

Short communication

Low genetic diversity in *Ozobranchus jantseanus* (Hirudinida: Ozobranchidae) in Japan: Possibility of introduction with their host turtles



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ABSTRACT

Leeches belonging to the genus *Ozobranchus* are ectoparasitic on sea and freshwater turtles. The freshwater species *O. jantseanus* has been recorded from China and Japan. *Ozobranchus jantseanus* inhabiting Japan is considered to be a non-indigenous species, because their primary host, the Reeves' pond turtle, *Mauremys reevesii*, is thought to have been introduced in the last few centuries from adjacent Asian countries. To assess whether the Japanese populations of *O. jantseanus* were likely to have been introduced, their genetic diversity was investigated using mitochondrial cytochrome *c* oxidase subunit I sequences. The very low sequence diversity as well as the historical record of this species from Japan suggest that Japanese populations of *O. jantseanus* may have been artificially introduced along with their host turtles. Molecular phylogenies of COI showed that two marine *Ozobranchus* species formed a clade together with the freshwater *O. jantseanus*.

The proboscideate leech genus *Ozobranchus* de Quatrefages, 1852 includes chelonian-specific ectoparasites, and comprises seven species [1]. Two of them, *O. branchiatus* (Menziés, 1791) and *O. margoi* (Apáthy, 1890), are parasites of sea turtles; four species, *O. jantseanus* Oka, 1912, *O. shipleyi* Harding, 1909, *O. papillatus* Kaburaki, 1921 and *O. polybranchus* Sanjeeva Raj, 1951, are indigenous to the Asian Region, and specific to freshwater turtles [2,3]. *Ozobranchus quatrefagesi* (Poirier and Rochebrune, 1884) from West Africa has been reported to infest crocodiles [4], but its systematic status is unclear.

Ozobranchus jantseanus is a freshwater species, and has been reported only from China and Japan [5,6]. It was originally described based on a specimen collected from Wuchang (Wuhan District), China [7]. Chinese individuals are known to infest Reeves' pond turtle, *Mauremys reevesii* (Gray, 1831) [5]. The primary host for the Japanese populations of *O. jantseanus* is also *M. reevesii*; but it has also rarely been recorded from the endemic Japanese pond turtle, *M. japonica* (Temminck and Schlegel, 1838), and from the red-eared slider, *Trachemys scripta elegans* (Wied, 1839), native to the southern United States [6,8,9].

When discussing *O. margoi*, Nishimura [10] briefly mentioned the possibility that *O. jantseanus* inhabiting Japan might be an introduced species, and this has been repeated in other publications [6,8]. Suzuki

et al. [11] showed that Japanese populations of *M. reevesii*, the primary host of *O. jantseanus*, were introduced from China and Korea. Accordingly, if they were introduced, *O. jantseanus* leeches inhabiting Japan would be parasitic primarily on the introduced *M. reevesii*, rather than the native *M. japonica*. To assess whether the Japanese populations of *O. jantseanus* had been introduced with their primary host or not, we analyzed the diversity of cytochrome *c* oxidase subunit I (COI) sequences, from *O. jantseanus* samples collected from various localities in Japan.

Representing the distribution of *O. jantseanus* in Japan, a total of 53 individuals from 11 populations were collected and analyzed in this study (Table 1; Fig. 1). All specimens were parasites of *M. reevesii*, and they were identified as *O. jantseanus* based on the following diagnostic morphological characters [5]: somites XIII–XXV biannulate, (a1 + a2) > a3; and branched-branchiae in 11 pairs, each pair arising from lateral of (a1 + a2) of somites XIII–XXIII. When possible, the turtle species were identified by the last author (Dai Suzuki). Almost all the specimens were relaxed by the gradual addition of absolute ethanol to freshwater for morphological identification. For DNA extraction, a piece comprising less than a quarter of the caudal sucker was removed, and then preserved in 99% ethanol. The remainder of the body was fixed in 10% formalin and preserved in 70% ethanol. Examination and

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Table 1

Samples of *Ozobranchus jantseanus* used for molecular analyses. Locality numbers (see Fig. 1) are accompanied by collection locality, voucher, INSDC accession numbers, and COI haplotype (see Fig. 1). Acronym: KUZ, Zoological Collection of Kyoto University.

Sample #	Locality	Individual #	Voucher	COI INSDC #	Haplotype
1	Ichihara, Chiba Pref.	7	KUZ Z1847–Z1853	LC215673–LC215679	a
2a	Minato-ku, Tokyo	1	KUZ Z1859	LC215685	d
2b		2	KUZ Z1860–Z1861	LC215686, LC215687	a
3	Gifu, Gifu Pref.	1	KUZ Z1814	LC215640	a
4	Joyo, Kyoto Pref.	5	KUZ Z1809–Z1813	LC215688–LC215692	a
5	Naruto, Tokushima Pref.	10	KUZ Z1816–Z1825	LC215642–LC215651	a
6	Manno, Kagawa Pref.	5	KUZ Z1826–Z1830	LC215652–LC215656	a
7	Matsue, Shimane Pref.	5	KUZ Z1831–Z1835	LC215657–LC215661	a
8	Miyoshi, Hiroshima Pref.	6	KUZ Z1836–Z1841	LC215662–LC215667	c
9a	Hatsukaichi, Hiroshima Pref.	4	KUZ Z1842–Z1845	LC215668–LC215671	c
9b		1	KUZ Z1846	LC215672	b
10	Fukuoka, Fukuoka Pref.	1	KUZ Z1815	LC215641	a
11	Iki Island, Nagasaki Pref.	5	KUZ Z1854–Z1858	LC215680–LC215684	a

dissection of the leech samples were performed using a stereoscopic microscope (Leica M125). Specimens analyzed in this study have been deposited in the Zoological Collection of Kyoto University (KUZ).

The COI sequences (1267 bp) were determined using the modified methods described in Nakano and Lai [12]; one of the primers for COI, LCO-in, was replaced by LCO-inOzo (5'-GCTGCAGCAATTACAATACTT-3'; this study). In total 53 COI sequences were newly obtained and deposited with the International DNA Database Collaboration (INSDC) through DNA Data Bank of Japan (Table 1). Additionally, a partial COI sequence (1267 bp) concordant with our data set was obtained from the complete mitogenome (KY861060) of a Chinese *O. jantseanus* specimen, which was collected from Jieyang, southern China, deposited with INSDC [13]. The alignment of COI was trivial, as no indels were observed. Relationships between the obtained COI haplotypes were estimated by statistical parsimony network using PopART v. 1.7 [14].

The phylogenetic position of *O. jantseanus* was estimated based on the obtained COI sequences with the aid of the COI sequences of *O. branchiatus* (16 sequences: GU985465, GU985466, KF728206–KF728213, and KJ451399–KJ451404) and *O. margo* (six

sequences: AF003268, GU985467, HM590711, and KJ451405–KJ452407) obtained from INSDC. Two proboscoidate piscicolids, *Pontobdella muricata* (Linnaeus, 1758) (AY336029) and *Stibarobdella tasmanica* (Hickman, 1942) (DQ414343) and one proboscoidate glossiphoniid, *Theromyzon tessulatum* (O.F. Müller, 1774) (AY047318), OTUs were included as outgroup taxa according to the results of previous molecular phylogenetic analyses [15–17]. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). Details for the reconstruction procedures are provided in Nakano and Lai [12]. The ML analysis with nonparametric bootstrapping (BS) with 1000 replicates was conducted based on the non-partitioned dataset. The best-fit partition scheme and models for each partition for the BI and Bayesian posterior probabilities (PPs) were selected as follows: for the 1st position of COI, GTR + I; for the 2nd position, F81 + I; and for the 3rd position, HKY + G; conducted for 1 million generations, and the tree was sampled every 100 generations; the first 3001 trees were discarded based on the results of the parameter estimates and convergence.

The COI data obtained from *O. jantseanus* samples showed low

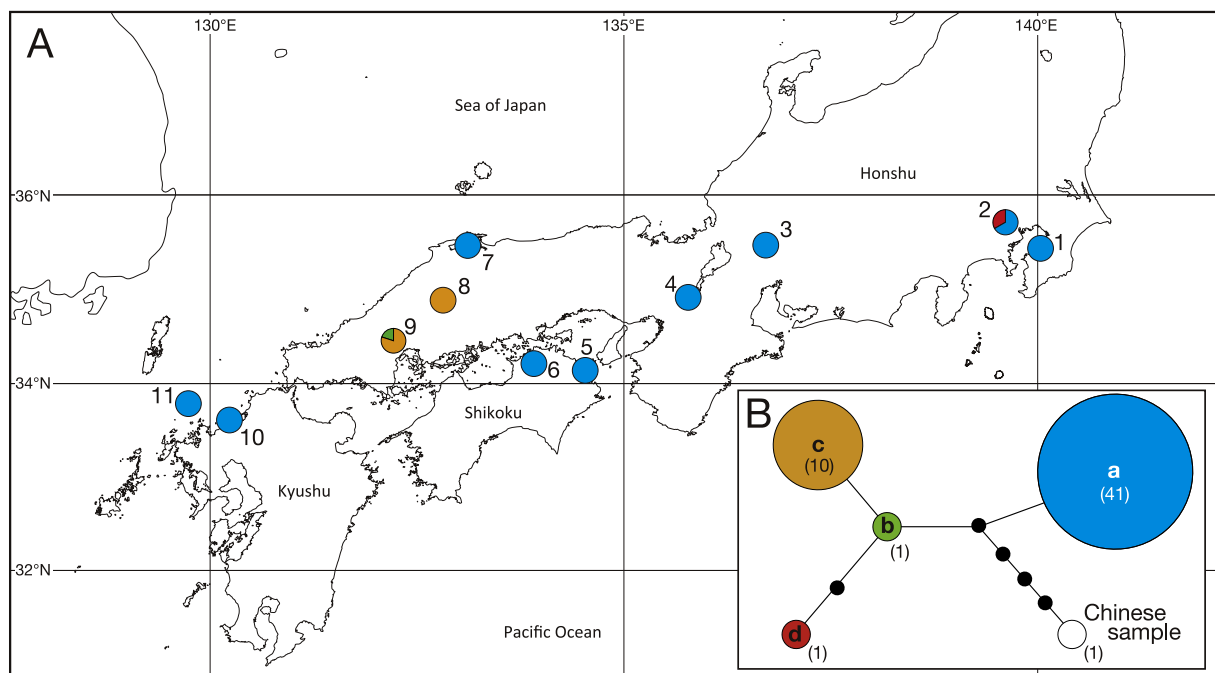


Fig. 1. Map showing sampling localities, and haplotype network of *Ozobranchus jantseanus*. A) Map showing the 11 sampling localities in the present study (see Table 1). Each colored circle indicates respective haplotype of mitochondrial cytochrome c oxidase subunit I (COI). B) Statistical parsimony network of COI haplotypes. Filled circles indicate missing haplotypes. Each numeral in parentheses denotes the sample size of the respective haplotype.

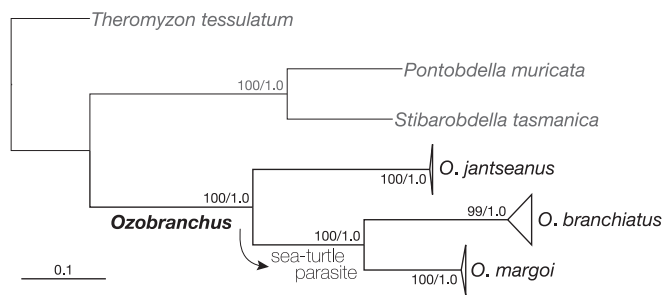


Fig. 2. Bayesian inference tree for 1267 bp alignment positions of mitochondrial cytochrome c oxidase subunit I marker. Numbers on nodes indicate bootstrap values for maximum likelihood and Bayesian inference posterior probabilities.

genetic diversity within the Japanese populations of this species: 5/1267 (0.39%) sites were polymorphic, with only two states recovered at each polymorphic site. Only four COI haplotypes were present in the Japanese populations, and the number of nucleotide differences between any two haplotypes ranged from one to four substitutions (Fig. 1B). The dominant haplotype (“a” in Fig. 1B) was observed in 41 samples, from 9/11 localities. Two geographically unique haplotypes (“b” and “c”) were observed in samples from two localities in Chugoku District (locality numbers 9 and 8 in Fig. 1A): most of the specimens ($n = 10$) had haplotype c; haplotype b was present in a single individual. A third geographically unique haplotype (“d”) was present in a specimen from the locality in Tokyo where dominant haplotype samples were also collected (locality number 2 in Fig. 1A). The COI genetic divergence between the Japanese populations and the Chinese individual was 0.40–0.55%, ranging from five to seven substitutions (Fig. 1B).

The observed low COI genetic diversity in the Japanese *O. jantseanus* supports the hypothesis that the Japanese populations of the species may have been artificially introduced to Japan with their host turtles. In addition to the species' low genetic diversity and high host specificity to *M. reevesii*, several other factors support this hypothesis: 1) Oka [18] did not include *O. jantseanus* in his 1910 synopsis of Japanese leech species; 2) Yamauchi and Suzuki [6] stated that the oldest specimens of Japanese *O. jantseanus* were collected in 1916; and 3) the oldest report of *O. jantseanus* collected from Japan was published in 1922 [19]. This suggests that *O. jantseanus* may have been introduced to Japan at the beginning of the 20th century.

Suzuki et al. [11] showed that individuals of *M. reevesii* inhabiting western Japan belonged to the same genetic group, as defined by mtDNA. These populations belong to the lineage originating from the Korean Peninsula, where *O. jantseanus* has never been recorded. Suzuki et al. suggested that these populations might initially have been introduced to northern Kyushu in the late 18th century and then gradually spread over western Honshu, while the eastern Japanese populations (as well as the central Kyushu population) of *M. reevesii* might have been introduced independently from continental China.

In contrast to the genetic structure of their host species, the observed leeches' COI sequence data showed that the geographically unique COI haplotype group (haplotypes b and c) occurred in western Honshu, while most of the remaining localities were occupied by a single dominant haplotype (a). This discordance between the population-genetic structure of the leeches and that of their primary host species suggests that the demographic history of the Japanese populations of *O. jantseanus* may be different from that of *M. reevesii* inhabiting Japan. It should be also noted that none of the detected COI haplotypes were completely identical to the known haplotype of the Chinese *O. jantseanus* specimen. Genetic data of *O. jantseanus* populations from continental China, the putative native range of this species, should be properly assessed to evaluate the present conclusion that the Japanese populations of this species were non-indigenous. Whether *O. jantseanus* was unintentionally introduced to Japan or not, its present genetic

diversity suggests that the Japanese populations are undergoing rapid expansion in the archipelago. Additional *O. jantseanus* samples from China and more sensitive markers, e.g. microsatellites, would be required to elucidate biogeographic and demographic histories of *O. jantseanus*.

The obtained phylogenies of COI (ML, $\ln L = -3816.163$, not shown; BI, mean $\ln L = -3511.578$ (Fig. 2)) recovered monophyly of three *Ozobranthus* species (BS = 100, PP = 1.0). The monophyly of the Japanese *O. jantseanus* was not recovered. The marine species *O. branchiatus* and *O. margo* formed a monophyletic lineage (BS = 100, PP = 1.0), suggesting a single origin of host specificity to sea turtles, in *O. branchiatus* and *O. margo*, although phylogenetic positions of the other freshwater ozobranthid species should be elucidated to reveal the origins of freshwater and marine taxa. Most of the known ozobranthid species have been reported as being ectoparasitic on freshwater turtles [1,20]; the number of branchiae is also highly variable in the freshwater species (cf. 5 pairs in *O. margo*, and 7 pairs in *O. branchiatus*): 5 pairs in *Unoculobranthiobdella expansa* Lobo et al., 1998 [20]; 8 pairs in *Bogabdella diversa* Richardson, 1969 [3]; 11 pairs in *O. jantseanus*, *O. shipleyi* and *O. papillatus* [2]; and 20 pairs in *O. polybranchatus* [2]. *Ozobranthus jantseanus* has also been reported to tolerate very low temperatures [21]. Thus, freshwater ozobranthid species are key taxa to our understating of how the chelonian-leech parasitism has evolved. Systematic and phylogenetic analyses of the remaining freshwater ozobranthid species should be carried out to elucidate the group's evolutionary history.

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