981161	GU981328	GU981374	GU981420	GU981040	GU981087	GU981198	GU981123	GU981456
Episoriculus	*leucops*	18950	China, Yunnan	Epileu07	GU981283	GU981237	GU981162	GU981329	GU981375	GU981421	GU981041	GU981088			
Episoriculus	*leucops*	18944	China, Yunnan	Epileu08	GU981284	GU981238	GU981163	GU981330	GU981376	GU981422	GU981042	GU981089			
Episoriculus	*macrurus*	19722	China, Yunnan	Epimac01	GU981285	GU981239	GU981164	GU981331	GU981377	GU981423	GU981043	GU981090	GU981199	GU981124	GU981457
Episoriculus	*macrurus*	19723	China, Yunnan	Epimac03	GU981286	GU981240	GU981165	GU981332	GU981378	GU981424	GU981044	GU981091	GU981200	GU981125	GU981458
Episoriculus	*macrurus*	19700	China, Yunnan	Epimac04	GU981287	GU981241	GU981166	GU981333	GU981379	GU981425	GU981045	GU981092			
Episoriculus	*macrurus*	18939	China, Yunnan	Epimac06	GU981288	GU981242	GU981167	GU981334	GU981380	GU981426	GU981046	GU981093	GU981201	GU981126	GU981459
Episoriculus	*macrurus*	19678	China, Yunnan	Epimac12	GU981289	GU981243	GU981168	GU981335	GU981381	GU981427	GU981047	GU981094	GU981202	GU981127	GU981460
Episoriculus	*macrurus*	19679	China, Yunnan	Epimac14	GU981290	GU981244	GU981169	GU981336	GU981382	GU981428	GU981048	GU981095			
Nectogale	*elegans*	19712	China, Yunnan	Neceleg1	GU981291	GU981245	GU981170	GU981337	GU981383	GU981429	GU981049	GU981096	GU981203	GU981128	GU981461
Nectogale	*elegans*	19713	China, Yunnan	Neceleg2	GU981292	GU981246	GU981171	GU981338	GU981384	GU981430	GU981050	GU981097			
Nectogale	*elegans*	19714	China, Yunnan	Neceleg7	GU981293	GU981247	GU981172	GU981339	GU981385	GU981431	GU981051	GU981098	GU981204	GU981129	GU981462
Nectogale	*elegans*	19715	China, Yunnan	Neceleg8	GU981294	GU981248	GU981173	GU981340	GU981386	GU981432	GU981052	GU981099			

Neomys	*anomalus*	IZEA 5524	Switzerland	Neomano1											DQ630243*	DQ630159*		
Neomys	*fodiens*	IZEA 1368	Yugoslavia, Popova Sapka	Neomfod0											DQ630245*	DQ630162*		
Neomys	*fodiens*	65298	Hochsauerlandkreis, German	Neomfod1	GU981295	GU981249	GU981174	GU981341	GU981387	GU981433	GU981053	GU981100	GU981205	GU981130	GU981463			
Notiosorex	*crawfordi*	NSC2	USA,Texas	Notcraw1									DQ630269*	DQ630188*				
Sorex	*alpinus*	IZEA 5444	Switzerland, Pont-de-Nant	Soralpi1									DQ630238*	DQ630154*				
Sorex	*bedfordiae*	19680	China, Yunnan	Sorbedf1	GU981296	GU981250	GU981175	GU981342	GU981388	GU981434	GU981054	GU981101	GU981206	GU981131	GU981464			
Sorex	*cinereus*	99.9.21.1	USA	Sorcine2									DQ630253*	DQ630170*				
Sorex	*excelsus*	MSI 4456	China, Qinghai	Sorexce1									DQ630240*	DQ630156*				
Sorex	*fumeus*	SEF.I	USA, Pennsylvania	Sorfume2									DQ630270*	DQ630190*				
Soriculus	*nigrescens*	19707	China, Yunnan	Sorinig1	GU981297	GU981251	GU981176	GU981343	GU981389	GU981435	GU981055	GU981102						
Soriculus	*nigrescens*	19708	China, Yunnan	Sorinig3	GU981298	GU981252	GU981177	GU981344	GU981390	GU981436	GU981056	GU981065	GU981207	GU981132	GU981465			
Soriculus	*nigrescens*	19709	China, Yunnan	Sorinig6	GU981299	GU981253	GU981178	GU981345	GU981391	GU981437	GU981057	GU981103	GU981208	GU981133	GU981466			
Soriculus	*nigrescens*	19710	China, Yunnan	Sorinig7	GU981300	GU981254	GU981179	GU981346	GU981392	GU981438	GU981058	GU981104						
Soriculus	*nigrescens*	19711	China, Yunnan	Sorinig8	GU981301	GU981255	GU981180	GU981347	GU981393	GU981439	GU981059	GU981105	GU981209	GU981134	GU981467			
sorex	*saussurei*	SESA2	Mexico, Guerrero	Sorsaus1									DQ630247*	DQ630164*				

* Sequences used from previous study.

2.3. Phylogenetic analyses and molecular divergence dating

Sequences of all genes were edited using DNASTAR Lasergene Seqman and EditSeq version 7.1, and aligned with Clustal X 1.83 (Thompson et al., 1997) using default settings and further checked by eye. Ambiguous regions in 12S and 16S were excluded from phylogenetic analysis. BRCA1 and ApoB genes of sixteen samples from a previous study (Dubey et al., 2007) were added to the nDNA data sets.

All sequences were combined into four data sets representing the four independently evolving loci (ApoB, BRCA1, RAG2, and the combined mtDNA genes), and analyzed separately using Bayesian phylogenetic analysis, assuming separate models for each codon position, in addition to separate partitions for the mtDNA 12S and 16S genes (i.e., "partitioned" Bayesian analysis; Brandley et al., 2005). The appropriate model of DNA evolution for each partition was determined using the likelihood-ratio test calculated by MrModeltest v2.3 (Nylander, 2004). Substitution models for all partitions are provided in Supplementary Material Appendix S1. With one exception (see Section 3) all Bayesian analyses consisted of 10 million generations, using four chains, sampled every 1000 generations, and used the default priors (including a random starting tree). To determine convergence, we constructed cumulative posterior probability plots for each analysis using the "cumulative" function in AWTY (Nylander et al., 2008). These plots indicated that excluding the first 2 million generations as burn-in was sufficient to ensure convergence. We repeated the analysis four times for each data set, and analyzed the results using the "compare" function in AWTY. If each of the four analyses converged on the same posterior distribution, posterior probabilities of each clade were calculated from the combined results (Sukumaran and Linkem, 2009). Posterior probabilities (PP) $\geqslant 0.95$ are considered statistically (i.e., "strongly") supported (Huelsenbeck and Rannala, 2004).

To give our paleoclimatic analysis a temporal framework, we used simultaneous Bayesian phylogenetic and molecular dating estimation using BEAST v1.5.1 (Drummond and Rambaut, 2007). An advantage of Bayesian molecular dating methods is the user's control of prior probabilities of age calibrations. Instead of using a point estimate, a variety of distributions can be used to accommodate uncertainty in the age of the fossil calibration (Ho, 2007). Moreover, the use of "relaxed" molecular clocks allows each branch of the phylogeny to evolve at a different, but relative rate, thus relaxing the unrealistic assumptions of the "strict" molecular clock (Drummond et al., 2006).

We limited this molecular divergence dating analysis to the combined ApoB and BRCA1 data sets for three primary reasons. Firstly, our phylogenetic results (see Section 3) indicate no statistically significant incongruence among these two loci, yet significant difference between these loci and the mtDNA, and the placement of *Episoriculus fumidus* in the RAG2 analysis. Secondly, the nuclear data includes far more outgroup species that are important for calibration age constraints. Finally, one of the nuclear genes (RAG2) contains far fewer taxa than ApoB and BRCA1; as there is essentially no research examining the effect of missing data on divergence time estimation, we chose to not include this locus in this analysis.

Each BEAST analysis used partition-specific models for each codon position of the two genes (see above), coalescent starting tree, birth–death tree prior, uncorrelated lognormal relaxed molecular clock, the program's default prior distributions of model parameters (with the exception of GTR substitution rates in which we used a uniform [0,100] distribution), and lognormal age distributions of the most recent common ancestor of the three clades used for calibration (see below). Analyses were run for 20 million generations, and were sampled every 10,000th generation. The analyses were

Table 2
Primers used in PCR and sequencing.

Locus	Partitions	Primer name	Primer sequences	Sense/anti-sense	Cited source
12s rRNA	12sa	L613	ACACAAAGCATGGCACTGAA	Sense	Mindell et al. (1991)
		H1478	TGACTGCAGAGGGTGACGGGCGGTGTGT	Anti-sense	Kocher et al. (1989)
		L613_hk1	GGCGGGCGAGCAAAGCACTGAAAATG	Sense	This study
		H1478_hk1	TGATTGGTGGAGGGTGACGAGCGGTGTGT	Anti-sense	This study
	12sb	12sb_L1	CGGACATAAAAACGTTAGGTCAAGG	Sense	This study
		12SB_H1	TCGGTTCATGGATAGCTCGTCTG	Anti-sense	This study
		12SB_H2	CCAGCTATCACCAGGCTCGGTAG	Anti-sense	This study
16s rRNA	16sa	16sar	CGCCTGTTTATCAAAAACAT	Sense	Simon et al. (1991)
		16sbr	CCGGTCTGAACTCAGATCACGT	Anti-sense	Simon et al. (1991)
	16sb	16SB_L1	CGGCGATAAGTCGTAACAAGGTAAGC	Sense	This study
		16SB_L2	GGACCCCTTGTACCTTTTGCATAATG	Sense	This study
		16SB_H1	TAACAGTTGTCACTGGGCAGGCAGT	Anti-sense	This study
	16sc	16SC_L1	CGGAAGAAGTAAAAGGAACTCGGC	Sense	This study
		16SC_L2	CGGCAGCAGAAATACTGTTAATATGAGT	Sense	This study
		16SC_H1	GGCGGATGTTGTTAGAGAGAGGAAT	Anti-sense	This study
Cyt *b*	cyt*b*	L14724	CGAAGCTTGATATGAAAAACCATCGTTG	Sense	Irwin et al. (1991)
		H15915	GGAATTCATCTCTCCGGTTTACAAGAC	Anti-sense	Irwin et al. (1991)
		L14724_hk3	GGACTTATGACATGAAAAATCATCGTTG	Sense	This study
		H15915_hk3	GATTCCCCATTTCTGGTTTACAAGAC	Anti-sense	This study
ND2	nd2	nd2L_hk1	CGGCGATAGAGTAAATAATAGAGGTT	Sense	This study
		nd2H_hk1	GATTGAAGCCAGTTGTTTAGGGTA	Anti-sense	This study
		nd2H_hk2	GAAGGTAGATTGAAGCCAGTTGTT	Anti-sense	This study
ND4	nd4	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Sense	Arevalo et al. (1994)
		ND4_hk1	GAATACCAAAAGCACCCGTAGAAGC	Sense	This study
		PII	TACTTTTACTTGGAGTTGCA	Anti-sense	Arevalo et al. (1994)
		Leu	GGCTATTACTTTTATTTGGAGTTGCACC	Anti-sense	Parkinson et al. (2000)
		Leu_hk1	GGCTATTACTTTTATTTGGAGTTGCACC	Anti-sense	This study
ND5	nd5	ND5L_hk1	GGCCGAGAAAGATTGCAAGAACTG	Sense	This study
		ND5H_hk1	TCAGGCGGTGGTATACGACGTGTT	Anti-sense	This study
		ND5H_hk2	AGGCGGTGATTTTTCATGTCATAAGTC	Anti-sense	This study
COI	COI	COI_L1	GGGCTTTACAGTCTAATGCTTAACCTC	Sense	This study
		COI_L2	GCTAAATACCCTAAACAACTGGCTTC	Sense	This study
		COI_H2	GTGACCGAAGAATCAGAAAAGATGTT	Anti-sense	This study
ATP6		ATP6_L1:	GCCTTGAGAAACAAAATGAAC	Sense	This study
		ATP6_H1:	GGACTTGGGTTTACTATGTGAT	Anti-sense	This study
		ATP6_H2:	GTATATGTTTTCGGTTGCCTT	Anti-sense	This study
BRCA1	BRCA1	B1f	TGAGAACAGCACTTTATTACTCAC	Sense	Dubey et al. (2006)
		B1r	ATTCTAGTTCCATATTGCTTATACTG	Anti-sense	Dubey et al. (2006)
		Brca1 F2	GAGATTCCCAAGAGATGACTTG	Sense (internal primer)	This study
		Brca1 R2	ACGTTTCTTGATAAAATCTTCAGG	Anti-sense (internal primer)	This study
ApoB	ApoB	vWFe-A2ag	GTGCTGAAGGTCTTCGTGGTG	Sense	Dubey et al. (2007)
		vWFe-B2ag	GTGACCATGTAGACCAGGTTAGG	Anti-sense	Dubey et al. (2007)
		ApoB R2	CTAATATTTCCCAGGGCTG	Anti-sense (internal primer)	This study
		ApoB F2	AGGACCTTTAAAATTCCAGG	Sense (internal primer)	This study
		ApoB F3	GCAATCATTTTATTTAAGTC	Sense	This study
		ApoB F4	GCCCGCCAATCATTTTATTTAAGTC	Sense	This study
		ApoB F5	CATACATGGTGAAGCCAATCTGG	Sense	This study
		ApoB F6	GCCAGACTTGAAGAAATTCTTGAG	Sense	This study
		AopB R3	GCCATAAGCAACAATATCTGTTTG	Anti-sense	This study
		ApoB R4	TCTCAATGACAGATGAAGAGGATGT	Anti-sense	This study
		ApoB R5	TTTCTGGTCAAACTTGAGGTGC	Anti-sense	This study
		ApoB R6	ACGCATTACTTAGAGACAGAGTTGTG	Anti-sense	This study
Rag2	Rag2	RAG2-F1	GATTCCTGCTAYCTYCCTCCTCT	Sense	This study
		RAG2-R2	CCCATGTTGCTTCCAAACCATA	Anti-sense	This study
		RAG2 F2	GGAGATGTTCCTGAAGCCAGAT	Sense (internal primer)	This study
		RAG2 R2	AGGCACTGGAAACTGAGATTCCT	Anti-sense (internal primer)	This study

repeated eight times (current versions of BEAST use a single MCMC chain) and convergence was assessed using AWTY (see above). To ensure proper rooting, we constrained the monophyly of subfamily Soricinae.

All fossil calibration age constraints were treated as lognormal distributions (Ho, 2007). The following fossils were used as age constraints: (1) The oldest Soricinae–Crocidurinae ancestors lived about 20 million year ago (Ma) (Reumer, 1989, 1994). We set a lognormal distribution so that the earliest possible sampled is 20 Ma (offset = 20) and the older 95% credible interval (CI) includes 25 Ma (Reumer, 1989) (mean = 0, standard deviation = 0.98); (2) The oldest Blarinellini was in both Europe and North America in the Early-Middle Miocene (Harris, 1998; Rzebik-Kowalska, 1998), and the oldest Blarinini was in the Barstovian of the United States (Repenning, 1967). We therefore set the earliest possible sampled age to 15 Ma (Buffetaut, 2002; Cheneval and Ginsburg, 2000); the older 95% CI encompasses the MN3 (20 Ma) (Agusti et al., 2001; Ziegler, 1989, 1994) (mean = 0, standard deviation = 0.98); 3. The oldest known Pliocene *Otisorex* (the subgenus of *Sorex* distributed in North America) inhabited North America approximately 3.5 Ma (Maldonado et al., 2001). We set the earliest possible sampled age to 3.5 Ma and the older 95% CI to 5 Ma in Early Pliocene (mean = 0, standard deviation = 0.25).

2.4. Bayesian ancestral state re-constructions

We used Bayesian ancestral state re-construction analyses to estimate whether the transition to an aquatic lifestyle evolved

independently in *Neomys* and *Chimarrogale + Nectogale*. This method is advantageous because it explicitly incorporates uncertainty in tree topology as well as providing posterior probabilities of reconstructed states. We coded species as binary data (non-aquatic = 0, aquatic = 1), and employed an MCMC analysis in BayesTraits v1.0 (Pagel et al., 2004) using the posterior distribution of the time-calibrated ApoB + BRCA trees from our BEAST analyses. We ran the analysis for 1.1×10^7 generations (excluding 10^6 generations as burn-in), sampling every 1000 generations, and restricting the forward and reverse rate to be the same (i.e., $q_{01} = q_{10}$). Posterior probabilities for selected nodes were calculated by taking the mean of the posterior probabilities inferred for these nodes calculated for each generation. Only nodes with significant clade posterior probability (i.e., $\geqslant 0.95$) were considered.

We infer independent evolution of an aquatic ecology in both clades only if we estimate significant posterior probability (i.e., $\geqslant 0.95$) for a non-aquatic ecology in the different recent common ancestors of *Neomys* and *Chimarrogale + Nectogale*.

3. Results

3.1. Phylogenetic relationships

We obtained 46 mitochondrial sequences comprising 7822 bp, and 29 nuclear sequences comprising 2007 bp. GenBank Accession No. are from GU981014 to GU981439 and additional sequences were from Ohdachi et al. (2006) and Dubey et al. (2007) (Table 1). The mtDNA Bayesian analyses were unusual in that most analyses suffered from extremely slow convergence, and/or converged on local, suboptimal posterior distributions (mean $-\ln L \sim 54{,}900$). We therefore ran additional analyses with a tree prior with a "better" estimate of Nectogalini phylogeny than the default random tree. One option would have been to use the maximum likelihood (ML) tree as a starting tree, but we were concerned about excessively biasing the prior. Instead of using the ML tree, we took a compromise approach and inferred ML trees of four separate ML bootstrap pseudoreplicates of the mtDNA data set using RAxML v7.0.4 (Stamatakis, 2006). One of these bootstrap trees was used as a starting tree for each of the four Bayesian analyses. The mean $-\ln L$ improved to $\sim$54,760, and we will limit our discussion of the mtDNA phylogeny to this tree (Fig. 2d).

The phylogenetic analyses of the mtDNA and three nuclear loci all supported the phylogenetic relationships of multiple clades (Fig. 2). The support for monophyly of Nectogalini was statistically significant (i.e., PP $\geqslant$ 0.95) in all analyses. All loci inferred a sister relationship between *Chimarrogale* and *Nectogale*, but this was significantly supported only in the mtDNA (PP = 1.0) and BRCA1 (PP = 1.0) analyses. Moreover, these analyses inferred the polyphyly of *Episoriculus*, supporting the East Himalayan species as a distinct clade from *E. fumidus* with statistically significant support in the analyses of the mtDNA, ApoB, and RAG2 data sets.

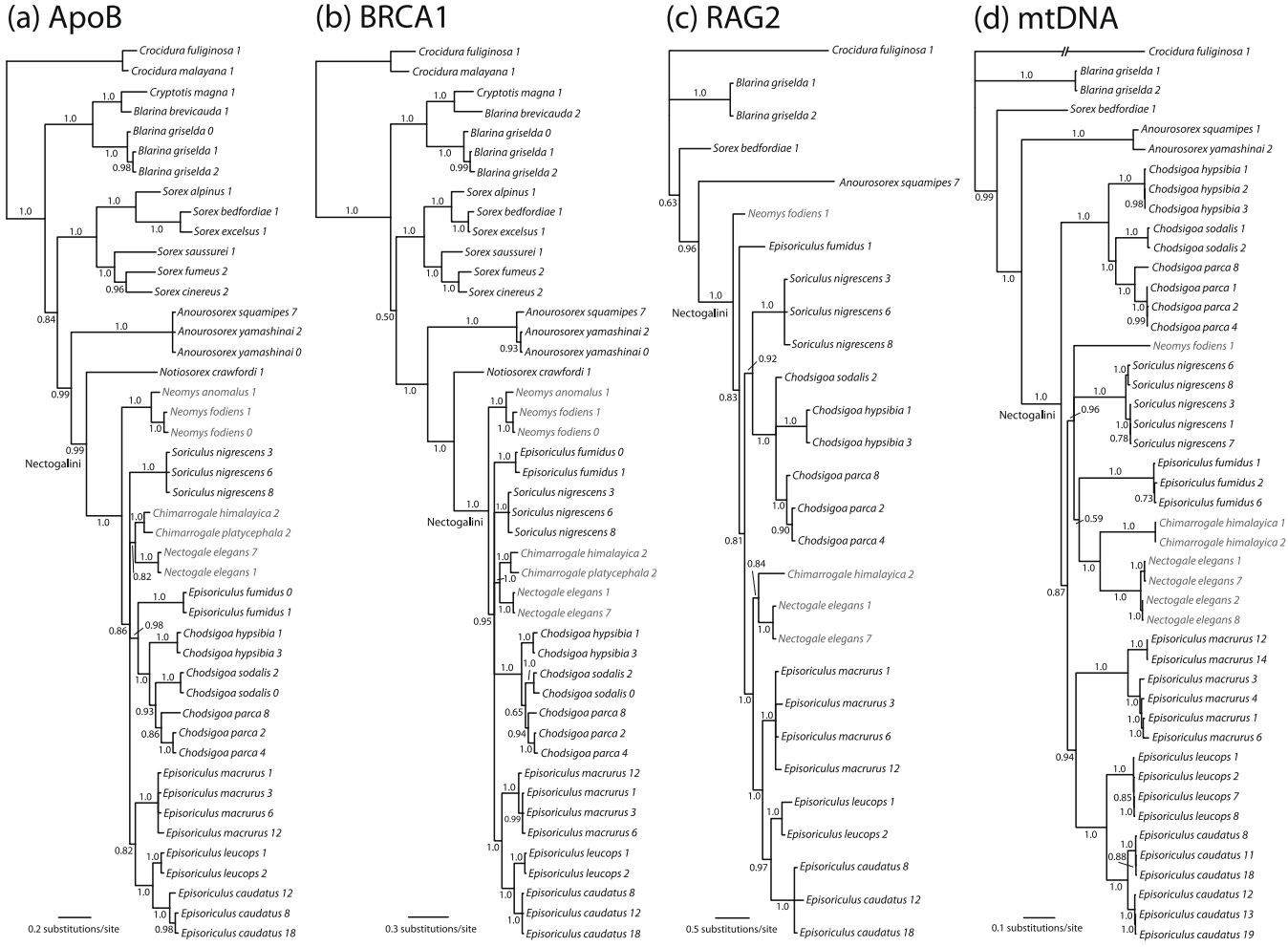

Fig. 2. Results of Bayesian phylogenetic analyses of three nuclear (a–c) and mtDNA (d) data sets. Branch lengths are means of the posterior distribution. Node numbers indicate Bayesian posterior probabilities. Taxa shaded in grey are aquatic.

Although the three nuclear loci inferred topologically incongruent trees, in only one case was a conflicting relationships significantly supported. The ApoB data significantly supported *Episoriculus fumidus* as the sister lineage to *Chodsigoa* (PP = 0.98), while the RAG2 data excludes *E. fumidus* from a clade containing *Chodsigoa* and other species (PP = 1.0). Another example of marginally strong incongruence was the sister relationship between *Soriculus* and *Chodsigoa* inferred by the RAG2 data set (PP = 0.92); the relationships of these genera were unresolved in the BRCA1 and ApoB analysis. Finally, all three nuclear loci inferred a basal split in Nectogalini between *Neomys* and all other genera. However, this was only significantly supported by the BRCA1 analysis (PP = 0.95).

Overall, the phylogeny inferred from the mtDNA data was considerably different from that inferred by any of the nuclear loci.

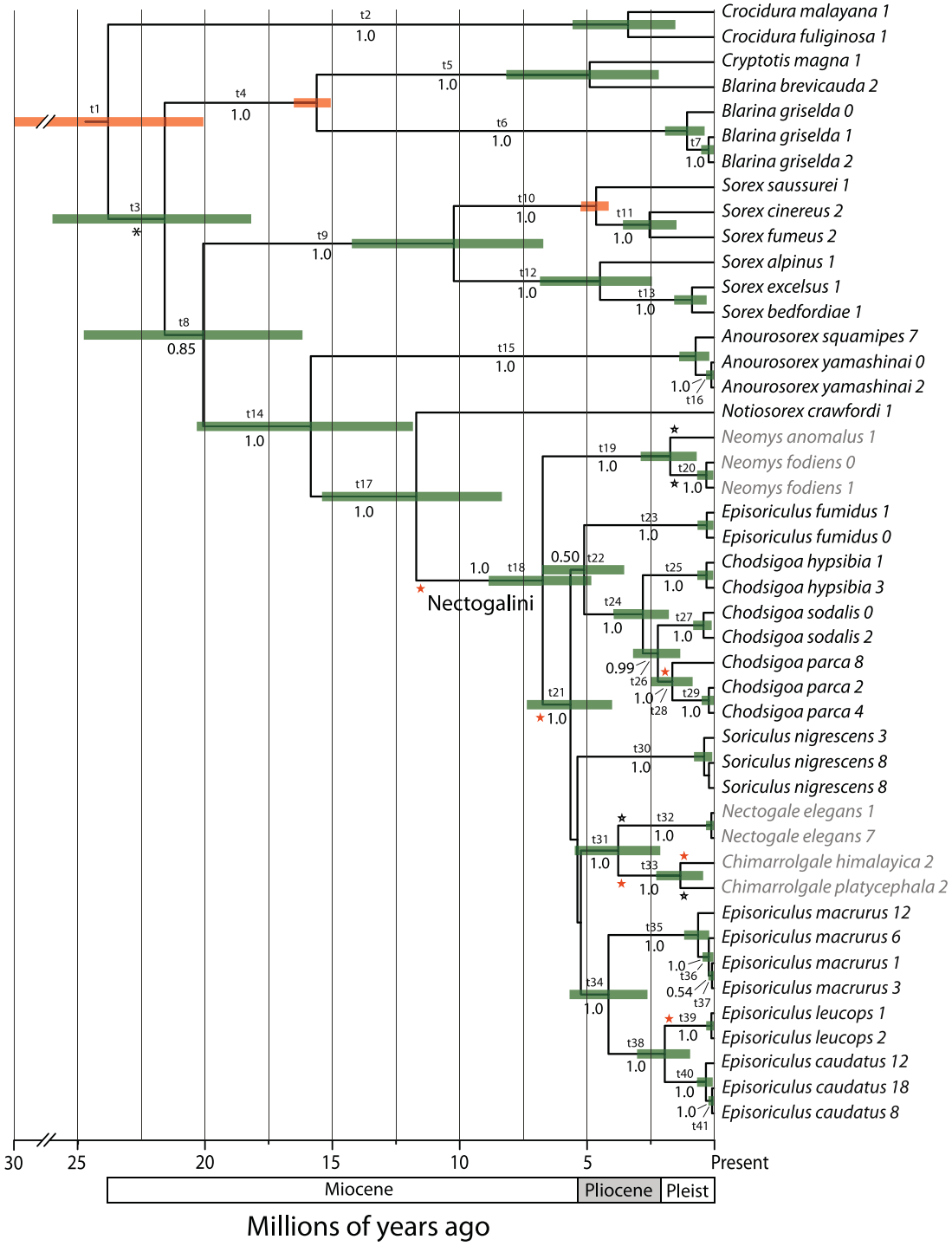

Fig. 3. Chronogram from the partitioned Bayesian analysis of the combined ApoB and BRCA1 nuclear genes using a relaxed molecular clock. Branch lengths represent time. Node bars indicate the 95% CI for the clade age. Orange bars represent nodes whose age was calibrated with fossil taxa. Numbers below the nodes indicate Bayesian posterior probabilities. The t_x designations above the nodes refer to median ages and 95% CI for each node in Table 3. A node with a red pentagon indicates fossil records of this lineage coincide (or nearly so) with age estimated by Bayesian divergence time analyses when a black pentagon indicates they are not congruent with each other. The asterisk indicates this node was constrained to be monophyletic. Taxa shaded in grey are aquatic. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Moreover, one of these incongruent relationships was strongly supported. Whereas all three nuclear loci inferred *Neomys* as the sister taxon to other Nectogalini genera, the mtDNA phylogeny instead placed this lineage in a significantly supported clade including *Chimarrogale*, *Episoriculus fumidus*, *Nectogale*, and *Soriculus* (PP = 0.96; Fig. 2d).

3.2. Molecular divergence dating

All eight partitioned BEAST analyses of the combined ApoB and BRCA1 data, using a lognormal uncorrelated relaxed molecular clock, converged on a similar posterior distribution within 4×10^6 generations. The 95% CI of the standard deviation of the uncorrelated lognormally distributed rates is 0.223–0.524, thus demonstrating sufficient lineage rate heterogeneity to reject a strict molecular clock (Drummond and Rambaut, 2007). The results of the consensus of the post burn-in trees, including 95% credible intervals of estimated divergence times, are provided in Fig. 3 and Table 3. The phylogeny was very similar to the Bayesian analyses of the individual nuclear loci (above). There was strong support for a monophyletic Nectogalini (PP = 1.0) splitting from its sister lineage (95% CI = 8.3–15.4 Ma), as well as a monophyletic crown Nectogalini (PP = 1.0) radiating 4.8–8.8 Ma. *Neomys* was significantly supported as the sister taxon to other nectogalines (PP = 1.0). As with the previous Bayesian analyses of the individual nuclear loci (above), the remaining relationships, representing the base of the non-*Neomys* clade, were characterized by extremely short, poorly supported interior branches coinciding with the Miocene–Pliocene boundary (~4–7 Ma; Fig. 3). *Episoriculus fumidus* formed the sister group to the genus *Chodsigoa* with very poor support (PP = 0.50, 3.52–6.74 Ma). The sister relationship between *Chimarrogale* and *Nectogale* was significantly supported (PP = 1.0, 2.1–5.46 Ma). *Episoriculus macrurus* forming a sister group of *E. caudatus* and *E. leucops* was significantly supported too (PP = 1.0, 2.61–5.67 Ma).

Table 3
Divergence times of lineages estimated from Bayesian phylogenetic analyses of nDNA genes using a lognormal relaxed molecular clock with 95% credible interval (CI). Node numbers are represented in Fig. 3.

Node	Age	Lower 95% CI	Upper 95% CI
t1	–	–	–
t2	3.22	1.51	5.55
t3	21.12	18.16	25.96
t4	15.47	15.04	16.49
t5	4.69	2.16	8.15
t6	0.98	0.36	1.92
t7	0.17	0.00	0.31
t8	19.73	16.15	24.73
t9	10.03	6.70	14.21
t10	4.59	4.13	5.21
t11	2.52	1.47	3.57
t12	4.33	2.44	6.83
t13	0.80	0.29	1.56
t14	15.70	11.81	20.30
t15	0.66	0.18	1.35
t16	0.07	0.00	0.31
t17	11.56	8.32	15.38
t18	6.63	4.81	8.84
t19	1.63	0.68	2.87
t20	0.26	0.02	0.66
t21	5.57	4.00	7.34
t22	5.04	3.52	6.74
t23	0.24	0.02	0.64
t24	2.74	1.77	3.93
t25	0.25	0.03	0.65
t26	2.15	1.32	3.17
t27	0.37	0.09	0.81
t28	1.59	0.84	2.49
t29	0.16	0.00	0.48
t30	0.34	0.06	0.78
t31	3.71	2.10	5.46
t32	0.07	0.00	0.31
t33	1.25	0.42	2.26
t34	4.09	2.61	5.67
t35	0.57	0.18	1.17
t36	0.17	0.02	0.45
t37	0.06	0.00	0.22
t38	1.87	0.93	3.02
t39	0.07	0.00	0.30
t40	0.28	0.05	0.66
t41	0.05	0.00	0.22

Table 4
Posterior probabilities of reconstructed ancestral states for selected clades. Clade identifications refer to those used in Fig. 3.

	Posterior probability	
Clade	Non-aquatic	Aquatic
t8	0.95	0.05
t14	0.94	0.06
t17	0.91	0.09
t18	0.76	0.24
t19	0.00	1.00
t21	0.98	0.02
t31	0.01	0.99

3.3. Ancestral state re-construction

Bayesian ancestral re-construction analyses infer significant support for the ability to utilize aquatic habitats in the most recent common ancestor (MRCA) of sampled *Neomys* populations (t19 in Fig. 3; PP = 1.0) and *Chimarrogale* + *Nectogale* (t31; PP = 0.99) (Table 4). Although these analyses suggest that the MRCA of the *Neomys* lineage and other Nectogalini lineages was not aquatic (t18; PP = 0.76), the posterior probability of a non-aquatic ecology is significantly (or marginally insignificantly) supported in deeper nodes (t8, t14, t17; PP = 0.91–0.95). The posterior probability for a non-aquatic ecology in the closest, well-supported MRCA of *Chimarrogale* + *Nectogale* (t21) is significantly supported (PP = 0.98).

3.4. Palaeontology of Nectogalini

The fossil localities are provided in Supplementary Material Appendix S2, and fossil localities in East Asia are represented in Fig. 4. The European record includes five genera: *Asoriculus*, *Macroneomys*, *Neomysorex*, *Nesiotites* and *Neomys* (Rzebik-Kowalska, 1998). Only the latter two genera survived the Last Glacial Maximum (LGM), but *Nesiotites* became extinct around 3000 years ago. One fossil species belonging to the genus *Asoriculus* was found in Morocco, North Africa (Butler, 1998; Geraads, 1995). All five living genera in Asia have fossils records (Storch et al., 1998). Historically, *Chodsigoa*, *Episoriculus*, and *Soriculus* were classified into a single genus *Soriculus*. Thus, some of the fossil *Soriculus* taxa in fact represent other genera (e.g. *Soriculus* praecursus, see Section 4.2).

4. Discussion

4.1. Data incongruence and the phylogeny of Nectogalini

Although previous phylogenetic studies (Dubey et al., 2007; Ohdachi et al., 2006) did much to improve our understanding of evolutionary relationships of Nectogalini shrews, these studies also inferred some conflicting or poorly supported relationships or did not sample heavily within Nectogalini. To remedy this, we employed Bayesian analyses including four independently evolving loci from mitochondrial and nuclear for up to 14 species representing all six genera. Our results clarified several phylogenetic rela-

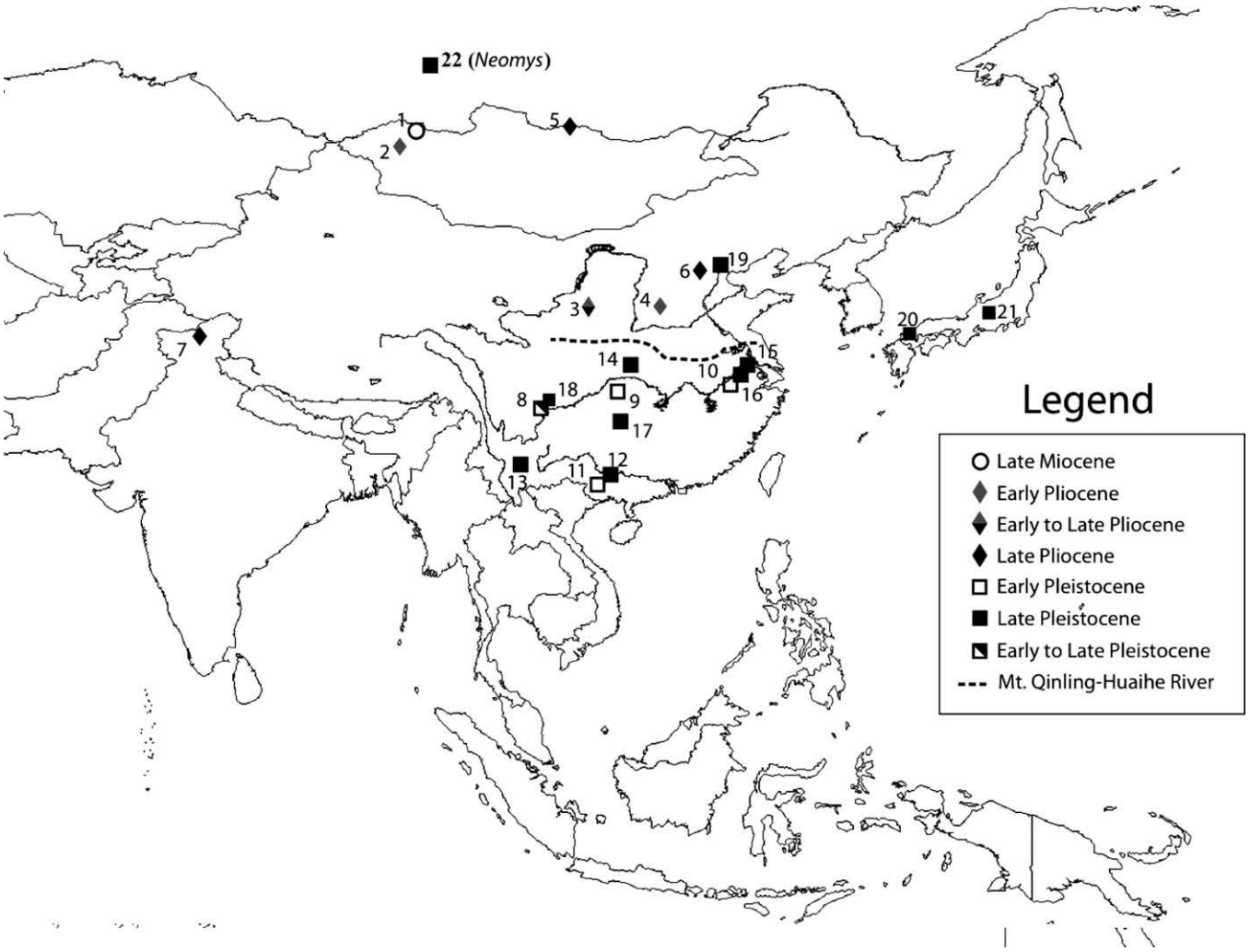

Fig. 4. Localities of Nectogalini shrews in East Asia from Late Miocene to Pleistocene. 1, Khirgiz-Nur; 2, Chono-Khariakh; 3, Lintai, Gansu; 4, Yushe, Shanxi; 5, Shama; 6, Yuxian, Hebei; 7, Kashmir; 8, Wushan, Chongqing; 9, Jianshi, Hubei; 10, Fanchang, Anhui; 11, Chongzuo, Guangxi; 12, Tiandong, Guangxi; 13, Chenggong, Yunnan; 14, Yunxi, Hubei; 15, Hexian, Anhui; 16, Wuhu, Anhui; 17, Huayuan, Hunan; 18, Geleshan, Chongqing; 19, Choukoutien, Beijing; 20, Ube; 21, Honshu; 22 Irkutsk.

tionships that were unresolved or conflicting in previous analyses including the placement of *Neomys* (and paraphyly of the aquatic genera) and the polyphyly of *Episoriculus*. However, before discussing these results, we first address the significant incongruence between the trees inferred by the three nuclear loci and the mtDNA.

Given the existence of phenomena such as ambiguous RNA alignment (Gillespie, 2004), ancient hybridization (Good et al., 2008), mutational rate, incomplete lineage sorting (Edwards, 2009; Lyons-Weiler and Milinkovitch, 1997), explosive speciation (Krause et al., 2008), or different inheritance pathways between nuclear and mtDNA (Doyle, 1997), it should be unsurprising that different loci would sometimes infer topologically incongruent phylogenies. However, the degree of significantly supported incongruence between the ApoB + BRCA1, RAG2, and mtDNA data sets is nonetheless striking. One potential explanation for this complex tree space is potential mis-alignment of the rRNA of mtDNA. However, reanalysis of the mtDNA excluding the 12S and 16S RNA data inferred a phylogeny that was topologically identical to the full mtDNA data set (Fig. 2d), but with much higher overall clade support (not shown). In other words, excluding RNA resulted in more support for the relationships that were incongruent with the three nuclear genes.

Instead, we hypothesize that explosive speciation (i.e., rapid radiation) is a feasible explanation for the discrepancy between the nuclear and mtDNA data. Our time-calibrated Bayesian analysis indicated rapid cladogenesis at Miocene–Pliocene boundary (Fig. 3), where most of the lineages of the extant genera diversified. We noted that these branches are also very short and poorly supported in Bayesian analyses of the individual loci (not enforcing a relaxed molecular clock; Fig. 2). Given such a rapid speciation event, the diversification of lineages will too rapid for sufficient phylogenetically informative DNA substitutions to evolve, making phylogenetic re-construction difficult (e.g. Poe and Chubb, 2004; Xiong et al., 2009). A second hypothesis is that relatively recent radiations may not provide sufficient time for complete lineage sorting, thus further obscuring the phylogenetic interrelationships of these lineages (Jackson et al., 2009). These two hypotheses are not mutually exclusive, but distinguishing between them will require additional nuclear loci (Edwards, 2009; Townsend, 2007). Regardless of the actual source of the incongruence, the ApoB and BRCA1 data sets infer congruent phylogenetic histories and will serve as our current "best" estimate of Nectogalini shrew relationships (Fig. 3).

The genus *Episoriculus* together with *Chodsigoa* was previously included in *Soriculus* as subgenera (Ellerman and Morrison-Scott, 1951; Hoffmann, 1985), which was accepted by paleontologists (e.g. Qiu and Storch, 2005; Storch et al., 1998). However, Repenning (1967) found remarkable differences in mandibular and

dental characters among the three taxa, and elevated them to full generic status. This assignment was accepted by Hutterer (1994) and Motokawa et al. (1998,1997) The three "subgenera" were considered to be monophyletic (Flynn and Wu, 1994), which was not supported by Ohdachi et al. (2006).

Our analyses of nuclear loci showed that the three genera are paraphyletic and can be split into four lineages: (i) the three mainland *Episoriculus* species, *E. caudatus*, *E. leucops* and *E. macrurus*; (ii) the Taiwan Island endemic species *E. fumidus*, (iii) *Soriculus nigrescens* and (iv) genus *Chodsigoa*. *Soriculus* is a monotypic genus (but see Motokawa, 2003), and its phylogenetic position is ambiguous in a previous phylogenetic analysis (Ohdachi et al., 2006) as well as the current study (Fig. 2). Ohdachi et al. (2006) did not determine the phylogenetic relationship of *E. fumidus* with strong support. Dubey et al. (2007) inferred *E. fumidus* (the only representative of the genus in their study) as the sister group of genus *Chodsigoa* with strong support, which was only supported by the ApoB gene tree (Fig. 2a). Although we too cannot place *E. fumidus* with strong support from every locus, our data nonetheless strongly supports non-monophyly of the genus *Episoriculus* (inclusive of *E. fumidus*). As a consequence, the current taxonomy of *Episoriculus* might not adequately reflect the evolutionary history of the genus and underestimates the phylogenetic diversity. Since the type species is *E. caudatus* (Hutterer, 2005), generic status should be given to *E. fumidus*, although we defer formally making this taxonomic change until completion of a thorough morphological analysis. All our four gene trees suggested that *E. macrurus* is the sister lineage to *E. caudatus* and *E. leucops*.

4.2. Relaxed molecular clock vs. the paleontological record

In the Bayesian relaxed molecular clock analysis, the divergence times largely coincide with the fossil record. All times estimated by this analysis have been presented as the median and 95% CI of the posterior distribution of ages (Table 3, Fig. 3): (1) Nectogalini and Notiosoricini separated at approximately 11.56 Ma (8.32–15.38), which is congruent with previous study (Dubey et al., 2007), but slightly older than the oldest fossil record of this tribe in Europe in Late Miocene (MN10, 8.7–9.7 Ma; Fejfar and Sabol, 2005); (2) the earliest divergence in extant Nectogalini lineages occurred 6.63 Ma (4.81–8.84) in Late Miocene, which is concordant with the fossil record in Europe and the oldest fossil record in Asia (Neomyini gen. indet.) both in the Latest Miocene (Rzebik-Kowalska, 1998; Storch et al., 1998); (3) the *Chimarrogale* and *Nectogale* lineages diverged around 3.71 Ma (2.1–5.46), congruent with the oldest fossil record of *Chimarrogale* sp. in the Early Pliocene in Asia (Storch et al., 1998); (4) the oldest fossil of *Chimarrogale himalayica* was discovered in Early Pleistocene deposits in Sichuan, China (Storch et al., 1998) and is concordant with the divergent time of *C. himalayica* and *C. platycephala* at 1.25 Ma (0.42–2.26); (5) *Episoriculus caudatus* and *E. leucops* diverged at about 1.87 Ma (0.93–3.02), and is congruent with the first fossil of *E. leucops* from the Early Pleistocene (Storch et al., 1998); and (6) the lineage leading to *Chodsigoa* diverged from its sister lineage ~5.04 Ma (3.52–6.74), and the oldest *Chodsigoa* fossil is from the Early Pliocene around 4 Ma (Zhang and Zheng, 2001), while the oldest fossils of *C. hypsibia* and *C. parca* are both in the Early Pleistocene. The latter coincides with the molecular dating of *C. parca* separated from *C. sodalis* at 2.15 Ma (1.32–3.17).

Thus, several lineages in Nectogalini diverged earlier than the fossil record indicates. This is not surprising given that DNA data records the maximum time of divergence while the fossil record provide a minimum age (Benton and Donoghue, 2007; Dubey et al., 2007). However, more fossils coincide (or nearly so) with ages estimated by the Bayesian divergence time analyses (Fig. 3; Table 3), therefore strengthening our conclusion that the divergence dates estimated by both the molecular and fossil data accurately reflect the time of divergence. In this way, we are able to confidently reconstruct the biogeography of nectogalines.

The fossil record reveals that the shrew fauna of Europe was represented by *Neomys* and at least four fossil nectogaline genera (Supplementary Material Appendix S2, also see Section 4.4), but Asia was inhabited only by other nectogalines, a hypothesis also supported by our phylogenetic analyses (Fig. 2). Also, our divergence time analyses estimate the age of divergence for the European and Asian lineages to be ~6.63 Ma. Thus, according to fossil records, it seems there were no transcontinental exchanges between Asia and Europe from Latest Miocene to Late Pleistocene (Supplementary Material Appendix S2).

Furthermore, the divergence date analyses demonstrate that the "deep" divergences in Asian genera occurred between 4 and 7 Ma, around M/P boundary (Table 3, Fig. 3). The fossil record indicates that during this period, the distribution of Nectogalini was quite different from there current distribution center in the East Himalaya–Hengduan Mountains regions (Fig. 4, Hutterer, 2005). The distribution pattern and evolutionary history suggests that the distribution center of Asian groups today is a living museum (Thorne, 1999), at least at the generic level.

Since the oldest fossil of tribe Nectogalini appeared in Europe, and subsequent fossils were found from Europe to Asia (Supplementary Material Appendix S2), two parsimonious biogeographic scenarios of nectogalines are compatible with the results of our phylogenetic analyses. In the first scenario, the ancestral species of Nectogalini migrated from Europe along Asia Minor to Central Asia, India, Southwest China, and then, eastward to Taiwan, northward to Middle and North China and Japan. The second scenario is that the ancestral species migrated eastward to Western Siberia and southward along northern China to southwest China, Indochina and Japan. The latter scenario is strongly supported by the fossil record (Fig. 4). The oldest fossils of the tribe Nectogalini in Asia were found in Transbaikalia from the Late Miocene, fossils in North China was from the Early Pliocene, and occurrence of this clade in south China was in the Early Pleistocene. However, no fossils are known from north China in the Early Pleistocene though one fossil was found in Choukoutien in the Late Pleistocene. Two more lines of evidence also support this scenario. First, several small mammals including Soricinae shrews (e.g. *Anourosorex*, *Blarinella*) were found in Lufeng, and Yuanmou, Yunnan, China from the Late Miocene, but no Nectogalini species were found in either of the two sites (Ni and Qiu, 2002; Qiu et al., 1985). Second, fossils in northern of China are morphologically more plesiomorphic than those in southern China. For example, the fossil species *Soriculus praecursus* in the Early Pliocene in Yushe, Shanxi, preserved some "primitive" characters of Nectogalini, and may represent an ancient clade of Asian groups (Flynn and Wu, 1994). Thus, a dispersal route from Europe to Asia through West Siberia and southward is more probable even though a Late Pliocene fossil species was found in Kashmir (Storch et al., 1998).

4.3. Implication for global climate change on the history of Nectogalini shrews

Why was there no transcontinental exchange between Europe and Asia in Nectogalini from Latest Miocene to Early Pleistocene? Nectogalini shrew species prefer moist or even wet environments. An arid or even semiarid environment will most likely serve as a barrier to their dispersal. Global climatic changes occurred in the Late Miocene (Fortelius et al., 2002, 2006; Janis, 1993). In Europe, it is well-known as the Messinian salinity crisis (e.g. Hsü et al., 1977; Krijgsman et al., 1999). In Asia, aridification of the Asian inland in the Late Miocene had been supported by several studies (e.g. An et al., 1999; Guo et al., 2004; Xu and Fang, 2008). This

drying event in Northwest China began at 8.4 Ma, and strengthened around 6.4 and 5.3 Ma (Xu and Fang, 2008). Thus, the first strengthening of the aridification may explain this divergence event as valid obstacle to migrating for the ancestors of Nectogalini.

Also, a rapid radiation around the M/P boundary might be a general event for many animals. A global cooling and drying trend around the Miocene/Pliocene boundary has been well-documented (e.g. García-Alix et al., 2008; Xu and Fang, 2008). This global climatic change, and the following turnover of vegetation and habitat (Cerling et al., 1997), may be one of the most significant reasons for this wave of species radiation. This radiation includes Asian groups of Nectogalini as well as bears (Krause et al., 2008), cats (Johnson et al., 2006), primates (Kumar et al., 2005), procyonids (Koepfli et al., 2007), woodpeckers (Fuchs et al., 2007) and cyprinoids (Perez-Rodriguez et al., 2009).

What factors are responsible for the southward migration of Asian Nectogalini shrews? We propose that cooling and desiccating events play a key role. Under this scenario, global climatic changes around the M/P boundary caused shrews to retreat to more southern latitudes in Europe (Reumer, 1989). In Asia, fossils of Nectogalini in the Late Miocene deposits were found exclusively in Transbaikalia (around 50° north latitude), but were present in more southern latitude areas in the Early Pliocene such as Lintai, Gansu, China and Yushe, Shanxi, China (around 34–37° north latitude) (Fig. 4). Thus, it seems likely that the climatic changes not only resulted in the radiation of Nectogalini but also caused simultaneous retreating of Asian groups to more southern latitudes.

The global cooling and desiccating event around the P/P boundary (about 2.4–1.8 Ma) has also been well-documented (e.g. Bonnefille, 1983; Demenocal, 2004; Fujiki and Ozawa, 2008; Lunt et al., 2008; Webb and Bartlein, 1992). In Europe, it caused the retreat of shrews to more southern latitudes and diminished both species diversity and abundance (Reumer, 1989). In Asia, this event may be also responsible for extinction of Nectogalini in Transbaikalia (Alexeeva and Erbajeva, 2005), Gansu (An et al., 1999) and Shanxi (Li et al., 2004). In the Early Pleistocene, fossils have been found only south of the Qinling Mountains and Huaihe River, the boundary of Oriental Region and Palearctic Region. Furthermore, the retreat of Nectogalini was not an isolated event but was relevant to the Cenozoic mammalian faunal regions evolution in China. The differentiation of mammals in China began during the Miocene, and became more distinct in the Pliocene. In the Pleistocene, the boundary of the Oriental and Palearctic Regions had been very clear (Qiu and Li, 2005; Tong et al., 1996). Thus, the evolution of Nectogalini is concordant with the evolution of mammalian faunal regions and reflects the global climatic changes as well as elevation of the Qinghai-Tibet Plateau (Jin et al., 2009).

Like many other animals and plants (Hewitt, 2000), the shrews in Europe were also strongly influenced by climatic situation in the Pleistocene and Holocene. In Nectogalini, the Pleistocene ice age may have induced the extinction of the widespread genus *Asoriculus*. Also, the humid and warm climate since the end of the Pleistocene may be responsible for the speciation of some *Sorex* shrews (Reumer, 1989). Accordingly, the ice age, especially the LGM, must have strongly influenced the Asian nectogalines. We speculate it may have lead to retreating of these groups to Japan, Taiwan, Southwest China and even more southern latitude regions in Southwest Asia as refugia where they are primarily distributed today (Hutterer, 2005). After the LGM, the warm and humid climate might have allowed *Chimarrogale* to spread to most areas of Middle and South China (see Section 4.4).

4.4. Adaptation of three aquatic shrews

Our molecular phylogenetic analysis infer strong support for the paraphyly of the aquatic shrews *Chimarrogale*, *Nectogale*, and *Neomys* (Figs. 2 and 3). Moreover, Bayesian ancestral state reconstructions (Table 4) infer significant support for the hypothesis that the transition to aquatic environments by *Neomys*, and the lineage leading to *Chimarrogale* + *Nectogale*, evolved independently. Although the posterior probability of a non-aquatic state is not well-supported in the immediate MRCA of *Neomys* and other Nectogalini species, (PP = 0.76), there is strong support for this in deeper nodes of the tree. More importantly, however, the posterior probability of a non-aquatic state in the closest well-supported ancestor of *Chimarrogale* + *Nectogale* (t21) is not only significant (PP = 0.98), but this node is exclusive of the non-aquatic ancestor that gave rise to the *Neomys* lineage. Thus, the ability to utilize aquatic environments in these two lineages derived from different, non-aquatic ancestors.

Paleontological evidence also supports the paraphyly of Nectogalini water shrews. To date, the fossils of *Chimarrogale* and *Nectogale* have only been found in China and Japan (Supplementary Material Appendix S2). The oldest fossil of water shrews in Asia was *Chimarrogale* sp. in Gansu, China from the Early Pliocene (Storch et al., 1998). The oldest fossil of *Neomys* was discovered in Uryv, Russia from the Late Pliocene (Rzebik-Kowalska, 1998), and most *Neomys* fossils were found in Europe. The only *Neomys* fossil found in Asia was *Neomys* fodiens, a modern species, in Irkutsk, Russia from the Late Pleistocene (Rzebik-Kowalska, 2008). So it is conceivable that *Neomys* and *Chimarrogale* + *Nectogale* originated in Europe and Asia independently.

Therefore, there exist at least two independent derivations of an aquatic lifestyle in *Neomys* and the lineage leading to *Chimarrogale* + *Nectogale*, thus suggesting a strong selective pressure to adapt to aquatic environments.

What factors contributed to this transition to an aquatic niche? The ancestor to extant Nectogalini shrews may have been preadapted to inhabiting aquatic habitats. It is well-known that soricines have high metabolic rates (Taylor, 1998). These higher metabolic rates may serve as an adaptation to vigorous exercise in cold water, such as diving and foraging (Churchfield, 1990). On the other hand, because Nectogalini shrews, in general, inhabit damp environments, Reumer (1984) hypothesized that the extinct genus *Asoriculus* was also adapted to moist or wet environments. This point of view is widely accepted by subsequent authors (García-Alix et al., 2008; Rofes and Cuenca-Bescós, 2006), though questioned by Popov (2003). Furthermore, fossils of *Asoriculus* coexist in geological deposits with aquatic animals including hippopotamus, beaver, and duck (Rofes and Cuenca-Bescós, 2006). The genus *Asoriculus* may have become extinct by the Middle Pleistocene (Supplementary Material Appendix S2) was explained as the result of unstable climatic conditions (Rofes and Cuenca-Bescós, 2006). Because it is one of the oldest discovered Nectogalini taxa, we speculate that *Asoriculus* was an inefficient aquatic forager (at least, not as efficient as *Neomys*) and this may explain why *Asoriculus* became extinct in the Pleistocene while *Neomys* increased its distribution and lived through the LGM.

The benefit of aquatic life is obvious. High metabolic rate leads to high energy budgets for an individual (Genoud, 1988). Soricines consume at most as much food as three times their body weight in 24 h and can only survive a few hours without feeding (Whitaker, 2004), thus necessitating a large and regular food supply. Although *Neomys* consumes mainly terrestrial prey, and aquatic prey comprise only 11–27% of their diet (Churchfield and Rychlik, 2006; Churchfield et al., 2006), there is evidence that *Neomys* consumes more aquatic food when terrestrial food supply is scarce (Castién, 1995). Thus, the ability to forage in aquatic environments could reduce both intraspecific and interspecific competition and help *Neomys* persist through a harsh climate with a more stable food supply, especially when terrestrial food is scarce (e.g. the winter or periods of global cooling). This ability may have helped *Neomys*

live through the Quaternary glaciation, making it the only surviving genus in Europe. Although few studies have been conducted with the Asian water shrews, we note that the distribution of genus *Chimarrogale* is the largest among Asian Nectogalini, and it is the only genus distributed to the eastern coastal area of China and Indonesian Islands. It is possible that this genus' aquatic life mode has contributed to its adaptive capacity and dispersal ability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.03.039.

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