Identification of Two Tentative Strains of European Subspecies of the Eurasian River Otter *Lutra lutra lutra* (Linnaeus, 1758) from the Partial Mitochondrial Cytochrome *b* Gene

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ABSTRACT

The Eurasian river otter *Lutra lutra* is the most widely distributed otter species. The European subspecies *L. lutra lutra* (Linnaeus, 1758) kept in European zoos has four strains; two of which (the A- and B-lines) have been introduced into zoos and aquariums in Japan. As the genetic backgrounds of, and differences between, these two strains were unknown, we identified partial nucleotide sequences (307bp) of the mitochondrial cytochrome *b* gene from an A-line and a B-line individual, *A-line cyb* and *B-line cyb*, respectively, kept in an aquarium in Japan. The nucleotide sequence of the *A-line cyb* and *B-line cyb* showed 98.7% identity and are distinguishable by the restriction fragment length polymorphism analysis. *A-line cyb* was 100% identical to the cytochrome *b* of *L. lutra lutra*, whereas *B-line cyb* failed to show 100% identity to the known nucleotide sequences on the database. The phylogenetic analysis of our sequences with those from a previous study revealed that the two strains had already been introduced into Japan by at latest the 1990s. These results suggest that there are slight differences between the A- and B-line individuals used in the present study. However, further investigations, including re-evaluation of the taxonomy of *L. lutra* subspecies, are needed to clarify the differences between the two strains.

Key words: conservation genetics, cytochrome *b* gene, Eurasian river otter, *Lutra lutra lutra*, RFLP

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INTRODUCTION

The Eurasian river otter *Lutra lutra* is the most widely distributed otter species; its habitat ranges across Europe, including parts of Asia and North Africa. The International Union for Conservation of Nature (IUCN) Red List has categorized *L. lutra* as “near threatened” [1]. Because of the wide habitat range of *L. lutra*, up to 11 subspecies have been reported, including the Japanese river otter *L. lutra nippon* (Imaizumi et Yoshiyuki, 1989) and the Hokkaido subspecies *L. lutra whileleyi* (Gray, 1867); the extinction of both of which was recently declared by the Ministry of the Environment of Japan [2]. The lineage relationships of *Lutra* subspecies are

not well defined: for example, some taxonomists treat *L. lutra nippon* as the independent species *L. nippon*.

As of 2013, both the Chinese subspecies *L. lutra chinensis* (Gray, 1837) and the European subspecies *L. lutra lutra* (Linnaeus, 1758), also known as European Otter, were being kept in Japanese zoos and aquariums [3]. According to the European Studbook for the European Otter (*L. lutra lutra*) [4], the captive *L. lutra lutra* population is separated in four lines; the A-line otters are *L. lutra lutra* with 100% known pedigree, the B-line otters are descended from animals bred many years ago at Norfolk Wildlife Park, Great Witchingham, Norfolk, UK, where both *L. lutra lutra* and the Southeast Asian subspecies *L. lutra barang* (Cuvier, 1823) were kept; it is uncertain whether they were ever crossed, the I-line otters are the Iberian ecotype, held in some Spanish zoos especially with Spanish reintroduction in mind, not included within the A-line, and

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the U-line is unassigned line. The genetic backgrounds of, and differences between, these strains are unknown. Among these lines, the A- and B-line *L. lutra lutra* have been introduced into Japanese zoos and aquariums from European zoos. The policy of the European Endangered Species Programme (EEP) of the European Association of Zoos and Aquariums (EAZA) is to breed only A-line *L. lutra lutra* and to remove B-line animals from the EEP [4]. However, currently only one A-line animal and 10 B-line animals are being kept in Japanese zoos and aquariums.

A breeding program is to be prepared by the Conservation Committee of the Japanese Association of Zoos and Aquariums [3]. To establish a proper breeding program we need to clarify the genetic backgrounds of both A- and B-line *L. lutra lutra*. Therefore, here, we determined and compared the partial nucleotide sequences of cytochrome *b* genes of individuals from the A- and B-lines of *L. lutra lutra*. We also used restriction fragment length polymorphism (RFLP) to develop a simple method of distinguishing these two strains.

MATERIALS AND METHODS

DNA samples were obtained from the blood of a male and a female *L. lutra lutra* kept at Aquamarine Fukushima in Fukushima, Japan. Both individuals originated from European zoos and their strains were clear: the male was A-line and the female was B-line. DNAs were isolated by using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). A fragment of the mitochondrial cytochrome *b* gene was amplified by PCR (PCR System 9700, Applied Biosystems, Carlsbad, CA) with a Takara Ex Taq Reaction Kit (Takara Bio, Shiga, Japan), using conserved primers [5] used previously for successful identification of the *L. lutra* mitochondrial cytochrome *b* nucleotide sequences [6]. The primers used were;

F1: CATCCAACATCTCAGCATGATGAAA

R1: CCCCTCAGAATGATATTTGTCCTCA

The PCR conditions were as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55 °C for 0.5 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min, using 10 ng of template DNA. The PCR products were ligated into the pCR 2.1-TOPO vector by using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Nucleotide sequences were confirmed by DNA sequencing with an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). Ten clones from each individual were sequenced.

The nucleotide sequences were compared with all other known *L. lutra* mitochondrial cytochrome *b* nucleotide sequences by using the Basic Local Alignment Search Tool (BLAST). Partial *L. lutra* mitochondrial cytochrome *b* sequences previously reported by Suzuki et al. (1996) [6] and not uploaded to the DDBJ (the DNA Data Bank of Japan)/EMBL (the European Molecular Biology Laboratory)/GenBank databases were also compared. The deduced amino acid partial sequences were obtained from the nucleotide sequences and compared.

A phylogenetic tree was constructed by using the neighbor-joining method [7] with ClustalX 2.0 and viewed with the NJplot 2.3 program [8]. A sequence of small-clawed otter *Aonyx cinerea* [6] was used as an outgroup. Bootstrap values were estimated by using 1000 replications [9].

Partial sequences of the *Lutra* mitochondrial cytochrome *b* gene were amplified as described, and 12 µl of the amplified sample was subjected to endonuclease digestion using the four-base recognition enzyme *Afa* I. Digestion was performed at 37°C for 12h. DNA fragments were separated on a 1.5% agarose gel and stained with ethidium bromide; the fragments were made to fluoresce by exposure to ultraviolet light and photographed.

RESULTS

The 307-bp sequences of mitochondrial cytochrome *b* were determined from the A-line individual (*A-line cyb*) and the B-line individual (*B-line cyb*) (Fig. 1). Single base substitutions were detected at four positions in *B-line cyb*, which had 98.7% identity to *A-line cyb*. Another sequence as found in both the A-line and the B-line; it was considered to be a pseudoform (*pseudo cyb*) according to the previous studies [6, 10]. The data were registered in the DDBJ/EMBL/GenBank databases and assigned the accession numbers AB933268 (for *A-line cyb*), AB933269 (for *B-line cyb*), and AB935512 (for *pseudo cyb*).

Comparison with previously reported *L. lutra* mitochondrial cytochrome *b* sequences from a number of specimens obtained from Japan (the subspecies of which were not known) revealed that *A-line cyb* had 100% identity to the sequences from two of these individuals. One originated from Latvia and had been kept in a Japanese zoo, and the other was killed by a motor vehicle in Asahikawa in 1989. *B-line cyb* also had 100% identity to the sequences from two other individuals of *L. lutra*, which had originated from China and Europe and had been

A-line cyb	1	TTTGGATCCCTACTAGGAACCTTGCTTAATCCTTCAGATTCTTACAGGTT	50
B-line cyb	1	G.....C.....	50
pseudo cyb	1	C...T.T..C...T...C.....A..C.....	50
		F G S L L G T C L I L Q I L T G	
A-line cyb	51	TATTTTTAGCCATACACTACACATCAGACACAACCACAGCCTTCTCATCA	100
B-line cyb	51		100
pseudo cyb	51	...C...T.....T.....	100
		L F L A M H Y T S D T T T A F S S	
A-line cyb	101	GTCGCACACATCTGCCGAGACGTCAACTACGGCTGGATTATCCGATACAT	150
B-line cyb	101	T.....	150
pseudo cyb	101	..TA.....T.A...T.C...T..T....A..C.....T..	150
		V A H I C R D V N Y G W I I R Y M	
A-line cyb	151	ACACGCAAACGAGCCTCCATATTCCTTCATCTGCCTGTTCTACATGTAG	200
B-line cyb	151		200
pseudo cyb	151	...T..C...A.....A.....TG..A...	200
		H A N G A S M F F I C L F L H V	
A-line cyb	201	GACGCGCCTGTACTACGGATCTTATATATTCCTGAAACATGAAACACT	250
B-line cyb	201	A.....	250
pseudo cyb	201	...A.....A.....T.....C.....TC	250
		G R G L Y Y G S Y M F P E T W N T	
A-line cyb	251	GGTATTATTCCTACTATTCGCAACCATAGCAACAGCATTCATAGGTTACGT	300
B-line cyb	251		300
pseudo cyb	251	A..C...C.....T...GTT.....T...	300
		G I I L L F A T M A T A F M G Y V	
A-line cyb	301	ATTACCA	307
B-line cyb	301		307
pseudo cyb	301	T.....	307
		L P	

Fig. 1 Comparison of the nucleotide sequences of mitochondrial cytochrome *b* (*A-line cyb* and *B-line cyb*) and its pseudoform (*pseudo cyb*) in our A- and B-line individuals of *L. lutra lutra*. Nucleotides that are identical to the *A-line cyb* sequence are denoted with a period (.). The *Afa* I cutting site is arrowed. The deduced amino acid partial sequence was indicated on the last line.

kept in Japanese zoos [6]. In addition, *A-line cyb* had 100% identity to a number of complete nucleotide sequences of European *L. lutra lutra* cytochrome *b* gene, such as X94923 [11], EF689068 [12], AJ536010 [13], and AF057124 [14]. In contrast, among these latter sequences no sequence identical to *B-line cyb* was found; *B-line cyb* had 99% identity to *L. lutra* (FJ236015; Jang and Hwang, 2008, direct submission from Korea) and 95% to *L. sumatrana* (EF472347) [15]. Our *pseudo cyb* sequence had 100% identity to a pseudogene from the above-mentioned *L. lutra* individual killed in Asahikawa in 1989 [6].

The deduced amino acid partial sequences that we obtained from A- and B-line *cyb* had 100% identity (Fig. 1).

We constructed a phylogenetic tree from the sequences we obtained, with reference to the previously described sequences (Fig. 2). The *A-line cyb* and *B-line cyb* were close to parallel, whereas possible Korean strain of *L. lutra* (FJ236015) formed a different branch in conjunction with *L. sumatrana* (EF472347).

The 357 bp fragments of PCR products including the primer sequences were amplified from the DNA samples. The profiles of digestion with *Afa* I revealed three fragments of PCR product from the A-line individual, namely 237 bp and 120 bp found only in *A-line cyb* and the 357 bp of the monomorphic pseudogene. The PCR product from the B-line individual was monomorphic (Fig. 3).

DISCUSSION

We revealed that *L. lutra* mitochondrial cytochrome *b* partial nucleotide sequences from the A-line and B-line individuals had slight but apparent differences. *A-line cyb* had 100% identity to the gene previously found in *L. lutra* individuals with an unidentified bloodline found in Japan, but it was also completely identical to purebred *L. lutra lutra*, which means that this is the A-line pedigree. *B-line cyb* was previously found in *L. lutra* individuals with another unidentified bloodline in Japan and was slightly different from *A-line cyb* and also

Fig. 2 Phylogenetic tree constructed by the neighbor-joining method for mitochondrial cytochrome *b* in A- and B-line individuals of *L. lutra lutra*, with reference to the sequences previously described. The sequence from *Aonyx cinerea* was used as an outgroup. Numbers at each branching point are bootstrap values, as estimated by 1000 replications. Horizontal lines indicate genetic distance.

Fig. 3 RFLP fragment profiles of *Afa* I digestion of the 357-bp fragment of *Lutra* mitochondrial cytochrome *b* sequences determined from the A- and the B-line individual. 1: Marker (2000, 1000, 500, and 100 bp), 2: A-line *cyb* PCR product, 3: *Afa* I digestion product from A-line *cyb*, 4: B-line *cyb* PCR product, 5: *Afa* I digestion product from B-line *cyb*.

the sequences of possible Korean strain of *L. lutra* and *L. sumatrana*. However, it is premature to conclude that these differences are characteristic of the B-line. B-line individuals are descended from animals bred in a single zoo, where *L. lutra lutra* and *L. lutra barang* were kept together, that simply remains possible that there are several crossbred individuals with different genetic backgrounds in B-line. So further analysis, using more samples from B-line *L. lutra* individuals, is required. Cytochrome *b* genes are mitochondrial and are thus maternally inherited, so evaluation of the nuclear genome is also necessary to reveal the genetic background of B-line individuals. In addition, comparison with *L. lutra barang* is needed.

Our comparison with the results of the 1996 study by Suzuki et al. [6] revealed that *L. lutra* individuals with A- and B-line *cyb* have been kept in Japan since at least that year. It might therefore be useful for the management of *L. lutra* pedigrees

to check *Lutra* mitochondrial cytochrome *b* genes of the individuals kept in Japan, including *L. lutra chinensis*, because the individual originated from China had the same nucleotide sequence as the B-line individual in the present study.

Determination and comparison of complete sequence of cytochrome *b* genes might be more effective. But the assessment of the fragmentation patterns by using the RFLP method described here would be an easier technique than sequencing, and more suitable for on-site screening.

Our RFLP testing revealed three fragments from *A-line cyb*, because only *A-line cyb* had an *Afa* I restriction-enzyme cutting site. The pseudoform was amplified by PCR at the same time, but it, like *B-line cyb*, had no cutting site for *Afa* I (Fig. 1). A number of pseudoforms of *Lutra* mitochondrial cytochrome *b* genes have already been reported; these pseudoforms have been amplified with a yield of 30% to 40% of the total clones [6]. Moreover, in this study we sequenced 20 clones, and nine of them were sequences of the pseudoform (Data not shown). To discriminate *A-line cyb* from *B-line cyb* by using RFLP we can expect to find, in the case of *A-line cyb*, three fragments: a dense fragment of 357 bp and two faint fragments of 237 and 120 bp. Even so, the RFLP method used in the present study could discriminate *A-line cyb* from *B-line cyb* successfully. If more accurate identification is necessary, the combination of long- and short PCR could be used to avoid the detection of pseudoforms or mitochondrial DNA heteroplasmy.

The phylogenetic analysis of the subspecies of *L. lutra* from morphological research seemed still confusing. Although *L. lutra* is the most widely distributed otter species and up to 11 subspecies have been reported, details of the genetic relationships are still being examined [11–15]. After the lineage relationships of the subspecies become clear, there remains the question of the difference between the A- and B-lines and how to deal with these lines in terms of conservation programs in zoos and aquariums in Japan.

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原著 分類系統学

ミトコンドリアシトクロム *b* 遺伝子部分配列を用いた
ユーラシアカワウソヨーロッパ産亜種 *Lutra lutra lutra* (Linnaeus, 1758) の暫定的な系統判別法

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要 約

ユーラシアカワウソは、イタチ科カワウソ亜科のうちでもっとも広い分布域を持つ種である。ヨーロッパの園館で飼育されているヨーロッパ産亜種 (*Lutra lutra lutra*) には 4 つの系統があるとされ、そのうち A ラインと B ラインに属する個体が日本国内で飼育されている。しかしながら、この 2 系統の遺伝的差異は明らかとされていない。そこで、A ラインと B ラインに属する個体各 1 頭の血液より抽出した DNA を用い、ミトコンドリアシトクロム *b* 遺伝子の部分配列を決定した。A ラインと B ラインの個体から各々 307bp の配列が得られ (A-line *cyb*, B-line *cyb*)、両者は 98.7% の相同性を示した。また、2 つの配列は制限酵素断片長多型 (RFLP) 法によって判別が可能であった。A-line *cyb* は既報のヨーロッパ産亜種と 100% の相同性を示したが、B-line *cyb* はデータベース上のいずれの配列とも一致しなかった。国内で飼育されていた亜種不明のユーラシアカワウソから得られた既報の配列と比較を行ったところ、A-line *cyb*, B-line *cyb* とともに一致する配列が認められた。以上の結果より、A ラインと B ラインの個体を持つミトコンドリアシトクロム *b* 遺伝子にはわずかながら違いが認められたが、これを系統差と断言するためには、ユーラシアカワウソ亜種の分類の再検討を含め、更に詳細な解析が必要であると考えられた。

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