Phylogenetic Position of the Japanese River Otter Lutra nippon Inferred from the Nucleotide Sequence of 224 bp of the Mitochondrial Cytochrome b Gene

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Phylogenetic Position of the Japanese River Otter *Lutra nippon* Inferred from the Nucleotide Sequence of 224 bp of the Mitochondrial Cytochrome *b* Gene

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ABSTRACT—A 224 bp fragment of the mitochondrial cytochrome *b* gene has been amplified from a 30-year-old mummy-like specimen of the Japanese river otter *Lutra nippon* by polymerase chain reaction (PCR). The amplified products were subcloned in the SmaI site of pUC18 and sequenced. The sequence was different from those of the congeneric Eurasian otters *Lutra lutra* (Latvia) and *Lutra lutra* (China) in 7-9 nucleotides, all of which were located at the third position of a codon and identified as transitional differences A↔G or C↔T. The phylogenetic analysis using the 224 bp sequences of *Lutra nippon*, *Lutra lutra* (Latvia), *Lutra lutra* (China), *Aonyx cinerea* (Asian small-clawed otter), *Mustela sibirica* and *Mustela itatsi* (weasels) supports the recent morphological study that the Japanese river otter is not a subspecies of *Lutra lutra*, but a distinct species, *Lutra nippon*. We found that *Lutra nippon* and *Lutra lutra* contain the cytochrome *b*-like sequences, that appear to be a pseudo-form of cytochrome *b* gene. The sequences are characterized by the presence of deletion and termination codons, by the presence of several types of sequences with minor variations, and by the faster evolutionary rate compared with that of the mitochondrial cytochrome *b* gene. The genes would present in the nuclear DNA rather than in the mitochondrial DNA, as in the case of the nonfunctional cytochrome *b*-like sequences previously reported in a rodent.

INTRODUCTION

The Japanese river otter, a nearly extinct species, has long been thought to be a subspecies *whiteleyi* of the Eurasian otter *Lutra lutra*. Recently, Imaizumi and Yoshiyuki (1989) reexamined the morphological characters in detail, and gave a new species name *Lutra nippon*, to the Japanese otter from Shikoku and Honshu in Japan. They described that *Lutra nippon* is a primitive species with relatively longer tail and longer facial portion of cranium than other Eurasian species of *Lutra*. However, Wilson and Reeder (1993) considered it to be a junior synonym of *Lutra lutra*. To investigate the possibility for reintroduction of the Eurasian otters to Japan, it is inevitably needed to make clear the phylogenetic position of the Japanese otter. Thus we decided to analyze the sequence of DNA fragment amplified from mummy-like and stuffed specimens of *Lutra nippon*, under the condition that no living Japanese otters are available. We compare the sequence of *Lutra nippon* with those of several related taxa, *Lutra lutra* from Latvia and *L. lutra* from China (Eurasian otter), *Aonyx cinerea* (Asian small-clawed otter) and *Mustela sibirica* (Siberian weasel) and *Mustela itatsi* (Japanese weasel), and discuss the phylogenetic position of the Japanese river otter.

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MATERIALS AND METHODS

DNA analysis of Lutra nippon

DNA was isolated from the muscle (ca. 0.1 g) of a 30-year-old mummy-like specimen deposited at the Ehime Prefectural Museum, Japan, and from the muscle (ca. 0.1 g) of at least 30-year-old stuffed specimen at the Kochi Prefectural Office, Japan of *Lutra nippon* with a conventional phenol-chloroform method. The DNA was further purified by a spin column of S-400HR (Pharmacia). A 224 bp fragment of mitochondrial cytochrome *b* gene was amplified for 30 cycles each consisting of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for annealing and 1 min at 72°C for primer extension, using ca. 10 ng of template DNA, by PCR (Saiki *et al.*, 1988). The primers used are:

F1: CATCCAACATCTCAGCATGATGAAA (25mer)

R4: ATGTTTCATGTTTCGGTGAATATAT (25mer)

F1 is the "conserved" primer designed for amplification of mitochondrial cytochrome *b* gene (Kocher *et al.*, 1989), and R4 was based on the sequence of *Mustela itatsi*. As an enzyme, Taq DNA polymerase (Promega or AGS GmbH) was used. The amplified products were subcloned in the SmaI site of pUC18, and sequenced by the dideoxy chain termination method with BcaBEST DNA sequencing kit (Takara) and non-RI Uniplex DNA detection kit (Millipore) (Suzuki and Takagi, 1992). Several clones were also sequenced with a PRISM dye terminator cycle sequencing kit using Model 373-18 DNA sequencer (Applied BioSystems).

DNA analyses of Lutra lutra, Aonyx cinerea, Mustela sibirica *and* Mustela itatsi

DNAs were isolated either from the fresh muscle (*Lutra lutra* (Oji Zoo, Japan, originated from Latvia), *Aonyx cinerea* (Noichi Zoo, Japan), *Mustela sibirica* (obtained from Kochi, Japan) and *Mustela itatsi* (two individuals, obtained from Fukushima, Japan)) or from fresh

hairs (*L. lutra* (Asa Zoo, Japan, originated from China) and *L. lutra* (Noichi Zoo, imported from Europe)) with a conventional phenol-chloroform method. *Mustela sibirica* and *M. itatsi* were identified based on their sequences of cytochrome *b* gene reported by Masuda and Yoshida (1994). The subspecies of *Lutra lutra* were not considered. The DNA of *Lutra lutra* (killed by traffic accident in Asahikawa in 1989, Japan, and identified as *Lutra lutra*, but not *Lutra nippon* (Tsutsumi, 1990) was kindly supplied by Dr. Toshihiko Shiroishi of the National Institute of Genetics. A 307 bp fragment of mitochondrial cytochrome *b* gene was amplified by PCR with the "conserved" primers (Kocher

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Consensus                YTTYGGMTCY HKDMTMRGRA YYTRCYTAAT YMTTCARATY CTTACAGGTT TATTYTTAGC   60

L.nippon (Ehime)-c4      T..T..A..T CTAC.AG.A. CC.G.T.... CC....G..T .......... ....T.....   60
L.nippon (Ehime)-c5      T..T..A..T CTAC.AG.A. TC.G.T.... CC....G..T .......... ....T.....   60
L.lutra (Latvia)         T..T..A..C CTAC.AG.A. CT.G.T.... CC....G..T .......... ....T.....   60
L.lutra (Asahi.)         T..T..A..C CTAC.AG.A. CT.G.T.... CC....G..T .......... ....T.....   60
L.lutra (China)          T..T..A..C CTGC.AG.A. CC.G.T.... CC....G..T .......... ....T.....   60
L.lutra (Euro.)          T..T..A..C CTGC.AG.A. CC.G.T.... CC....G..T .......... ....T.....   60
Aonyx cinerea            C..T..A..T CTAC.CG.A. TC.G.T.... TC....A..T .......... ....C.....   60
Mustela sibirica         C..C..C..C CTTC.CG.A. TC.G.C.... TA....G..T .......... ....T.....   60
Mustela itatsi           C..C..C..C CTTC.CG.A. TC.G.C.... TA....G..T .......... ....T.....   60
L.lutra (Asahi.)-ps1     T..T..C..C TGTC.CA.A. TT.G.C.... CC....A..C .......... ....C.....   60
L.lutra (Asahi.)-ps3     T..T..C..C TTTC.CG.A. TT.G.C.... CC....A..C .......... ....C.....   60
L.lutra (Euro.)-ps1      T..T..C..C TGTC.CA.A. TT.G.C.... CC....A..C .......... ....C.....   60
L.lutra (Euro.)-ps34     T..T..C..C TGTC.CA.A. TT.G.C.... CC....A..C .......... ....C.....   60
L.nippon(Kochi)-ps17     T..T..C..C ATTA.CG.G. TT.A.C.... CC....A..C .......... ....C.....   60
L.nippon(Kochi)-ps7      T..T..C..C ATTA.CG.G. TT.A.C.... CC....A..C .......... ....C.....   60
L.nippon(Ehime)-ps7      T..T..C..C ATTA.CG.G. TT.A.C.... CC....A..C .......... ....C.....   60

Consensus                YATACACTAY WCRTCAGACA CARCYACAGC CWTYTCATYA GTYRCVCAYA TYTGYCRAGA  120

L.nippon (Ehime)-c4      C........C A.A..... ..A.C..... .T.C....C. ..CG.A..C. .T..C.G...  120
L.nippon (Ehime)-c5      C........C A.A..... ..A.C..... .A.C....C. ..CG.A..C. .T..C.G...  120
L.lutra (Latvia)         C........C A.A..... ..A.C..... .T.C....C. ..CG.A..C. .C..C.G...  120
L.lutra (Asahi.)         C........C A.A..... ..A.C..... .T.C....C. ..CG.A..C. .C..C.G...  120
L.lutra (China)          C........C A.A..... ..A.C..... .T.C....C. ..CG.A..C. .T..C.G...  120
L.lutra (Euro.)          C........C A.A..... ..A.C..... .T.C....C. ..CG.A..C. .T..C.G...  120
Aonyx cinerea            C........C A.G..... ..A.T..... .T.C....C. ..TA.G..T. .C..C.G...  120
Mustela sibirica         C........T A.A..... ..G.C..... .T.T....C. ..CA.C..T. .T..T.G...  120
Mustela itatsi           T........T A.A..... ..G.C..... .T.T....C. ..CA.C..T. .C..T.G...  120
L.lutra (Asahi.)-ps1     T........C T.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.A...  120
L.lutra (Asahi.)-ps3     T........C T.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.A...  120
L.lutra (Euro.)-ps1      T........C T.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.G...  120
L.lutra (Euro.)-ps34     T........C T.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.G...  120
L.nippon(Kochi)-ps17     T........C A.A..... ..A.C..... .T.C....T. ..TA.A..C. .C..T.G...  120
L.nippon(Kochi)-ps7      T........C A.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.G...  120
L.nippon(Ehime)-ps7      T........C A.A..... ..A.C..... .T.C....C. ..TA.A..C. .C..T.G...  120

Consensus                YGYCARYTAB GGCTRRATYA TBYRRTAYAT ACAYGCMAAY RGRGCYTYYA TATTCTTYAT  180

L.nippon (Ehime)-c4      C.T..AC..C ....GA..T. .TCGG..T.. ...C..A..C G.A..C.CT. .......C..  180
L.nippon (Ehime)-c5      C.T..AC..C ....GA..T. .TCGG..T.. ...C..A..C G.A..C.CT. .......C..  180
L.lutra (Latvia)         C.T..AC..C ....GG..T. .CCGA..C.. ...C..A..C G.A..C.CC. .......C..  180
L.lutra (Asahi.)         C.T..AC..C ....GG..T. .CCGA..C.. ...C..A..C G.A..C.CC. .......C..  180
L.lutra (China)          C.T..AC..C ....GG..T. .CCGA..C.. ...C..A..C G.A..C.CC. .......C..  180
L.lutra (Euro.)          C.T..AC..C ....GG..T. .CCGA..C.. ...C..A..C G.A..C.CC. .......C..  180
Aonyx cinerea            C.T..AC..C ....GA..T. .CCGA..C.. ...T..A..T G.G..C.CT. .......T..  180
Mustela sibirica         C.T..AC..T ....GA..C. .CCGA..C.. ...C..A..C G.A..T.CC. .......C..  180
Mustela itatsi           C.T..AC..T ....GA..C. .CCGA..T.. ...T..A..C G.A..C.CC. .......T..  180
L.lutra (Asahi.)-ps1     T.C..AT..T ...-.AA..C. .CCGA..T.. ...T..C..C A.A..C.CC. .......C..  180
L.lutra (Asahi.)-ps3     T.C..AT..T ....GA..C. .CCGA..T.. ...T..C..C A.A..C.CC. .......C..  180
L.lutra (Euro.)-ps1      T.C..GT..G ....GA..C. .CTGA..T.. ...T..C..T A.A..C.CT. .......C..  180
L.lutra (Euro.)-ps34     T.C..GT..G ....GA..C. .CTGA..T.. ...T..C..T A.A..C.CC. .......C..  180
L.nippon(Kochi)-ps17     C-T..AT..T ....GA..C. .GCAA..T.. ...T..C..T G.A..C.TC. .......C..  179
L.nippon(Kochi)-ps7      C-T..AT..T ....GA..C. .GCAA..T.. ...T..C..T G.A..C.TC. .......C..  179
L.nippon(Ehime)-ps7      C-T..AT..T ....GA..C. .GCAA..T.. ...T..C..T G.A..C.TC. .......C..  179
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Consensus	YTGCCTRTTC YTAYRYRTAG GRCRMGGYYT RTAYTRYGGR YCTTATATAT TCMCYGAAAC	240
L.nippon (Ehime)-c4	C.....G... C..CATG... .A.GC..CC. A..C.AC..A T...------ ----------	224
L.nippon (Ehime)-c5	C.....G... C..CATG... .A.GC..CC. A..C.AC..A T...------ ----------	224
L.lutra (Latvia)	C.....G... C..CATG... .A.GC..CC. G..C.AC..A T..... ..C.T.....	240
L.lutra (Asahi.)	C.....G... C..CATG... .A.GC..CC. G..C.AC..A T..... ..C.T.....	240
L.lutra (China)	C.....G... C..CATG... .A.GC..CC. A..C.AC..A T..... ..C.T.....	240
L.lutra (Euro.)	C.....G... C..CATG... .A.GC..CC. A..C.AC..A T..... ..C.T.....	240
Aonyx cinerea	C.....G... T..CACG... .A.GC..CC. A..C.AT..G T..... ..C.C.....	240
Mustela sibirica	C.....G... C..CACG... .G.GA..TT. A..T.AC..A T..... ..A.C.....	240
Mustela itatsi	C.....G... C..CACG... .G.GA..TT. A..T.AC..A T..... ..A.C.....	240
L.lutra (Asahi.)-ps1	C.....A... C..TGTA... .A.GA..CC. A..C.AT..A T..... ..C.C.....	240
L.lutra (Asahi.)-ps3	C.....A... C..TGTA... .A.GA..CC. A..C.AT..A T..... ..C.C.....	240
L.lutra (Euro.)-ps1	C.....A... C..CATA... .A.GA..CT. A..C.GT..A T..... ..C.C.....	240
L.lutra (Euro.)-ps34	C.....A... C..CATA... .A.GA..CT. A..C.AT..A T..... ..C.C.....	240
L.nippon(Kochi)-ps17	T.....A... C..CATG... .A.AA..CC. A..C.AC..A T...------ ----------	223
L.nippon(Kochi)-ps7	T.....A... C..CATG... .A.AA..CC. A..C.AC..A C...------ ----------	223
L.nippon(Ehime)-ps7	T.....A... C..CATG... .A.AA..CC. A..C.AC..A T...------ ----------	223

Consensus	ATGAAACAYY RGYATYATYY TAYTRTTYRM ARYYATAGCA ACWGCATTCA TAGGTTACDT	300
L.lutra (Latvia)	........CT G.T..T..TC ..C.A..CGC .ACC..... ..A..... ....G.	300
L.lutra (Asahi.)	........CT G.T..T..TC ..C.A..CGC .ACC..... ..A..... ....G.	300
L.lutra (China)	........CT G.T..T..TC ..C.A..CGC .ACC..... ..A..... ....G.	300
L.lutra (Euro.)	........CT G.T..T..TC ..C.A..CGC .ACC..... ..A..... ....G.	300
Aonyx cinerea	........TT G.C..C..CC ..C.A..CAC .ACT..... ..A..... ....G.	300
Mustela sibirica	........TC G.C..T..CT ..T.G..CGC .GTT..... ..T..... ....G.	300
Mustela itatsi	........TC G.C..T..CT ..T.A..CGC .GTC..... ..T..... ....G.	300
L.lutra (Asahi.)-ps1	........TC A.C..T..CC ..C.A..TGC .GTT..... ..A..... ....T.	300
L.lutra (Asahi.)-ps3	........TC A.C..T..CC ..C.A..TGC .GTT..... ..A..... ....T.	300
L.lutra (Euro.)-ps1	........TT A.C..T..CC ..C.A..TGA .GTT..... ..A..... ....A.	300
L.lutra (Euro.)-ps34	........TT A.C..T..CC ..C.A..TGA .GTT..... ..A..... ....A.	300

Consensus	HTTACCR	307
L.lutra (Latvia)	A.....A	307
L.lutra (Asahi.)	A.....A	307
L.lutra (China)	A.....A	307
L.lutra (Euro.)	A.....A	307
Aonyx cinerea	C.....A	307
Mustela sibirica	T.....G	307
Mustela itatsi	T.....A	307
L.lutra (Asahi.)-ps1	T.....A	307
L.lutra (Asahi.)-ps3	T.....A	307
L.lutra (Euro.)-ps1	T.....A	307
L.lutra (Euro.)-ps34	T.....A	307

Fig. 1. Comparison of the nucleotide sequences of the mitochondrial cytochrome *b* and cytochrome *b*-like sequences of *Lutra nippon* (Ehime and Kochi) with those of *L. lutra* (Latvia, China, Europe and Asahikawa), *Aonyx cinerea*, *Mustela sibirica* and *M. itatsi*. The sequence length, 224 bp for *L. nippon* and 307 bp for the others. Several representatives of the cytochrome *b*-like sequences (ps) are shown. Positions of the deletion and termination codon in the cytochrome *b*-like sequences are boxed. Euro., Europe; Asahi., Asahikawa.

et al., 1989),

F1: CATCCAACATCTCAGCATGATGAAA (25mer)

R1: CCCCTCAGAATGATATTTGTCCTCA (25mer)

The amplification conditions were as for in *Lutra nippon*. The amplified products were subcloned in the SmaI site of pUC18, and at least three clones were sequenced.

RESULTS AND DISCUSSION

We have amplified the 307 bp fragment of the mitochondrial cytochrome *b* genes from the Eurasian otters *Lutra lutra* (Latvia), *L. lutra* (China), *L. lutra* (Europe) and *L. lutra* (Asahikawa), the Asian small-clawed otter *Aonyx cinerea* and the weasels *Mustela sibirica* and *Mustela itatsi*, subcloned the amplified products in pUC18 and determined their sequences. The sequences of *L. lutra* (Europe) and *L. lutra* (Asahikawa) agreed completely with those of *L. lutra* (China) and *L. lutra* (Latvia), respectively. The sequences of two individuals of *Mustela itatsi* were identical, but different in three positions from that reported recently (Masuda and Yoshida, 1994), in the sequence positions 1-224 (see Fig. 1).

On the other hand, many attempts were made to amplify the DNA fragment of cytochrome *b* gene of *Lutra nippon* using various 10-50-year-old samples, such as the liver, heart and skin preserved in formalin, the tanned leather and the hairs of stuffed specimen, but all attempts were unsuccessful.

We have succeeded in amplifying the 224 bp fragment of the mitochondrial cytochrome *b* gene from a 30-year-old mummy-like specimen of *Lutra nippon* from Ehime (Fig. 1). Direct sequencing of the amplified products suggested the presence of more than two types of sequence. Therefore the products were subcloned in pUC18 and 30 clones were sequenced. Among them, nine clones were ambiguously identified to be the mitochondrial cytochrome *b* sequence of *Lutra nippon* since the sequence was apparently similar to the mitochondrial sequences from other *Lutra* species, of which eight clones had the same nucleotide sequence (represented by the sequence of *L. nippon* (Ehime)-c4 in Fig. 1) but the remaining one (the sequence of *L. nippon* (Ehime)-c5 in Fig. 1) was different in two positions (21: C→T and 92: T→A). The eleven clones had the cytochrome *b*-like sequences as described below. The remaining 10 clones, unexpectedly, had the almost identical sequence to the pig cytochrome *b* sequence, suggesting some serious DNA contamination occurred during storage of the mummy-like specimen.

We found that the DNA from a mummy-like *Lutra nippon* (Ehime) contains a cytochrome *b*-like sequence. Surprisingly, all of the amplified products from another *Lutra nippon* from Kochi (at least 30-year-old stuffed specimen) corresponded to the cytochrome *b*-like sequences, and no mitochondrial cytochrome *b* fragment was amplified. In addition, the cytochrome *b*-like fragments of 307 bp were amplified, with a yield of 30-40% of the total clones, from the DNAs prepared from the fresh liver or hairs of the Eurasian otters *L. lutra* (Europe and Asahikawa), but not from the DNAs from *L. lutra* (Latvia and China). Thus the presence of cytochrome *b*-like sequence is not an artifact during a long storage of DNA. The cytochrome *b*-like sequence are also found in the nuclear DNA in a rodent (Smith *et al.*, 1992). The cytochrome *b*-like sequences of *Lutra* are characterized by the presence of a deletion to cause a reading frame shift and termination codons (Fig. 1), by the presence of several types of sequences with minor variations (Fig. 1), and by the apparently faster evolutionary rate compared with the mitochondrial cytochrome *b* sequence (see Fig. 2). Thus the cytochrome *b*-like sequence

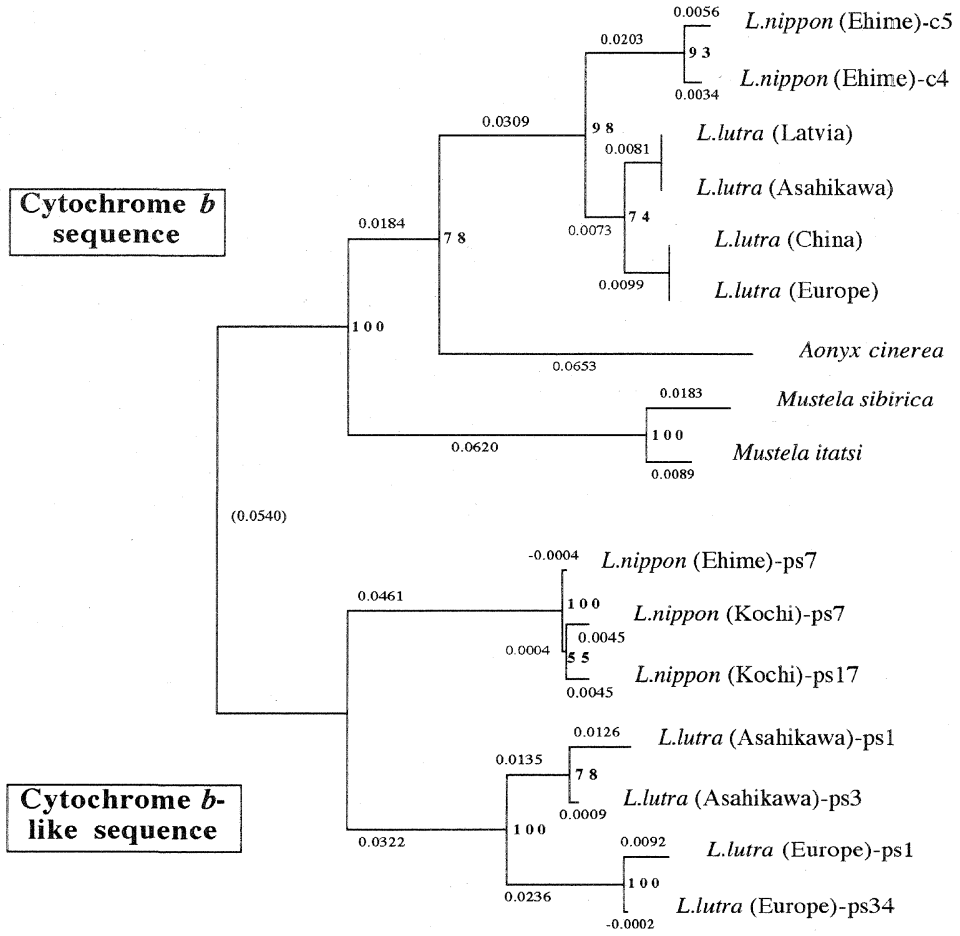

Fig. 2. A phylogenetic tree constructed from the sequence alignment (based on sequence 1-224 in Fig. 1) of the mitochondrial cytochrome *b* and cytochrome *b*-like sequences with the Neighbor-Joining method. The numbers at each branching point show bootstrap values (100 replications). ps represents the cytochrome *b*-like sequence.

appears not to function and is present as a pseudo-form. We have not examined whether the cytochrome *b*-like sequence of *Lutra* is present in the mitochondrial DNA or in the nuclear DNA.

The nucleotide sequences of the mitochondrial cytochrome *b* and cytochrome *b*-like genes of *Lutra nippon* are aligned with those of *L. lutra* (Latvia, China, Europe and Asahikawa), *Aonyx cinerea*, *Mustela sibirica* and *Mustela itatsi*, in Fig. 1. Of the sequence 1 to 224, *Lutra nippon* (C4) was different from those of *L. lutra* (Latvia) and *L. lutra* (China) in 9 and 7 nucleotides, respectively, all of which were located at the third position of a codon and identified as transitional differences A↔G or C↔T. The difference within *Lutra lutra* was 4 nucleotides. These nucleotide substitutions cause no amino acid replacement. On the other hand, there are 6 nucleotides difference in the two congeneric weasels *Mustela sibirica* and *Mustela itatsi* in this region. Thus the sequence difference between *Lutra nippon* and *Lutra lutra* (7-9 nucleotides: 3.6% in average) is larger than that (6 nucleotides) between the two *Mustela* species. This is also consistent with the idea that in the Mustelidae species, more than 3.5% difference in sequence of cytochrome *b* gene may correspond to the difference of distinct species (Masuda and Yoshida, 1994). The percentage difference between the nucleotide sequences are shown in Table 1.

Figure 2 shows a phylogenetic tree constructed from the sequence data obtained in this study with the Neighbor-Joining method in the PHYLIP package version 3.5c (Felsenstein, 1993). The same topology was reconstructed with the Fitch and Margoliash method (Felsenstein, 1993). The sequences were divided into two main clusters, one is for the mitochondrial cytochrome *b* genes and the other for cytochrome *b*-like sequences. As stated above, the cytochrome *b*-like sequences were amplified from the DNAs of *Lutra nippon* (2 individuals, from Kochi and Ehime), *L. lutra* (Europe) and *L. lutra* (Asahikawa). The branching pattern of the cytochrome *b*-like sequences of the two *Lutra* species was the same as in the mitochondrial cytochrome *b* genes, but the evolutionary rate for the cytochrome *b*-like sequences was at least two-times faster, in agreement with the general characteristics of pseudogenes. The tree also clearly shows that there is a large genetic difference between *Lutra nippon* and *Lutra lutra*, and this result is consistent with the recent morphological study that the Japanese river otter is not a subspecies of the Eurasian otter *Lutra lutra*, but a distinct species, *Lutra nippon* (Imaizumi and Yoshiyuki, 1989). However, to get a conclusive evidence for the phylogenetic relationship between the two species, longer DNA sequence of *Lutra nippon* must be determined. This would be done when the fresh sample of the Japanese river otter was obtained.

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Table 1. Percentage difference between the nucleotide sequences (224 bp) shown in Fig. 1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
	L.l. Lat.	*L.l.* Asahi.	*L.l.* China	*L.l.* Euro.	*L.n.* Ehime c4	*L.n.* Ehime c5	*A.c.*	*M.s.*	*M.i.*	*L.l.* Asahi. ps3	*L.l.* Asahi. ps1	*L.l.* Euro. ps34	*L.l.* Euro. ps1	*L.n.* Ehime ps7	*L.n.* Kochi ps7	*L.n.* Kochi ps17
B	0.00															
C	1.79	1.79														
D	1.79	1.79	0.00													
E	4.02	4.02	3.12	3.12												
F	4.46	4.46	3.57	3.57	0.89											
G	11.16	11.16	11.16	11.16	10.71	10.71										
H	12.95	12.95	11.61	11.61	13.39	13.39	14.73									
I	13.84	13.84	13.39	13.39	14.29	14.29	13.84	2.68								
J	14.29	14.29	14.73	14.73	15.62	15.62	16.52	16.52	14.73							
K	15.62	15.62	16.07	16.07	16.96	16.96	17.86	17.86	16.07	1.34						
L	15.62	15.62	16.07	16.07	16.96	16.96	16.96	17.41	15.62	4.46	4.02					
M	16.52	16.52	16.96	16.96	16.96	16.96	16.96	18.30	16.52	5.36	4.91	0.89				
N	14.35	14.35	14.80	14.80	15.25	15.25	16.59	16.59	14.80	8.52	9.87	9.42	10.31			
O	14.80	14.80	15.25	15.25	15.70	15.70	17.04	17.04	15.25	8.97	10.31	9.87	10.76	0.45		
P	14.80	14.80	15.25	15.25	15.70	15.70	17.04	17.04	15.25	8.97	10.31	9.87	10.76	0.45	0.90	

Lat., Latvia; Asahi., Asahikawa; Euro., Europe.

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