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RESEARCH ARTICLE

Establishing the foundation for an applied molecular taxonomy of otters in Southeast Asia

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Abstract Four species of otters (Mustelidae, Lutrinae) occur in Southeast Asia and are considered to be of conservation concern: *Aonyx cinerea* (Asian small-clawed otter), *Lutra lutra* (Eurasian otter), *Lutra sumatrana* (Hairy-nosed otter), and *Lutrogale perspicillata* (Smooth-coated otter). Among these, *L. sumatrana* is endemic to the region, yet little is known about its biology, and the precise distribution of all four species in Southeast Asia is not well known. Furthermore, the taxonomy and systematics of *L. sumatrana* and *L. perspicillata* have been the subject of controversy, which has implications for the legal protection and for conservation programs of these taxa. To resolve these controversies, we used a multigene data set comprised of segments from 13 nuclear and 5 mitochondrial loci (11,180 nucleotides) to evaluate the phylogenetic relationships of Asian Old World otters. Phylogenies were also estimated using two mitochondrial loci (1,832 nucleotides) obtained from two or more individuals of the four Southeast Asian species. The results from maximum parsimony, maximum likelihood and Bayesian inference

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showed that *L. sumatrana* and *L. lutra* are sister taxa, whereas *L. perspicillata* is sister to *A. cinerea*. Furthermore, the results from the two-mitochondrial gene analyses indicate that *L. sumatrana* is reciprocally monophyletic with respect to *L. lutra*, supporting the specific validity of the former taxon. Signs such as tracks and feces are often used in field surveys to provide information on the distribution and abundance of otters, but the accuracy of these methods may be compromised when several closely related species occur sympatrically. Therefore, the two-gene data set was used to develop a provisional set of diagnostic nucleotides that can be potentially used to identify the four species of Southeast Asian otters from noninvasively collected biological samples, such as feces.

Keywords Otter · Lutrinae · *Lutra sumatrana* · Molecular phylogeny · Molecular taxonomy · Southeast Asia · Conservation genetics

Introduction

Knowledge of the distribution and abundance of species is critical to developing effective conservation plans. Along with more traditional field-based survey methods, molecular methods are being increasingly employed to help establish the presence of particular species within a geographic region (Schwartz et al. 2007). Molecular methods are especially helpful in studying species such as those in the Mustelidae (Mammalia, Carnivora) that are elusive and cryptic in habit and thus difficult to observe directly. For such species, data from tracks, feces, and scent marking stations collected during surveys may provide indirect evidence that a particular species is present (e.g., Kruuk et al. 1993). When multiple species occur sympatrically, however, accurate species identification from these signs may be difficult, even when species are distantly related (e.g., Davison et al. 2002). Reliably distinguishing species using signs becomes even more challenging when multiple, closely related species occupy the same area. Under such circumstances, molecular methods have been shown to be especially valuable in identifying different species, usually from fecal DNA samples (Hansen and Jacobsen 1999; Farrell et al. 2000; Riddle et al. 2003; Gómez-Moliner et al. 2004; López-Giráldez et al. 2005; Pilot et al. 2007).

Four species of otters (Mustelidae, Lutrinae) occur sympatrically in Southeast Asia: *Aonyx cinerea* (Asian small-clawed otter), *Lutra lutra* (Eurasian otter), *Lutra sumatrana* (Hairy-nosed otter), and *Lutrogale perspicillata* (Smooth-coated otter), following the classification of Wozencraft (2005). According to the IUCN/SSC Otter Specialist Group action plan (Mason and Macdonald 1990), three of the species (*A. cinerea*, *L. lutra*, and *L. perspicillata*) are of local conservation concern, whereas *L. sumatrana* is considered of global conservation concern. Further, the most recent IUCN Red List (IUCN 2006) categorizes *A. cinerea* and *L. lutra* as nearly threatened, *L. perspicillata* as vulnerable, and *L. sumatrana* as data deficient. Although mammal references with a larger regional coverage (e.g., Lekagul and McNeely 1988; Corbet and Hill 1992; Payne et al. 1985; Melisch 1995) indicate all four otter species occur throughout Southeast Asia, their precise distribution in this region is not well known (Foster-Turley and Santiapillai 1990; Sivasothi and Nor 1994; Conroy et al. 1998; Poole 2003). As is the case for otter species elsewhere and for many species in Southeast Asia in particular (Sodhi et al. 2004), all four species face pressures from habitat loss, pollution, retaliatory killing due to assumed or actual human-otter food competition (Melisch and Lubis 1998) and direct hunting and trade (Mason and Macdonald 1990; Rudyanto and Melisch 1994). Consequently, developing methods to increase knowledge about the ecology of these species is a high priority for their conservation.

Among the four species of otters found in Southeast Asia, *L. sumatrana* is of special concern, as it is the only species endemic to the region. Almost nothing is known about the biology of this species; hence its designation as "data deficient" in the IUCN Red List (IUCN 2006). Records for this species, museum or otherwise, are sparse since it was first described (Gray 1865; see Sivasothi and Nor 1994). Only recently has this species become the focus of intensive research efforts, when individuals were rediscovered during the 1990s in Malaysia (Sebastian 1995) and Thailand (Kanchanasaka 2001). Since then, the presence of Hairy-nosed otters has also been confirmed in Vietnam (Nguyen et al. 2001), Cambodia (Poole 2003), and Sumatra, Indonesia (Lubis 2005). Initial field studies (Kanchanasaka 2001) indicate that the tracks of *L. sumatrana* and *L. lutra* are quite similar, thereby making

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it difficult to distinguish the two species using this method. Moreover, the appearance and composition of feces (or spraints) from *L. sumatrana* overlaps with those from *L. lutra* and *L. perspicillata*, making species identification using this evidence equivocal at best (Kanchanasaka 2001). DNA analysis of fecal samples may provide an alternative means to unambiguously differentiate individuals from these three species. However, diagnostic molecular characters for all four species in the region must be discovered before such molecular analyses can be implemented.

Another important aspect relevant to the conservation of these otters is their taxonomy, especially with regards to the Hairy-nosed and Smooth-coated otters. Both species have had a complicated taxonomic history (see Sivasothi and Nor 1994). Although there is now general agreement that *L. sumatrana* is a valid species, this was not always the case. Due to the high similarity in behavior, body size and morphology of *Lutra lutra* and *L. sumatrana*, some authors classified the latter as a subspecies of *L. lutra*, possibly related to the Indo-Malayan *Lutra lutra barang* (e.g., Davis 1978; see Sivasothi and Nor 1994). The Smooth-coated otter has at times been placed in the genus *Lutra* (e.g., Chasen 1940; Harris 1968). However, classification of Smooth-coated otters into the genus *Lutrogale* is meant to reflect the distinctive differences in behavior and morphology this species exhibits compared to otters in the genus *Lutra* (Duplaix 1975; Hwang and Larivière 2005). Different taxonomic classifications for the same set of species can potentially affect conservation priorities and actions by altering the perceived "evolutionary value" of taxa (e.g., Avise and Nelson 1989; Daugherty et al. 1990; Bowen et al. 1991; also see Thompson 1997). For example, recognition of *L. sumatrana* as a distinct species elevates its conservation status considerably as opposed to its classification as a distinct subspecies of the widespread *L. lutra*. In general, taxonomic classifications that reflect phylogenetic relationships as accurately as possible convey more information and thus have a greater scientific and conservation value. Therefore, understanding the phylogenetic relationships among otters, and of Hairy-nosed and Smooth-coated otters in particular, will help to provide a foundation for more informed conservation management decisions.

Here, we use a multigene data set that expands upon our previous analyses (Koepfli and Wayne 1998, 2003) to investigate the molecular systematics of otters to establish a more precise taxonomy, especially with regards to Old World taxa. Furthermore, the phylogenetic relationships and genetic divergence of *Aonyx cinerea*, *Lutra lutra*, *L. sumatrana*, and *Lutrogale perspicillata* were examined in greater depth through the use of two mitochondrial DNA (mtDNA) gene regions from the multigene data set and samples of two or more individuals from each of the four taxa. The mitochondrial data set was used to develop a provisional set of diagnostic characters for identifying noninvasively collected biological samples from the four species found in Southeast Asia. Finally, we discuss the evolutionary and conservation significance of our findings.

Materials and methods

Study design

To estimate the phylogenetic relationships among otters distributed in Eurasia and to assess the specific status of the Hairy-nosed otter within the context of the phylogenetic species concept (e.g., Cracraft 1983), we assembled two data sets of DNA sequences for phylogenetic analysis that differed in taxon representation and sequence composition. The first data set (hereafter referred to as the multigene data set) contained 15 taxa that included 11 otter species and four outgroup species: American mink (*Neovison vison*), long-tailed weasel (*Mustela frenata*), zorilla (*Ictonyx striatus*), and striped weasel (*Poecilogale albinucha*). These four species were used as outgroups, based on their close relationship to the Lutrinae, as inferred from previous and ongoing molecular systematic studies of the Mustelidae (Koepfli and Wayne 2003; Sato et al. 2004, 2006; Flynn et al. 2005; Koepfli et al. unpublished data). This data set was composed of 15 sequence segments from 13 nuclear genes and three segments from five mitochondrial genes (11,180 bp total, after exclusion of 9 bp from one of the nuclear gene segments; see below) (see Supplementary Table 1).

The second data set (hereafter referred to as the two-gene data set) contained 59 terminals, including multiple individuals of *Aonyx cinerea*, *L. lutra*, *L. sumatrana*, and *Lutrogale perspicillata*. The three otter species in the genus *Lontra* were used as outgroups based on previous research that showed this clade of otters are the sister group to the clade comprised of Old World river otters and the sea otter (Koepfli and Wayne 1998, 2003). This data set was comprised of sequences from two of the mitochondrial genes also used in the 15 taxa data set, *CYTb* and *NADH5* (1,832 bp total). This data set was also used to establish diagnostic nucleotide differences among the four species known to be distributed in Southeast Asia (see below).

Sample collection

Tissue or hair samples were obtained from wild-caught Hairy-nosed otters in Thailand ($n = 2$) and Vietnam ($n = 2$). Hair samples from Smooth-coated otters were collected from four individuals at three different zoos: (1)

one male and one female from Khao Khiew Zoo, Chon Buri Province, Thailand, both of which originated from southern Thailand; (2) one male from Angkor Zoo, Siem Reap Province, Cambodia; and (3) one female from Tampok Zoo, southwest of Phnom Penh, Cambodia. At all three zoos, Smooth-coated otters were never kept with other species of otters, including Asian small-clawed otters, with which they are known to hybridize (Melisch and Foster-Turley 1996). Forty-one tissue samples were collected from Eurasian otters from the following localities and/or countries: Orkney Islands ($n = 3$), Western Wales ($n = 2$) and East Anglia ($n = 2$), United Kingdom; Mogeely ($n = 1$) and Rathcormac ($n = 1$), Ireland; Portugal ($n = 4$); Badajoz, Spain ($n = 2$); Brittany, France ($n = 2$); Germany ($n = 2$); Waldviertel, Austria ($n = 2$); Jutland, Denmark ($n = 2$); Norway ($n = 3$); Hungary ($n = 3$); Belarus ($n = 2$); Kavala, Greece ($n = 1$); Israel ($n = 1$); Primorski Province, Russia ($n = 1$); Novosibirsk Zoo, Russia ($n = 1$); and South Korea ($n = 6$). Nearly all of the Eurasian otter samples were from individuals that had been found road-killed or had died from other causes. Sample information for other otter species used in this study can be found in Koepfli and Wayne (1998, 2003).

Laboratory methods

Total genomic DNA was extracted from hair or tissue samples using phenol chloroform methods, followed by ethanol precipitation (Sambrook et al. 1989) or using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Fifteen nuclear and three mitochondrial gene segments were amplified using published primers (see Supplementary Table 1) and the polymerase chain reaction (PCR). PCR was carried out in an MWG-Biotech Primus 96 Plus thermal cycler with the following conditions: 28–30 cycles of 94°C for 30 s, 50–56°C for 30 s, 72°C for 45 s, and one cycle of 72°C for 5 min. Each 50 µl reaction contained 35.7 µl sterile double-distilled water, 5 µl 10× PCR buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of both 25 pM/µl forward and reverse primers, 0.3 µl *Taq* polymerase (Sigma-Aldrich, St. Louis, MO), and 1 µl of 0.1–1 µg genomic DNA. All PCRs included a negative control (no DNA). PCR products were run on and excised from 1% agarose/Tris-acetic acid-EDTA gels and purified using an Ultra Clean Kit (MoBio Laboratories, Solana Beach, CA). PCR products were then cycle sequenced using the original amplification primers and either the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Fullerton, CA) or the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing reactions were then run through either an 8-capillary CEQ 2000XL DNA Analysis System or a 48-capillary Applied Biosystems 3730 DNA Analyzer.

The DNA extract from hair samples of *L. perspicillata* did not yield a sufficient amount of DNA for direct amplification of the nuclear gene segments. We therefore whole-genome amplified these samples using the GenomiPhi V2 DNA Amplification Kit (Amersham Biosciences, Little Chalfont, UK). PCR tests with several of the nuclear primers resulted in successful amplification for one of the *L. perspicillata* and this sample was then used to amplify the 15 nuclear gene segments. To ensure that the whole-genome amplification process had not introduced any errors into our target sequences, we amplified the whole genome from the same individual a second time and then amplified and sequenced several nuclear loci to compare the sequences from the two samples. All sequences were identical from the two samples. Finally, we were unable to obtain clean sequence for the first ~244 bp of the *CYTb* gene from the two *L. perspicillata* samples from Thailand and question marks were used to represent missing data for these two individuals.

The *CHRNA1* PCR product from *Lutra sumatrana* was found to contain a heterozygous 19 bp insertion and deletion (indel) that resulted in poorly resolved chromatograms when the PCR product was directly sequenced. Therefore, prior to cycle sequencing, the PCR product was cloned using a TOPO TA Cloning Kit for Sequencing and transformed in One Shot Top10 competent *Escherichia coli* cells (Invitrogen Corporation, Carlsbad, CA). Positive clones were picked and cultured overnight and plasmid DNA was then isolated using a Wizard Miniprep Kit (Promega, Madison, WI).

Data analyses

Sequence chromatograms were checked for accuracy and edited using Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, MI). New sequences for six gene segments (*APOB-29*, *CHRNA1*, *FES*, *GHR*, *RHO1*, and *CYTb*) were combined with sequences from previous studies (Koepfli and Wayne 1998, 2003). Multiple alignments of exon and mitochondrial coding sequences were facilitated by translating these sequences into amino acids and comparing them with the orthologous human gene sequence. Multiple sequence alignments for all other gene segments were generated using Clustal X (Thompson et al. 1997), with default settings, and manually adjusted. For the segments that contained both exon and intron sequences, alignments were verified by checking that GT-AG splice junctions occurred at exon–intron boundaries (Breathnach et al. 1978). A 9-bp region of the *WT1* gene segment, corresponding to a poly-A track of various lengths in different

species, could not be aligned unambiguously and was excluded from the analyses. This reduced the multigene data set to 11,180 bp. Gaps, corresponding to indels, were introduced into the alignments of seven of the gene segments (*CHRNA1*, *FES*, *GHR*, *GNAT1*, *PLCB4*, *WT1*, and *12S-16S*). Novel sequences generated for this study were deposited in Genbank (see Supplementary Table 2 for accession numbers).

Sequences were concatenated into two data sets (the multigene and two-gene data sets, see above). For the two-gene data set, sequences from four otter species represented by multiple individuals (*Aonyx cinerea*, $n = 2$; *L. lutra*, $n = 41$, *L. sumatrana*, $n = 4$, and *Lutrogale perspicillata*, $n = 4$) were collapsed to haplotypes using the program Collapse v1.1 (available from http://www.darwin.uvigo.es). This resulted in a data set containing 30 ingroup terminals.

Phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) methods. We used PAUP* 4.0b10 (Swofford 2002) to reconstruct maximum parsimony and maximum likelihood phylogenetic trees. For parsimony searches, all characters were equally weighted. Gaps were coded as present or absent (1 or 0), regardless of length, to utilize their potential phylogenetic signal (Barriel 1994). We performed heuristic searches using 100 random sequence additions, with one tree held at each step during stepwise addition, tree-bisection-reconnection branch swapping, steepest descent option not in effect, no upper bound for MaxTrees, and MulTrees option in effect. Jackknife analyses were conducted to evaluate the robustness of clades, using 10,000 replicates, with all uninformative characters excluded (Cunningham 1997), 50% of the characters deleted in each replicate and the same heuristic search conditions as described above. Branch support (BS) and partitioned branch support (PBS) for clades (Bremer 1988) were also calculated using TreeRot v2 (Sorenson 1998).

The GTR + I + G and the GTR + I models were selected as the best-fitting models of DNA substitution for the multigene and two-gene data sets, respectively, using the Akaike Information Criterion (AIC) as implemented in Modeltest v3.7 (Posada and Crandall 1998). These models and their estimated parameters were specified in maximum likelihood heuristic searches, using the same search conditions as used in the parsimony searches and gaps coded as missing. Bootstrap analyses were performed using 500 pseudoreplicates and the same search conditions as previously described, except only 5 random sequence additions were used.

We used MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian phylogenetic analysis. The AIC as implemented in MrModelTest v2.2 (Nylander 2004) selected the HKY + G model and GTR + I model for the multigene and two-gene data sets, respectively. We analyzed the multigene data set under three different partitioning strategies to examine the effect data partitioning has on the estimated topology: (1) unpartitioned, where all nucleotide positions in the multigene data set evolve under the same nucleotide substitution model (i.e., HKY + G); (2) two partitions, where the nuclear portion and mitochondrial portion of the multigene data set evolve under two different substitution models (HKY + I + G and GTR + I + G, respectively); and (3) 18 partitions, where the 18 gene segments evolve under their respective model of substitution, as determined using MrModelTest. For the last strategy, the GTR model was selected for each of the three mitochondrial gene segments, the K80 model was selected for three of the nuclear gene segments (*CHRNA1*, *RAG1*, *WT1*), the HKY model was selected for eight nuclear gene segments (exon 26 and exon 29 of *APOB*, fragment 2 of *BRCA1*, *FES*, *GNAT1*, *PLCB4*, *RAG2*, and *TMEM20*), the GTR model was selected for three nuclear gene segments (fragment 1 of *BRCA1*, *GHR*, and *RHO1*), and the SYM model was selected for the *COL10A1* segment. Rate heterogeneity among nucleotide sites was accounted for by including I, G, or I + G parameters in these models. The following set of priors were used for the multigene data set: a Beta prior for the transition/transversion rate ratio, a Dirichlet prior for base frequencies, a uniform prior for the gamma shape parameter, all topologies equally probable, and unconstrained branch lengths with an exponential probability density. For the two-gene data set, we used Dirichlet priors for the six substitution rates of the GTR model, a Dirichlet prior for base frequencies, a uniform prior for the proportion of invariable sites, all topologies equally probable, and unconstrained branch lengths with an exponential probability density. For the multigene and two-gene data sets, two independent runs of four Metropolis-coupled MCMC chains were conducted for 5×10^6 generations, sampling trees every 500 generations. For each independent run of the two data sets, the first 1,000 trees were discarded as burn-in. Tracer plots (Rambaut and Drummond 2003) and potential scale reduction factors (PSRF) of 1.00 showed that log-likelihood scores and model parameters had converged on a stationary distribution following burn-in. Further, the effective sample size values for estimates of the posterior distribution of the tree likelihood and model parameters were greater than 200, as determined with Tracer 1.3 (Rambaut and Drummond 2003). For each data set, the two independent MCMC runs generated a combined total of 18,002 trees. However, we were conservative in estimating consensus trees and clade credibility values, and thus based these estimates on a total of 15,002 trees for each data set.

We used the two-gene mtDNA data set and population aggregation analysis (Davis and Nixon 1992) to establish diagnostic nucleotide differences for the four otter species distributed in Southeast Asia. Nucleotide changes that were exclusive to each of the four species were used to identify

"pure" (fixed) diagnostic characters (Davis and Nixon 1992). Using this approach, we establish a character based DNA barcode for each species that is independent of a tree-based approach to species identification (DeSalle et al. 2005).

Results

Phylogenetic analysis of the multigene data set

Phylogenetic analysis of the 11,180 bp multigene data set using ML and BI resulted in trees with identical topologies (Fig. 1). Moreover, consensus tree topologies were identical among the three partitioning strategies implemented in the Bayesian analyses. The results show that *Lutra sumatrana* and *Lutrogale perspicillata* are members of a clade containing other Old World river otters (*Aonyx*, *Lutra*, and *Hydrictis*) and the sea otter (*Enhydra*) (node 4). Within this clade, *L. sumatrana* is placed as sister to *L. lutra* (node 8) whereas *Lutrogale* is placed as sister to *Aonyx cinerea* (node 7). The Old World clade is sister to a clade comprised of river otter species from the New World (*Lontra*) (node 5). Finally, the phylogenetic analyses support the placement of the giant otter (*Pteronura*) from

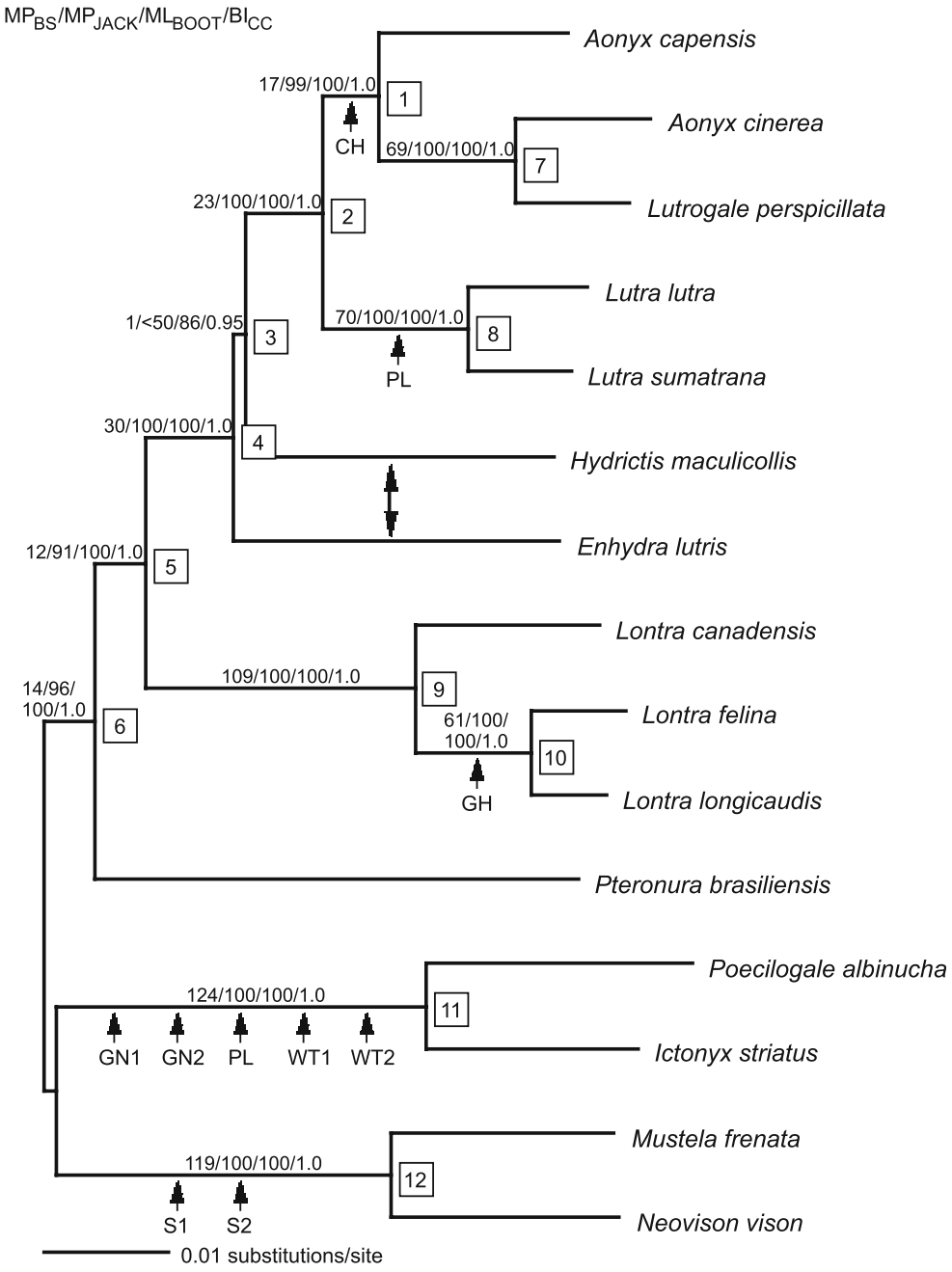

Fig. 1 Phylogenetic tree based on maximum likelihood analysis of the multigene data set using the GTR + I + G model (−ln L = 32688.380). Numbered boxes denote nodes. Branch support (MP_{BS}) and jackknife proportions (MP_{JP}) from MP, bootstrap proportions from ML (ML_{BP}) and clade credibility values from BI (BI_{CC}) are shown above internodes from left to right. The clade credibility values shown are based on the unpartitioned analysis. Single-headed arrows below internodes denote clades supported by parsimony-informative indels from particular gene segments. Gene segments are abbreviated as follows: CH = *CHRNA1*, GH = *GHR*, GN = *GNAT1*, PL = *PLCB4*, S = *12S–16S rRNA*, WT = *WT1*. Numbers following abbreviation indicate more than one indel for that particular gene segment. Double-headed arrow indicates that *Enhydra lutris* and *Hydrictis maculicollis* switch their positions in the MP tree. Branch lengths are proportional to number of substitutions per site (see scale bar)

South America as the sister to the ((*Aonyx*, *Lutrogale*, *Lutra*), (*Lontra*)) clade (node 6).

Gap/indel coding (see Materials and methods) for parsimony analysis reduced the multigene data set from 11,180 to 11,166 bp. This data set contained 1,779 variable characters and 1,172 parsimony-informative characters. Phylogenetic analysis resulted in a single most parsimonious tree of 3,353 steps and a retention index (RI) of 0.57. This tree was similar to the ML and BI trees in all respects except that the positions of *Enhydra* and *Hydrictis* were switched. In the MP tree, *Enhydra* was sister to the clade containing *Aonyx*, *Lutra*, and *Lutrogale*, whereas in the ML and BI trees, this position was occupied by *Hydrictis*. However, the branch separating *Enhydra* and *Hydrictis* (node 3) is the shortest internode within the ingroup and is also the least robust, with a branch support value of 1 and <50% jackknife value in the MP tree, and a bootstrap value of 86% in the ML tree (Fig. 1). The clade credibility value of node 3 was 0.95 in the unpartitioned and 18 partition Bayesian consensus trees, whereas this node was unresolved in the two-partition analysis. All other phylogenetic relationships recovered in MP, ML, and BI analyses were supported by maximum or near maximum jackknife, bootstrap, and clade credibility values, respectively. Further, several clades were supported by parsimony-informative indels. The clade that includes the two species of *Aonyx* and *Lutrogale* was supported by a 3 bp insertion within the *CHRNA1* intron, a 1 bp deletion in *PLCB4* was a synapomorphy for *L. lutra* + *L. sumatrana* and a 2 bp deletion within the *GHR* intron supported the *Lontra felina* + *L. longicaudis* clade. Finally, PBS analyses showed that the more rapidly evolving mitochondrial gene segments contributed more phylogenetic information to the support of the multigene phylogeny than the slower evolving nuclear gene segments (Table 1). Among the 15 nuclear gene segments, *WT1*, *RAG1*, and fragment 2 of *BRCA1* were the most informative. The number of gene segments contributing congruent phylogenetic signal (i.e., positive PBS values) to the 12 nodes of the MP phylogeny ranges from 3 to 18 (median = 11 gene segments). The relatively low amount of conflicting phylogenetic signal, as indicated by negative PBS values, indicates that congruence among the data partitions is generally high (Table 1).

Phylogenetic analysis of the two-gene data set

Analysis of the two-gene data set revealed 20 haplotypes among the 41 individuals of *L. lutra* sampled from throughout Eurasia (Table 2). Most haplotypes were restricted to single localities, although several localities

Table 1 Partitioned branch support (PBS) values for the 12 nodes shown in Fig. 1

Partition	Node												% of Total
	1	2	3	4	5	6	7	8	9	10	11	12	
APOB26	3	1	0	0	0	−1	1	2	5	1	9	9	4.6
APOB29	0	0	0	0	0	3	−1	1	1	0	2	5	1.7
BRCA1F1	0	0	−1	−1	0.5	5	5	1	1	0	7	7	3.8
BRCA1F2	0	4	0	3	2	3	1	3.5	4	0	11	12	6.7
CHRNA1	3	−1	−1	0	−1.5	1	1	1	7	0	3	1	2.1
COL10A1	1	0	0	1	0	1	0	0	3	0	5	5	2.5
FES	0	1	0	3	0	0	0	3	4	0	3	9	3.5
GHR	0	2	0	1	2.5	1	3	2	10	3	6	7	5.8
GNAT1	0	0	0	1	0	0	0	2	4	0	12	2	3.2
PLCB4	0	1	0	0	−1	−1	0	1	1	1	5	1	1.2
RAG1	0	4	1	5	2	3	2	4.5	6	1	6	13	7.3
RAG2	1	0	0	2	0	1	0	1	2	0	6	1	2.2
RHO1	0	0	0	1	0	1	0	1	0	1	3	1	1.2
TMEM20	2	0	0	1	0	0	2	4	4	1	4	3	3.2
WT1	0	4	2	3	4	1	2	2	8	1	14	10	7.9
CYTB	1	8	0	6	4	−4	17	16.5	26	20	7	18	18.4
NADH5	3	−1	0	4	−1.5	−2	13	15.5	8	16	17	3	11.6
12S/16S	3	0	0	0	1	2	23	9	15	16	4	12	12.9
Total nodal BS	17	23	1	30	12	14	69	70	109	61	124	119	

Total nodal branch support (BS) values are from Fig. 1 and % of Total indicates relative contribution of phylogenetic information from each gene segment to the support of the MP multigene phylogeny

Table 2 Distribution of 20 haplotypes among 41 samples of *L. lutra*

Locality	Haplotype																				Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Orkney I., UK	3																				3
Ireland		2																			2
W. Wales, UK	2																				2
East Anglia, UK	1	1																			2
Portugal		2		2																	4
Spain				1	1																2
France		2																			2
Germany		1				1															2
Denmark							2														2
Austria		2																			2
Norway								1	1	1											3
Hungary		2									1										3
Belarus												1	1								2
Greece														1							1
Israel															1						1
Russia																1	1				2
South Korea																		4	1	1	6
Total	3	14	1	3	1	1	2	1	1	1	1	1	1	1	1	1	1	4	1	1	41

(e.g., Norway) were represented by multiple haplotypes. Haplotype 2, however, was found in Eurasian otters distributed across a wide area in Western Europe (Table 2). Haplotypes from *L. lutra* differed from one another by 1–21 bp (0.05–1.15% uncorrected distance), with the three haplotypes identified among six Eurasian otters from South Korea showing the most differences. Among the four individuals sampled from *L. sumatrana*, we found two haplotypes that differed by 4 bp (0.22%), with one haplotype occurring in the two individuals from Thailand and the other occurring in the two individuals from Vietnam. Three haplotypes that differed by 2–8 bp (0.11–0.50%) were found among the four individuals of *Lutrogale perspicillata*, with haplotype 3 being shared between two individuals, one from Cambodia and the other from Thailand. The other two haplotypes (*L. perspicillata* 1 and 2) were found in Smooth-coated otters from Thailand and Cambodia, respectively. Finally, the two individuals of *Aonyx cinerea* represented two different haplotypes that differed by 1 bp (0.06%).

Haplotypes from each of the four species formed well-supported monophyletic groups in MP, ML, and BI analyses, using the three species of *Lontra* as outgroups (Fig. 2). Within *Lutra lutra*, the three haplotypes from South Korea (*L. lutra* 18, 19, and 20) form a well-supported subclade that is joined with a haplotype (17) from a Eurasian otter sampled from East Anglia, UK. Most *L. lutra* haplotypes (1–16) are joined together in another subclade, with low to moderate support. Phylogenetic relationships among species in *Aonyx*, *Lutra*, and *Lutrogale* are congruent with relationships based on the much larger multigene data set. As in the phylogeny based on the latter data set, the relationships of *Enhydra* and *Hydrictis* differ according to the reconstruction method used. ML and BI analyses again place *Hydrictis* sister to the clade containing *Aonyx*, *Lutra*, and *Lutrogale*, whereas MP analysis (1 tree, 1,163 steps, RI = 0.82) joins *Enhydra* and *Hydrictis* into a clade. Nonetheless, both of these relationships received low support (Fig. 2).

Diagnostic nucleotides in the two-gene data set

Population aggregation analysis of the two-gene data set comprised of two or more individuals from each of the four species of otters showed that they could be distinguished by diagnostic nucleotides found in both the *CYTb* and *NADH5* genes (Tables 3, 4). As phylogenetic analyses showed that *Lutra lutra* was sister to *L. sumatrana* (Figs. 1, 2), these two species were distinguished by five nucleotides each with the *CYTb* gene and three (*L. lutra*) and four (*L. sumatrana*) nucleotides with the *NADH5* gene. Similarly, *Lutrogale perspicillata* and *Aonyx cinerea* were respectively distinguished by eight and five nonoverlapping nucleotides using the *CYTb* gene, and seven and 10 nucleotides using the *NADH5* gene. Among the 47

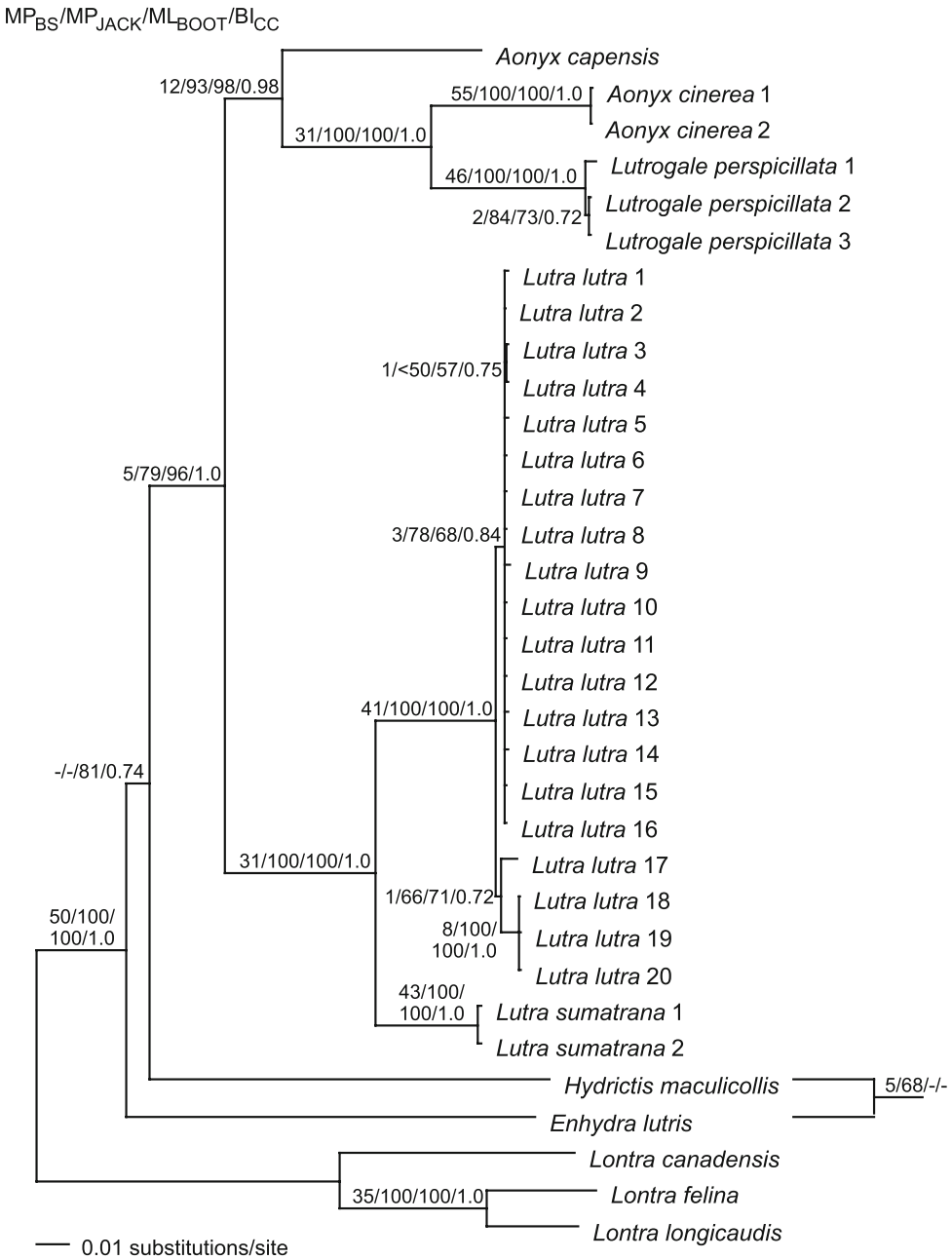

Fig. 2 Phylogenetic tree based on maximum likelihood analysis of the two-gene data set (*CYTb* and *NADH5*) using the GTR + I model ($-\ln L = 7497.784$). Branch support (MP_{BS}) and jackknife proportions (MP_{JP}) from MP, bootstrap proportions from ML (ML_{BP}) and clade credibility values from BI (BI_{CC}) are shown above or to the left of internodes. Numbers to the right of taxon names indicate haplotype number. *Enhydra lutris* and *Hydrictis maculicollis* are sister taxa in the MP tree (shown as a clade to the right of these two taxa) and this clade is then sister to the remaining Old World otters. See Table 2 for locality information of haplotypes from *Lutra lutra*. Branch lengths are proportional to number of substitutions per site (see scale bar)

diagnostic nucleotides for the four species, the majority of changes involved transition substitutions (41 transitions and 6 transversions).

Discussion

The present study builds upon previous work that dealt either directly or indirectly with the molecular phylogenetics of otters (Koepfli and Wayne 1998, 2003). In agreement with those studies, our results show that otter species are grouped into three primary lineages: one containing the sea otter and river otters of Eurasia and Africa (*Aonyx*, *Enhydra*, *Hydrictis*, *Lutra*, and now *Lutrogale*); another containing river otters from North and South America (*Lontra*); and a third that includes the giant otter (*Pteronura*), which is sister to the other two lineages and forms the first divergence within the extant Lutrinae. Except for the placement of *Enhydra* and *Hydrictis*, all methods of phylogeny reconstruction recovered the same robust topology with maximal or near maximal branch support (Fig. 1). This robustness stems from the large number of phylogenetically informative characters included in the multigene data set. Of the 1,172 parsimony-informative

Table 3 Diagnostic nucleotides (bolded) in the *CYTb* gene that distinguish the four species of otters distributed in Southeast Asia

	48	72	168	201	315	472	486	492	516	568	693	702	717	798	831	919	942	978	1033	1035	1053	1065	1140
Aonyx capensis	C	A	C	A	A	A	A	C	A	T	C	C	A	C	A	C	C	A	C	C	C	A	A
Enhydra lutris	.	.	A	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
Lontra canadensis	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lontra felina	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lontra longicaudis	.	.	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hydrictis maculicollis	.	.	A	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.
Aonyx cinerea 1	.	.	**G**	**G**	**G**	.	**G**	.	.	**C**	.	.	.	.	.	.	**T**	.	**T**	.	.	**C**	.
Aonyx cinerea 2	.	.	**G**	**G**	**G**	.	**G**	.	.	**C**	.	.	.	.	.	.	**T**	.	**T**	.	.	**C**	.
Lutra lutra 1	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 2	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 3	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 4	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 5	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 6	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 7	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 8	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 9	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 10	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 11	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 12	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 13	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 14	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 15	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 16	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 17	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 18	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 19	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra lutra 20	.	.	A	.	.	.	.	.	.	.	**A**	.	**T**	.	**G**	**T**	.	.	.	**T**	.	.	.
Lutra sumatrana 1	**T**	**G**	A	.	.	**G**	.	.	**G**	.	.	.	.	.	.	.	.	**G**	.	.	.	.	.
Lutra sumatrana 2	**T**	**G**	A	.	.	**G**	.	.	**G**	.	.	.	.	.	.	.	.	**G**	.	.	.	.	.
Lutrogale perspicillata 1	?	?	?	?	.	.	.	**T**	.	.	.	**T**	.	**T**	.	.	.	.	.	.	**T**	.	**G**
Lutrogale perspicillata 2	.	.	A	.	.	.	.	**T**	.	.	.	**T**	.	**T**	.	.	.	.	.	.	**T**	.	**G**
Lutrogale perspicillata 3	.	.	A	.	.	.	.	**T**	.	.	.	**T**	.	**T**	.	.	.	.	.	.	**T**	.	**G**

Nucleotide positions that are identical in state to the *Aonyx capensis* sequence are denoted with a period (.). Nucleotide position number based on position within the *CYTb* gene alignment (1–1140)

characters in the gap-coded multigene data set, 402 of these were contributed by the 15 nuclear gene segments and 770 were contributed by the three mitochondrial gene segments. The combination of slowly evolving nuclear sequences with fast evolving mitochondrial sequences results in well-resolved topologies with high statistical support for nearly all internal branches of the ingroup. However, despite the large amount of data included in the present study, the branch separating *Enhydra* and *Hydrictis* remains difficult to resolve. This difficulty may be related to the high level of homoplasy associated with such branches, as recently discussed for several well known clades whose resolution has been refractory, regardless of the amount of data applied (Rokas and Carroll 2006). Indeed, *Enhydra* and *Hydrictis* both have long external branches associated with a high level of homoplasy: of the 108 substitutions that occur along the branch separating *Enhydra* and *Hydrictis* in the MP analysis of the multigene data set, 91 (84%) of these are

Table 4 Diagnostic nucleotides (bolded) in the *NADH5* gene that distinguish the four species of otters distributed in Southeast Asia

	1	27	53	55	83	111	119	131	165	210	222	242	257	269	296	341	425	444	492	539	578	592	610	671
Aonyx capensis	T	C	C	T	C	A	C	A	A	C	C	C	A	A	A	A	T	G	A	C	A	A	T	A
Enhydra lutris	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lontra canadensis	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
Lontra felina	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lontra longicaudis	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
Hydrictis maculicollis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Aonyx cinerea 1	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	**G**	**C**	.	.	.	.	**G**	**G**	**C**	.
Aonyx cinerea 2	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	**G**	**C**	.	.	.	.	**G**	**G**	**C**	.
Lutra lutra 1	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 2	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 3	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 4	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 5	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 6	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 7	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 8	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 9	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 10	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 11	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 12	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 13	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 14	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 15	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 16	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 17	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 18	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 19	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra lutra 20	.	**A**	.	.	.	.	.	.	.	.	.	**T**	**G**	.	.	.	.	.	.	.	.	.	.	.
Lutra sumatrana 1	.	.	**T**	**C**	.	.	.	.	.	**T**	**T**	.	.	.	.	.	.	.	.	.	.	.	.	.
Lutra sumatrana 2	.	.	**T**	**C**	.	.	.	.	.	**T**	**T**	.	.	.	.	.	.	.	.	.	.	.	.	.
Lutrogale perspicillata 1	**C**	.	.	.	.	.	**T**	**G**	**G**	.	.	.	.	**T**	.	.	**C**	**A**	**G**	**T**	.	.	.	**G**
Lutrogale perspicillata 2	**C**	.	.	.	.	.	**T**	**G**	**G**	.	.	.	.	**T**	.	.	**C**	**A**	**G**	**T**	.	.	.	**G**
Lutrogale perspicillata 3	**C**	.	.	.	.	.	**T**	**G**	**G**	.	.	.	.	**T**	.	.	**C**	**A**	**G**	**T**	.	.	.	**G**

Nucleotide positions that are identical in state to the *Aonyx capensis* sequence are denoted with a period (.). Nucleotide position number based on position within the *NADH5* gene alignment (1–692)

homoplastic (with a CI <1.0). The majority of the homoplastic substitutions (78/91) derive from the mitochondrial sequence data. This condition can result in long-branch attraction artifacts for methods such as parsimony (Felsenstein 1978), which may explain why *Enhydra* and *Hydrictis* form a clade, albeit with low support, in the MP analysis of the two-gene data set (Fig. 2). Nevertheless, the short branch between *Enhydra* and *Hydrictis* suggests that the cladogenetic event that gave rise to these lineages occurred in rapid succession.

Phylogenetic relationships of *Lutra sumatrana*

The multigene and two-gene data sets indicate that *L. lutra* and *L. sumatrana* are sister species (Figs. 1, 2), thereby confirming the suspected close relationship between these two species. Maximal values of node support across different methods of phylogenetic analyses as well as a synapomorphic 1 bp indel in the PLCB4 gene fragment support this relationship. Our results contrast with prior studies that suggested *L. sumatrana* shared a common

ancestry with *Hydrictis maculicollis*, the latter formerly classified in the genus *Lutra*, based on morphometric similarity of cranial, postcranial, and/or soft anatomical features (Pohle 1919; Pocock 1921; Van Zyll de Jong 1972, 1987). Notably, however, Van Zyll de Jong (1987) also conducted cladistic analyses using 12 anatomical characters (with 44 states) and found that *L. lutra* and *L. sumatrana* were joined as sister species in these analyses. Willemsen (1992) suggested that *L. lutra* and *L. sumatrana* may share ancestry with the extinct *L. palaeindica* (Late Pliocene or Early Pleistocene), which was uncovered in the Siwalik Hills, India.

Using multiple samples from each species, the two-gene data set indicates that *L. lutra* and *L. sumatrana* are reciprocally monophyletic and their respective branches are well supported, thus supporting the specific status of *L. sumatrana*. Intraspecific versus interspecific comparisons of genetic distances further supports the distinction between these two species. The 20 haplotypes we found among the 41 samples of *L. lutra* differ from one another by 0.05–1.15% uncorrected genetic distance (1–21 substitutions) and the two haplotypes from *L. sumatrana* differ by 0.22% (4 substitutions). The difference between *L. lutra* and *L. sumatrana*, however, ranges from 5.68 to 6.17% (104–113 substitutions), clearly indicating that the intraspecific distances do not overlap with the interspecific distance. Further, *L. sumatrana* is distinguished from *L. lutra* by 9 (exclusive) diagnostic nucleotide characters in the two-gene data set (see below). Incidentally, the two species differ by 0.39% uncorrected genetic distance (32 substitutions) in the nuclear portion and 4.82% (139 substitutions) in the mitochondrial portion of the multigene data set.

Morphologically, *L. sumatrana* is distinguished from *L. lutra* by a hair-covered rhinarium (nose pad) and facial pelage coloration (Pocock 1941). Our sampling of *L. lutra* did not include the putative subspecies *L. l. barang*, which is thought to be distributed in Thailand, Vietnam, and Sumatra (type locality) (Pocock 1941; but see Sivasothi and Nor 1994). We were therefore unable to assess the relationship of this taxon to *L. sumatrana*. Even so, skull length measurements of *L. l. barang and L. sumatrana* from multiple localities indicate that the skull of *L. l. barang* is shorter (condylobasal length = 97–106 mm) than that of *L. sumatrana* (condylobasal length = 100–119 mm) (Pocock 1941). Sivasothi and Nor (1994) also noted that the skull of *L. sumatrana* was longer and flatter than that of *L. lutra*. Collectively, these morphological differences reinforce the hypothesis suggested by the genetic data that *L. sumatrana* and *L. lutra* are distinct species, although samples from *L. l. barang* will be necessary to corroborate this hypothesis.

Phylogenetic relationships of *Lutrogale perspicillata*

The phylogenetic affinities of the Smooth-coated otter (*L. perspicillata*) have long been a puzzle ever since the species was first described by Geoffroy St.-Hillaire (1826) and named *Lutra perspicillata*. Gray (1865) later reclassified the species as *Lutrogale macrodus*, but it was not until Pocock (1940) that the current name was first used. Since then, some authors have considered *Lutrogale* synonoymous with *Lutra* (e.g., Chasen 1940) or as a subgenus of *Lutra* (e.g., Ellerman and Morrison-Scott 1966; Harris 1968; Payne et al. 1985) whereas others recognize *Lutrogale* as a monotypic genus (Pocock 1941; Van Zyll De Jong 1972, 1987; Corbet and Hill 1992; Willemsen 1986, 1992; Wozencraft 2005). *Lutrogale* is distinguished from *Lutra* by having a short, smooth pelage, a highly arched skull with large orbits set more anteriorly and laterally, a shortened rostrum, larger teeth, and a tail that is dorsoventrally flattened distally with distinctive integumentary keels (Pohle 1919; Pocock 1941; Harris 1968; Willemsen 1980, 1992; Hwang and Larivière 2005). The characters of the skull, teeth and tail in *Lutrogale* are shared with *Pteronura* (the giant otter, which has a completely keeled tail) and morphometric or cladistic analyses of morphology, as well as comparisons of behavior and vocalizations among lutrines, have suggested a relationship between these genera (Van Zyll de Jong 1972, 1987; Duplaix 1980). Nonetheless, other studies have suggested that *Lutrogale* evolved from *Lutra*-like ancestors (Pohle 1919; Willemsen 1992).

Our results do not support either of these hypotheses and instead indicate that *Lutrogale* shares common ancestry with *Aonyx*, forming a highly supported sister relationship with *A. cinerea* (Figs. 1, 2). Placement of *Lutrogale* within *Aonyx* is also supported by a synapomorphic indel in the *CHRNA1* gene segment. The uncorrected genetic distance between *A. cinerea* and *Lutrogale* is 0.217% (18 substitutions) for the nuclear portion and 6.07% (175 substitutions) for the mitochondrial portion of the multigene data set. The pairing of these two species is surprising given their considerable morphological and ecological differences. The body mass of *Lutrogale* is two to three times larger than that of *A. cinerea* (7–11 vs. <3.5 kg, respectively) (Larivière 2003; Hwang and Larivière 2005). *Lutrogale* has well-developed claws and fully webbed forefeet and hindfeet whereas the claws are rudimentary in adults and the feet incompletely webbed in *A. cinerea*. These features correlate well with the diet and foraging mode of each species. *Lutrogale* is highly piscivorous, swimming after fish and catching them in their mouths (Kruuk et al. 1994; Sivasothi and Nor 1994; Kanchansaka 1997; Hwang and Larivière 2005). In contrast, *A. cinerea* mainly eats crabs and other shellfish and uses its sensitive forefeet to find its

prey under rocks or sediment within rivers and streams (Kruuk et al. 1994; Sivasothi and Nor 1994; Kanchansaka 1997; Larivière 2003).

Despite these differences between *Lutrogale* and *Aonyx cinerea*, our molecular results showing they are sister taxa are consistent with several observations. First, *A. cinerea* and *L. perspicillata* are known to hybridize in captivity (female *A. cinerea* x male *L. perspicillata*), resulting in viable hybrids intermediate in size between the two parent species (Melisch and Foster-Turley 1996). Both species have $2n = 38$ chromosomes, which is found in other lutrines and mustelids and may represent the ancestral karyotype number for the family (Wurster and Benirschke 1968; Couterier and Dutrillaux 1986). Second, both *Aonyx* and *Lutrogale* share a similar brain structure, with an enlargement of the lateral part of the posterior sigmoid gyrus, which suggests high tactile sensitivity of the forelimb and correlates with the foraging mode in *Aonyx* (Radinsky 1968; Willemsen 1980). Third, Pleistocene fossil remains from Java, Indonesia, consisting of robust teeth and attributed to the extinct taxa *Lutrogale palaeoleptonyx* and *L. robusta* suggest that these earlier forms fed mainly on shellfish (Willemsen 1986, 1992). This, combined with the fact that both *A. cinerea* and *A. capensis* (the Cape clawless otter of sub-Saharan Africa) both feed primarily on crabs (although both species supplement their diet with fish, Lubis et al. 1998; Larivière 2001), suggests that *L. perspicillata* evolved piscivory secondarily from ancestors that were durophagous.

The placement of *Lutrogale perspicillata* as sister to *Aonyx cinerea* renders *Aonyx* paraphyletic. Given that *Aonyx* (Lesson 1827) has priority over *Lutrogale* (Gray 1865), one suggestion for taxonomic revision would be that *Lutrogale* be reclassified as *Aonyx perspicillata*. However, the descriptive name *Aonyx*, meaning "without claw," would be inappropriate for *L. perspicillata* because of its well-developed claws. An alternative would be to reclassify *A. cinerea* and *L. perspicillata* in *Amblonyx* (Rafinesque 1832) (meaning "blunt claw"), based on our present findings that these are joined as sister species (Fig. 1). Our previous studies (Koepfli and Wayne 1998, 2003) showed that *A. capensis* and *Amblonyx cinereus* (now *Aonyx cinerea*) were sister taxa, but samples of *L. perspicillata* were not available at the time. Therefore, based on these results, we suggested that generic separation of *Amblonyx* and *Aonyx* was unwarranted (Koepfli and Wayne 1998, p. 413), and this was used by Wozencraft (2005) to place *Amblonyx cinereus* in *Aonyx*. This taxonomic scheme is no longer tenable, however, given the phylogenetic position of *L. perspicillata* in the present study. Consequently, we suggest that placement of the Asiatic *Aonyx cinerea* and *L. perpicillata* into *Amblonyx* reflects the monophyly of these species as well as their separation from the African *Aonyx capensis*. If *Amblonyx* is indeed resurrected for *A. cinerea*, the species epithet *cinereus* should be used because it agrees with the gender of the genus name, as first noted by van Zyll de Jong (1987). Furthermore, following the tribal classification of the Lutrinae proposed by Willemsen (1992), we suggest that *Lutrogale* be transferred from the Lutrini to the Aonyxini, which would then include *Aonyx capensis*, *Aonyx cinerea*, and *Lutrogale perspicillata*.

Diagnostic nucleotides for Southeast Asian otters

We have established a panel of diagnostic nucleotides for each of the four species of otters found in the region using *CYTb* and *NADH5* mitochondrial gene segments (Tables 3 and 4). These sets of DNA characters can be used as a character-based barcode (sensu DeSalle et al. 2005) to help identify if one or more otter species are present at a particular site, using DNA samples from feces, hair, and/or anal jelly (see Hájková et al. 2006). The diagnostic nucleotides can be assayed using direct sequencing of PCR products or through the design of species-specific primers. Alternatively, they can be used in conjunction with restriction enzymes to develop a species-specific PCR-RFLP assay, which has been found to be especially useful for analyzing low amounts of degraded DNA (e.g., Gómez-Moliner et al. 2004). The diagnostic nucleotides for the four species we report here should be considered provisional and are subject to corroboration or falsification as new sequences from additional individuals are added. This condition is common to any character-based taxonomic barcode, whether based on molecular or morphological characters (DeSalle et al. 2005).

Habitats and their biota throughout Southeast Asia are facing severe threats, especially from deforestation (Sodhi et al. 2004). In order to effectively design and implement conservation plans for any species, it is essential to know where they are found. Compared to other regions of the world where the distribution of otters is relatively well known, the distribution and abundance of the four species of otters in Southeast Asia is of immediate concern. We hope the description of diagnostic sequence sites for species identification will catalyze additional geographic surveys. When combined with information collected from tracks and camera traps (Kruuk et al. 1993), fecal DNA methods can provide a powerful tool in species identification and monitoring (Farrell et al. 2000; Davison et al. 2002).

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