Origin of the two major distinct mtDNA clades of the Japanese population of the oriental weather loach Misgurnus anguillicaudatus (Teleostei: Cobitidae)

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Origin of the two major distinct mtDNA clades of the Japanese population of the oriental weather loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae)

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Abstract. The oriental weather loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) inhabits the waters of East Asia including Japanese Islands. The Japanese population of *M. anguillicaudatus* includes two major mitochondrial DNA (mtDNA) clades, but their evolutionary origin is unknown. In this study, we conducted phylogeographic analyses of *M. anguillicaudatus* that were based on mtDNA cytochrome *b* sequences to clarify the evolutionary origin of the two distinct mtDNA clades. This newly obtained data were integrated with the mtDNA sequence data obtained in previous studies and reanalysed. The results showed that one major clade originated because of mtDNA introgression from a loach of the genus *Cobitis*. The geographic range of the populations carrying non-introgressed mtDNA tended to be limited to the peripheral areas of the Japanese Islands, whereas the range of the populations carrying introgressed mtDNA was spread over wide regions of the Japanese Islands. These distribution and divergence patterns suggested that *M. anguillicaudatus* populations carrying introgressed mtDNA have spread and replaced the range of populations carrying non-introgressed mtDNA.

Key words: introgression, cytochrome *b*, hybridization, *Cobitis*

Introduction

Mitochondrial DNA (mtDNA) introgression is the replacement of the whole mitochondrial genome of one species by that of another species without leaving any trace of a past hybridization in its nuclear genome. Mitochondrial introgression rarely occurs between distantly related species of freshwater fishes belonging to different genera (Freyhof et al. 2005, Šlechtová et al. 2008). Such mitochondrial introgression may cause confusion regarding the genealogy estimates of a species and lead to incorrect inferences of species history (Liu et al. 2010).

The oriental weather loach *M. anguillicaudatus* (Teleostei: Cobitidae) inhabits the muddy bottoms of creeks, ponds, wetlands and paddy fields across East Asia including the Japanese Islands. Recently, two genetically divergent mitochondrial clades have

been recognised within the Japanese population of *M. anguillicaudatus* by two independent phylogeographic studies using mtDNA sequences (Morishima et al. 2008, Koizumi et al. 2009). In the study by Morishima et al. (2008) that was based on the control region, the Japanese population of *M. anguillicaudatus* was divided into two distinct major mtDNA clades, of which one was closely related to the European *M. fossilis* and the East Asian *Paramisgurnus dabryanus*, while the other was only distantly related to other *Misgurnus* samples (Fig. 1A). The genetic divergence between these two major clades was large (average sequence divergence, 13 %) and it was equivalent to the intergeneric levels in other freshwater fishes. The study by Koizumi et al. (2009) that was based on the cytochrome *b* gene reported similar genetic relationships with a large

genetic divergence between the two major clades of *M. anguillicaudatus* (average sequence divergence, 15 %-18 %) (Fig. 1B). Both previous mitochondrial studies indicated that these two clades correspond to the two lineages that diverged early in the evolutionary history of *Misgurnus*. Our preliminary genetic study, which was based on the control region of some specimens used in the study of Koizumi et al. (2009), confirmed that the two major clades identified in these two independent studies had common genetic divergence background (data not shown). We called these two major clades 'Type I' and 'Type II' (Table 1, Fig. 1A, B). The distributions of the mtDNA Type I and Type II in the Japanese Islands showed that Type I was mostly restricted to the north-eastern region with discontinuous populations. In contrast, Type II was widely distributed throughout the Japanese Islands (Morishima et al. 2008, Koizumi et al. 2009, Shimizu & Takagi 2010, Fig. 2). In many locations, both mitochondrial types coexisted in the same populations. However, whether the two mitochondrial types represent genetic polymorphisms within *Misgurnus anguillicaudatus* or two biologically different *Misgurnus* species is unclear. Currently, the evolutionary origin of these two distinct clades has not yet been elucidated.

A recent molecular phylogenetic study of Cobitidae species using nucleic DNA gene sequences revealed that *Misgurnus* and its relatives (*Paramisgurnus* and *Koreocobitis*) and *Cobitis* (excluding *Cobitis misgurnoides*) and its relatives (*Niwaella*, *Iksookimia* and *Kichulchoia*) were reciprocally monophyletic when the nuclear DNA was studied (Šlechtová et al. 2008). However, with mtDNA data, the majority of samples of East Asian *Misgurnus* representing at least five species from Russia, China, Korea and Japan, were included in the *Cobitis* clade (Fig. 1C). This discrepancy between nuclear and mtDNAs was explained to be a result of hybridization and mtDNA introgression between an ancestral species of *Cobitis* and an ancestral species of *Misgurnus*. Another molecular phylogenetic study (Saitoh et al. 2010)
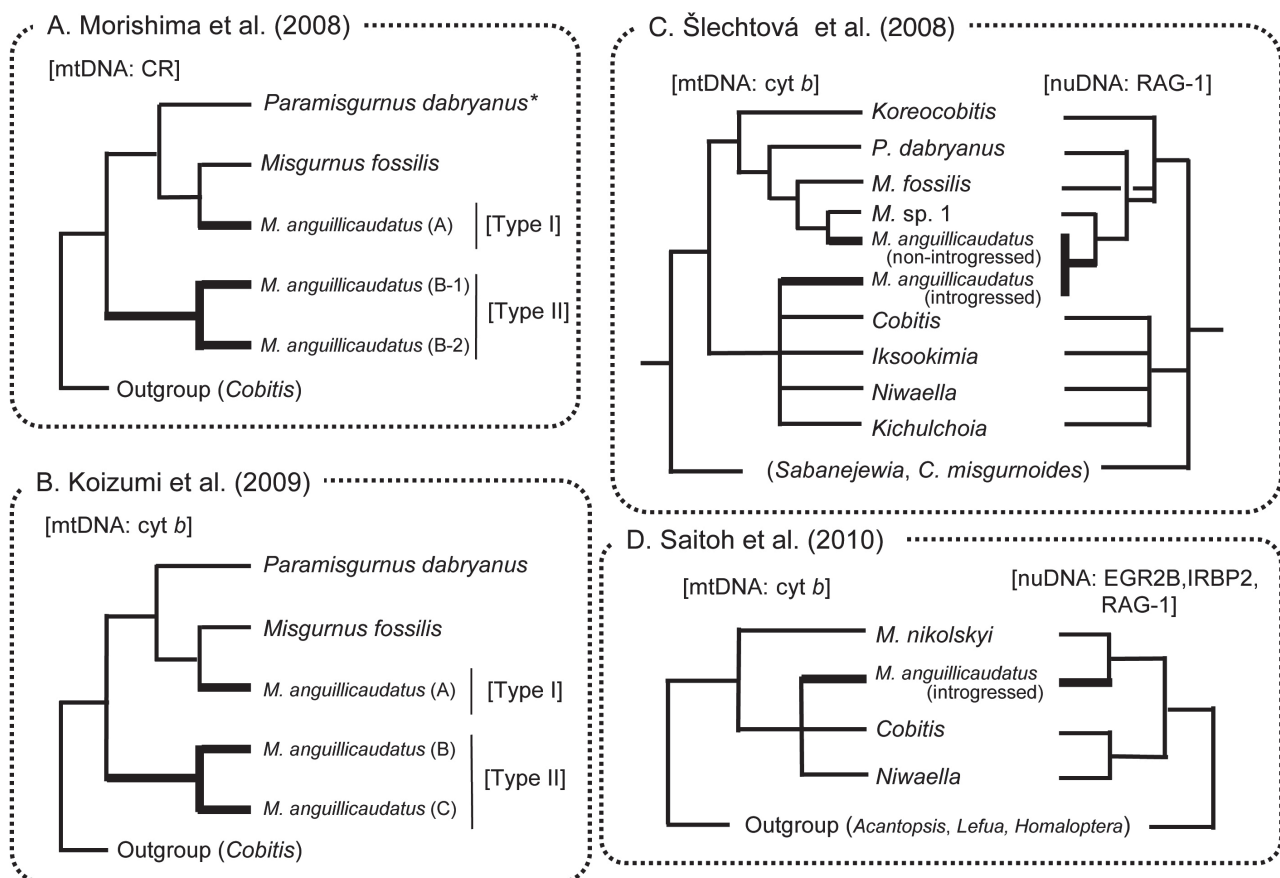

Fig. 1. *An overview of recent molecular genetic studies of* Misgurnus anguillicaudatus.

* Because of taxonomical confusion, Paramisgurnus dabryanus *has sometimes been called* Misgurnus mizolepis *by some Japanese researchers (Fujita 2007). Morishima et al. (2008) may also have used* M. mizolepis *instead of* P. dabryanus.

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also supported this mtDNA introgression hypothesis (Fig. 1D). Several morphological differences are observed between *Misgurnus* and *Cobitis* (and their relatives) with respect to body colouration and several osteological characters, and they have been traditionally considered taxonomically distinct groups (Nalbant 1963, 1994, Sawada 1982).

Considering the phylogenetic relationships mentioned above, the large mtDNA divergence within the Japanese *M. anguillicaudatus* populations may passively have been the result of this intergeneric mtDNA introgression event (Šlechtová et al. 2008) or the large mtDNA divergence may correspond to two different lineages that diverged early in the evolutionary history of *Misgurnus*. In this study, we examined the phylogenetic relationships between the two Japanese mtDNA types. We reported new findings suggesting introgression of *M. anguillicaudatus* and traced the evolutionary history of the Japanese Type I and Type II *M. anguillicaudatus* populations.
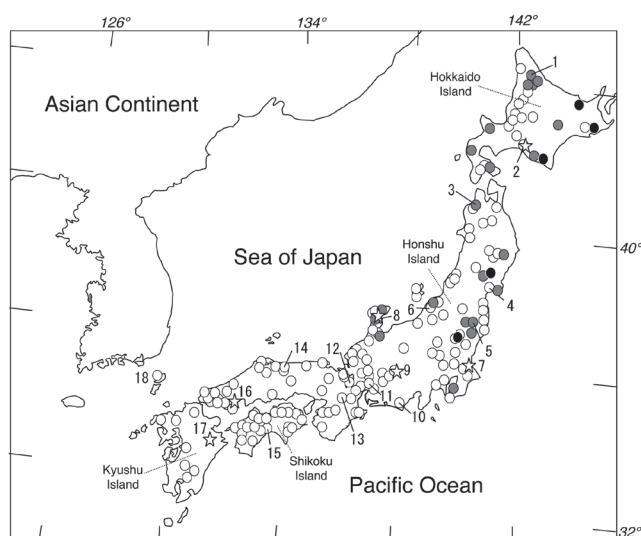
Fig. 2. Sampling localities of the specimens newly collected in this study (stars) and the geographic distribution of the two types of the mtDNA of Misgurnus anguillicaudatus *in Japan, following the findings of Morishima et al. (2008), Koizumi et al. (2009) and Shimizu & Takagi (2010). Solid circles, Type I; grey circles, Types I and II, sympatrically; open circles, Type II.*

Material and Methods

Misgurnus anguillicaudatus specimens were collected from the geographically distant areas in Japan (Table 1, Fig. 2). Total DNA was extracted from approximately 100 mg of ethanol-preserved muscle tissue, as described by Asahida et al. (1996). A mtDNA fragment encompassing the entire cytochrome *b* gene was amplified and sequenced. The utilised PCR primers and sequencing procedures that were followed were as described by Šlechtová et al. (2008) and Kitagawa et al. (2005), respectively. DNA sequence data were edited using the DNASIS program (Hitachi Software, Yokohama, Japan). These sequence data are available in GenBank (Table 1, accession nos. AB599977-AB599980 and AB614357-AB614359). Sequence data were compared with some of the reported cytochrome *b* sequences representing Types I and II (reported by Koizumi et al. 2009), with the introgressed and non-introgressed types) of *M. anguillicaudatus* (reported by Šlechtová et al. 2008), those from Chinese and Korean *M. anguillicaudatus* (downloaded from DNA data base), and those from three congeners and two *Cobitis* species, respectively (Table 1). Based on the molecular phylogenetic results of Šlechtová et al. (2008), two species of loaches *Sabanejewia balcanica* and *Pangio pangia* were also included as outgroups.

The phylogeny of loaches was inferred by the maximum likelihood (ML) method. The best fitting model of sequence evolution for this dataset was estimated using the program jModelTest 0.1.1 (Posada 2008). According to the Akaike information criterion, TrN93 + G + I (Tamura & Nei 1993) was selected. ML analysis was performed using PAUP* 4.0 (Swofford 2002), and bootstrap support (Felsenstein 1985) at each branch (1000 times) was calculated using PhyML 3.0 (Guidon & Gascuel 2003).

Results

An 1108-bp region of the cytochrome *b* gene was successfully amplified by PCR and sequenced in all specimens analysed (Fig. 3). In the phylogenetic tree, *M. anguillicaudatus* was divided into two major clades with high bootstrap supports (97.6 % and 93.2 %); one consisted of Japanese mitochondrial Type I and non-introgressed mitochondrial type of *M. anguillicaudatus*; this clade was closely related to other *Misgurnus* species, such as *M. nikolskyi*, *M. fossilis* and *Misgurnus* sp. 1 (with an average sequence divergence of 10 %). The second clade included Japanese mitochondrial Type II and introgressed type of *M. anguillicaudatus*, and this clade was closely related to the *Cobitis* species. The average sequence divergence between the latter clade and the *Cobitis* species was 14 %. The second clade was further divided into two highly supported subclades (99.7 % and 100.0 %) with an average sequence divergence of 9 %. Within one of these subclades, a highly supported monophyletic cluster (99.8 %) was formed with only specimens from Japan (Type II).

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**Table 1.** List of specimens used in the present study.

No. in Fig. 2	Species	Locality	Accession No. (cytochrome *b*)	References	Status*
1	*Misgurnus anguillicaudatus*	Nayoro, Hokkaido, Hokkaido Island, Japan	AB473261	Koizumi et al. (2009)	Type I
2		Yufutu, Hokkaido, Hokkaido Isl., Japan	AB599977	present study	
3		Gosyogawara, Aomori, Honshu Isl., Japan	AB473277	Koizumi et al. (2009)	Type I
4		Imaizumi, Miyagi, Honshu Isl., Japan	EF508556	Šlechtová et al. (2008)	introgressed
4		Imaizumi, Miyagi, Honshu Isl., Japan	EF508557	Šlechtová et al. (2008)	non-introgressed
5		Sugakawa Fukushima, Honshu Isl., Japan	AB473308	Koizumi et al. (2009)	Type I
6		Nagaoka, Niigata, Honshu Isl., Japan	AB473266	Koizumi et al. (2009)	Type II
7		Notojima, Ibaraki, Honshu Isl., Japan	AB614357	present study	
8		Notojima, Ishikawa, Honshu Isl., Japan	AB599978	present study	
8		Hannoura, Ishikawa, Honshu Isl., Japan	AB614358	present study	
9		Nakatsugawa Gifu, Honshu Isl., Japan	AB599979	present study	
10		Yoshida, Shizuoka, Honshu Isl., Japan	AB473306	Koizumi et al. (2009)	Type II
11		Yatomi, Aichi, Honshu Isl., Japan	AB473347	Koizumi et al. (2009)	Type II
12		Higashiomi, Shiga, Honshu Isl., Japan	AB473301	Koizumi et al. (2009)	Type II
13		Yamazoe, Nara, Honshu Isl., Japan	AB473359	Koizumi et al. (2009)	Type II
14		Kurayoshi, Tottori, Honshu Isl., Japan	AB473368	Koizumi et al. (2009)	Type II
15		Nangoku, Kochi, Sikoku Isl., Japan	AB473267	Koizumi et al. (2009)	Type II
16		Iwakuni, Yamaguchi, Kyushu Isl., Japan	AB614359	present study	
17		Tsushima, Nagasaki, Kyushu Isl., Japan	AB473402	Koizumi et al. (2009)	Type II
18		Bungo-ono, Oita, Kyushu Isl., Japan	AB599980	present study	
		Republic of Korea	EU670766	Bang & Lee, unpubl.	
		-	AF051868	Xiao & Zhang, unpubl.	introgressed
		Hejiang, China	AY625700	Tang et al. (2005)	
		Luonan, China	DQ105238	Tang et al. (2006)	introgressed
		Zigui, China	EF424603	Yang et al. unpubl.	
		Wuhan, China	EF508555	Šlechtová et al. (2008)	introgressed
		Wuhan, China	EF508558	Šlechtová et al. (2008)	introgressed
		Shandong, China	EF595980	Yang et al. unpubl.	
		Jiangxi, China	EU131139	Zeng et al. unpubl.	
	Misgurnus fossilis	Warmia-Masuria, Poland	EF508561	Šlechtová et al. (2008)	
	Misgurnus nikolskyi	Nenjiang, China	AB242171	Saitoh et al. (2006)	
	Misgurnus sp. 1	Khabarovsk, Russia	EF508566	Šlechtová et al. (2008)	
	Cobitis lutheri	Oa U, Republic of Korea	EF508498	Šlechtová et al. (2008)	
	Cobitis taenia	Niedersachsen, Germany	EF508508	Šlechtová et al. (2008)	
Out group:					
	Sabanejewia balcanica	Prešov, Slovakia	AY887851	Šlechtová et al. (2008)	
	Pangio pangia	West Bengal, India	EF508583	Šlechtová et al. (2008)	

* 'introgressed or non-introgressed' and 'Type I or Type II' were from the results by Šlechtová et al. (2008) and Koizumi et al. (2009), respectively.

Discussion

Previous mitochondrial studies on *M. anguillicaudatus* (Morishima et al. 2008, Koizumi et al. 2009) indicated that two major clades that corresponded to the different lineages diverged early in the evolutionary history of *Misgurnus*. The present phylogenetic relationships, however, reveal that the Japanese mitochondrial Type I corresponds to the non-introgressed type of *M. anguillicaudatus* and Type II corresponds to the introgressed one from the genus *Cobitis* (Fig. 3). In addition, we included other mitochondrial cytochrome *b* sequences of *M. anguillicaudatus* from the Asian continent (China and Korea), and all these sequences belonged to the introgressed clade that included Type II. This result confirms the findings of Šlechtová et al. (2008) that the distribution of the populations carrying introgressed mtDNA stretches over most of East Asia. Based on the present distribution pattern of the two types of mtDNA, the zoogeographic historical process of *M. anguillicaudatus* can be explained as follows. The genus *Misgurnus* most probably originated from the Asian continent because some of its congeners and relatives were found in the Asian continent. Subsequently here, a hybridization and subsequent mtDNA introgression occurred between an ancestral species of *Cobitis* and an ancestral species of *Misgurnus*. The timing of this mtDNA introgression event is estimated to be a period ranging from the basal point of the introgressed clade to the branching point of *Cobitis* on the tree (Fig. 3). By applying the recent estimation of the cytochrome *b* mutation rate for loaches (0.68 per million years ago,

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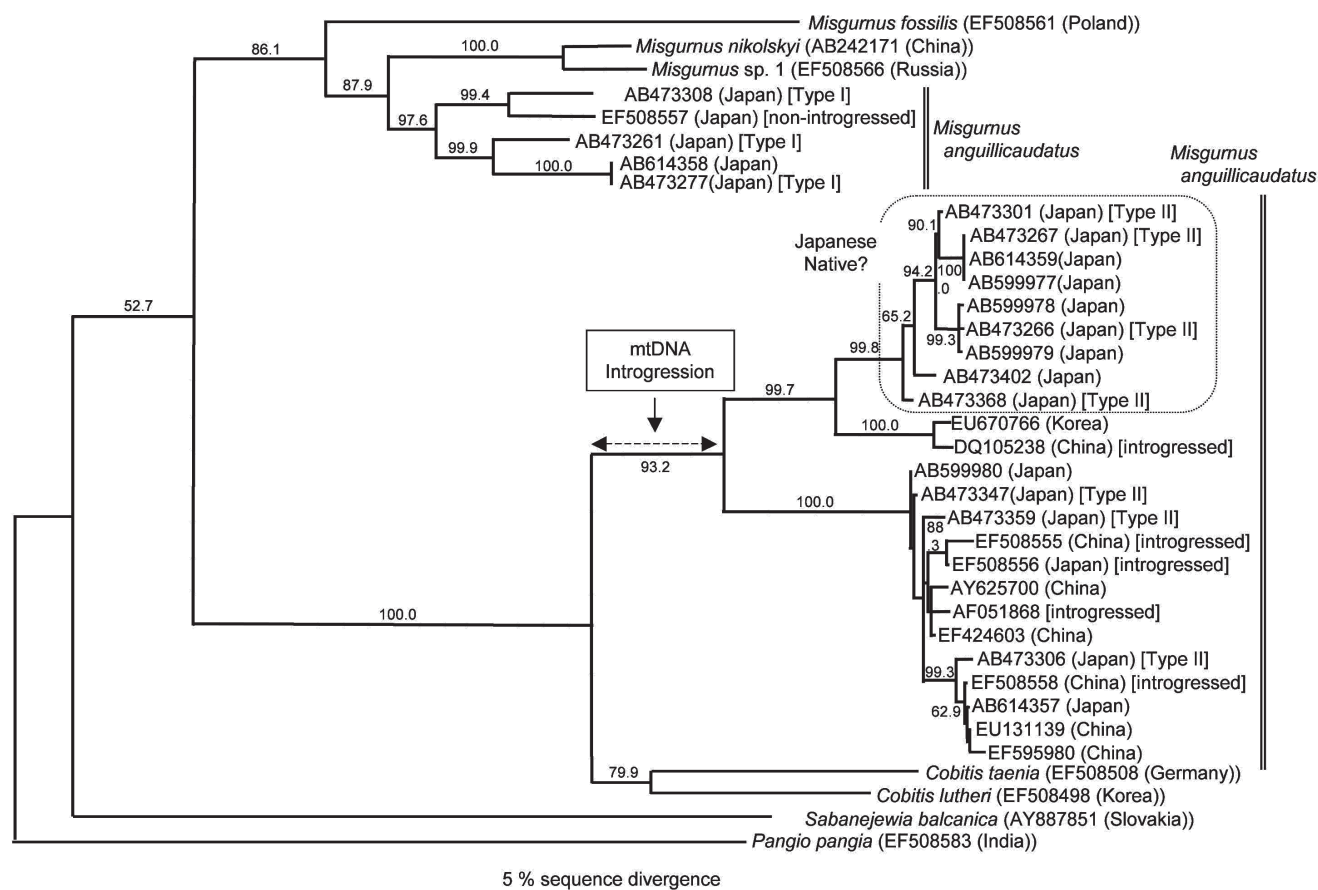
Fig. 3. Maximum likelihood gene tree based on cytochrome b sequence (1140 bp) among Misgurnus anguillicaudatus *and its related species (–ln L = 6554.28828). Bootstrap probabilities (Felsenstein 1985) with 1000 replications (%) are shown for each cluster, but values < 50 % are not shown. The broken line with arrows on both sides indicates the range of the period when mtDNA introgression from* Cobitis *to* M. anguillicaudatus *occurred.*

Doadrio & Perdices 2005) to these data (9 %-14 % sequence divergence), this event was estimated to have occurred about 7-10 million years ago. The introgressed mtDNA is widespread and found in several species of *Misgurnus* in East Asia, indicating that the introgression has occurred before the present-day species evolved. When the Japanese Islands were connected to, or were a part of the Asian continent, *M. anguillicaudatus* migrated into and widely dispersed into the present Japanese Islands region. At present, the population carrying introgressed mtDNA is present in the northernmost regions of the Japanese Islands. Following this hypothesis, the present mitochondrial Type I that is fragmentally distributed only in the north-eastern region should be considered a relic of *M. anguillicaudatus* non-introgressed mtDNA. A monophyletic cluster consisting only of the Japanese population was formed within the mitochondrial introgressed clade. Koizumi et al. (2009) reported the existence of some

geographical population structures within the Type II Japanese *M. anguillicaudatus*. These findings indicate the existence of Japanese native populations carrying introgressed mtDNA. Although some secondary dispersal events may have been accelerated by recent human activities mainly related to exploitation of paddy field areas (Nishimura 1974), some expansion of introgressed mtDNA must have been a natural historic event. A similar expansion pattern with mtDNA introgression by the native population was also reported in other Japanese loaches (*Cobitis biwae* complex) (Kitagawa et al. 2003). These succesfully introgressed DNAs may have a positive selective advantage, as suggested in chars (Glemet et al. 1998, Doiron et al. 2002).

Recently, large numbers of *M. anguillicaudatus* individuals have also been imported from other Asian countries to Japan for use as food materials, and they have been accidentally introduced into the Japanese wild populations (Yoshizato 2007,

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Shimizu & Takagi 2010). In this study, some Type II mtDNAs from various places in Japan clustered with some Asian continental mtDNA. This indicates that non-native *M. anguillicaudatus* have been already spread in Japan (Fig. 3). Thus, immediate actions are required to assess the current status of distribution and hybridization of the non-native population. Mabuchi et al. (2008) reported that the Japanese common carp *Cyprinus carpio* also had two mtDNA lineages: one is native, and the other has been artificially introduced from European and Asian countries. Presently, non-native mtDNA is widely spread throughout Japan, and the population of the native type is declining.

Our hypothesis is mainly based on the results of previous studies based on mtDNA or nuclear DNA (Morishima et al. 2008, Šlechtová et al. 2008, Koizumi et al. 2009, Saitoh et al. 2010, Shimizu & Takagi 2010). To test our hypothesis, genetic data based on common genes are required. In addition, it will be interesting to determine whether Type I-related (non-introgressed) mtDNA exists in the *M. anguillicaudatus* populations of the Asian continent, and further analysis using nuclear DNA for populations where Type I and II are sympatric is required.

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