

Title:

Rapid expansion of the distributional range and the population genetic structure of the freshwater amphipod *Crangonyx floridanus* in Japan

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Abbreviated form of the title:

Distributional expansion and population genetics of an amphipod

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ABSTRACT:

The freshwater amphipod *Crangonyx floridanus* (Amphipoda: Crangonyctidae) has considered to be recently introduced from North America to Japan, and now the recorded sites of collection cover nearly all over Japan excluding the northern part. In this study, we surveyed further areas outside the known distribution ranges and also examined the population genetic structure and the phylogenetic relationships between Japanese and North American populations based on nuclear (18S rRNA) and mitochondrial (COI) DNA sequences. We found that this amphipod already reached to Hokkaido, the northernmost Japan, and suggested the rapid expansion in a pattern of concentric circles from the central part of Japan. The genetic analysis showed the genetic homogeneity of Japanese population in contrast to the genetic diversification in North American *Crangonyx* populations. The process of introduction, establishment, and expansion of this amphipod in Japan may be explained as follows. A limited number of individuals from a North American native population were probably inadvertently introduced and established somewhere within the Kanto region. After that the local population size increased and the range of its distribution was rapidly expanded over Japan.

Key words: crangonyctid amphipods, alien species, DNA analysis, 18S, COI

INTRODUCTION

The crangonyctid amphipod, *Crangonyx floridanus* Bousfield (Amphipoda, Crangonyctidae), originating from North America, was unintentionally introduced to the freshwater habitats of Japan. In 1989, an odd and previously unseen freshwater amphipod was found in the Furutone-numa oxbow lake of the Tone-gawa River, which flows along the Chiba-Ibaraki prefectural boundary (Morino et al. 2004). This was the first record of the family Crangonyctidae within Japan. The amphipod was subsequently identified as being *C. floridanus*, by comparing specimens with other *Crangonyx* species upon utilising morphological characteristic diagrams (Morino et al. 2004). Since its discovery in 1989, the distribution of this alien amphipod has expanded rapidly within a short time frame of only about 20 years (Kanada et al. 2007). It was recently reported that this amphipod already inhabits a large portion (*i.e.*, 28 prefectures) of Japan, excluding the northern part. In this study, we surveyed further areas outside the known distribution ranges in addition to the complete survey of references of benthic fauna in Japan.

From our ecological studies of *C. floridanus* (Tanaka et al. 2007; Tojo et al. 2007, 2010), this species seems to have a physiological tolerance to a wider range of water temperature and water flow and a higher reproductive rate than Japanese native species. As this amphipod has rapidly expanded its distribution into new habitats as yet uninhabited by native amphipod species, it is also expected to significantly affect the native species with a similar niche. As a result, there is a significant risk of a collapse/disruption of the ecological balance that has been established for a long time. Therefore, consideration of possible countermeasures to prevent the further expansion and distribution of *C. floridanus* is very important. The resolved problems are that how *C. floridanus* was introduced to Japan, the introduction was once or multiple, if multiple there were several origins and routes. With these questions in mind, we have conducted a population genetic analysis of Japanese *C. floridanus* to compare it with North American *C. floridanus* and some other closely related species of *Crangonyx*.

MATERIALS AND METHODS

Surveys of distribution

In order to document and quantify the expansion progress of *Crangonyx floridanus*, we used the data of our own field surveys and the published data of benthic fauna surveys across Japan; *e.g.*, the reviewed paper of Morino et al. (2004) and Kanada et al. (2007), the data of ‘National Censuses on River Environments’, which was conducted by the Japanese Ministry of the Land Infrastructure and Transport, and the collection records of *C. floridanus* in the Japanese local journals. Obtained records were plotted on the map (Fig. 1).

DNA analysis

Specimens of Japanese *Crangonyx floridanus* for DNA analysis were collected from 17 localities across Japan (41 specimens from 11 prefectures) in 2007 and 2008. Of these 41 specimens, two were from the Furutone-numa oxbow Pond, near the Tone-gawa River, Abiko, Chiba (specimen nos. 9 and 10 in Table 1), which was the first place that this species was found in Japan in 1989: one was from the northernmost place, the Chitose-gawa River, Chitose, Hokkaido (specimen no. 1 in Table 1), and one was from an aquarium of the pet shop at Ota-ku, Tokyo (specimen no. 12 in Table 1). Most specimens were fixed in 99.5% ethanol in the field. The muscles of the thoracic segments were dissected in the laboratory to extract DNA (muscles of abdominal segments were occasionally used). The other specimens were incubated in a water tank at about 10°C with several fallen leaves immediately after collection in the field, and their muscles were dissected and finally fixed in 99.5% ethanol before DNA extraction.

Total DNA was extracted from the specimens and purified using the DNeasy^R Tissue kit (QIAGEN, Hilden). The 18S rRNA genes and the COI genes were amplified by a PCR method using the primer sets [18S: forward primer 18S700 (5'-GTCTGGTGCCAGCAGCCGCG-3'; Englisch and Koenemann 2001), reverse primer 18S1015R (5'-TTTGAGCACTCTGATTTACTCAAGG-3'; designed in this study), COI: forward primer LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'; Folmer et al. 1994), reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3'; Folmer et al. 1994)] PCR products were purified with Microcon^R Kit (MILLIPORE, Massachusetts). The purified DNA was sequenced directly by an automated method using the DYEnamicTM ET Terminator Cycle Sequencing Kit (GE Healthcare UK, Buckinghamshire) on an automated sequencer (ABI PRISM 377 Genetic Analyzer; Perkin Elmer/Applied Biosystems, California). The 18S rRNA sequence data of the 32 samples from 14 populations, and the COI sequence data of 36 samples from 15 populations, have been submitted to the DNA data Bank of Japan (DDBJ database), and their accession numbers are given in Table 1. The sequence data of *C. floridanus* from North American native habitats and other related species, *Crangonyx* spp., *Bactrus* spp. and *Synurella* sp., were taken from GenBank (accession numbers in Table 1) to make phylogenetic analyses.

All sequences were aligned automatically with Clustal W (Thompson et al. 1994) and MEGA 4 (Kumar et al. 2008), and then cross-checked by eye carefully. Phylogenetic analyses were performed by the neighbor-joining (NJ) method (Saitou and Nei 1987), implemented using the software PHYLIP version 3.57 (Felsenstein 1995), and the maximum parsimony (MP) method, implemented using the software MEGA version 4 (Tamura et al. 2007). Gaps and ambiguous sites were omitted from

data set of phylogenetic analyses. The NJ analyses employed matrices of genetic distances generated using Kimura's two-parameter method (Kimura 1980), and confidences of branches were assessed by 1,000 bootstrap resamplings. The MP analyses were performed for all tree searches using in the program MEGA for heuristic searches, and the majority rule consensus tree method. Bayesian analyses as conducted four times (independent runs started from different, randomly chosen trees) for each set of phylogenetic data using MrBayes (Huelsenbeck and Ronquist 2001). The Bayesian analysis was inferred for 18S [HKY substitution model was selected as the best substitution model based on the hLRT using MrModeltest v2.3 (Nylander et al. 2004); 5,000,000 generations], COI [GTR+G substitution model was selected as the best substitution model based on the hLRT; 5,000,000 generations], and nodal support was assessed by posterior probabilities estimated from the final 80% sampled trees.

RESULTS

Distribution patterns

We could add two new locality records of *Crangonyx floridanus*: seven individuals collected from the Iwaki-gawa River, the Jyohoku-ohashi bridge, Hirosaki, Aomori, by S. Kanada on 9 August 2008, and four individuals from the Chitose-gawa River, Aoba-Koen Park, Chitose, Hokkaido, by S. Kanada on 31 August 2008. All collection data of our field surveys and the previous reports showed that the distribution area of *C. floridanus* expanded from the central part to the peripheral regions of Japan just like in a pattern of concentric circles (Fig. 1).

DNA analyses

Sequences of 369-bp 18S rRNA region, excluding some base pairs near the primer sites, were all identical among 32 individuals from 14 localities (Table 1, Fig. 2). In the sequences of 624-bp COI region, excluding some base pairs near the primer sites, only two haplotypes were found among 36 individuals from 15 localities (Table 1, Fig. 3). These two haplotypes differed at two sites, one synonymous and the other nonsynonymous substitutions. One haplotype was obtained across Japan, but the other restricted to central Japan (Chiba and Kanagawa). Established invading *C. floridanus* in Japan was thus highly genetically homogeneous (genetic diversity = 0, nucleotide diversity = 0 in 18S rRNA; haplotype diversity = 0.157, nucleotide diversity = 0.00053 in COI).

The Bayesian dendrogram of *Crangonyx* species was estimated using *Bactrurus* spp. in 18S rRNA and *Synurella* sp. in COI as outgroups, with the bootstrap values in the NJ and MP methods (Figs. 2, 3). In both dendrograms, the monophyly of all Japanese populations can be strongly supported. However, there

were no individuals with the same sequences to Japanese *C. floridanus* even in North American *C. floridanus*. Moreover, *C. pseudogracilis*, originally inhabiting North America and now introduced in Europe, was an in-group of *C. floridanus* in both 18S and COI dendrograms.

DISCUSSION

Rapid dispersion of *Crangonyx floridanus* in Japan

The results of the surveys of the distribution of *Crangonyx floridanus* revealed that the time profile of initial observation records of this introduced amphipod in each region extended in a pattern of nearly concentric circles (Fig. 1). Furthermore, the result of the genetic analysis of the various Japanese populations of *C. floridanus* found throughout Japan, strongly suggested their genetic homogeneity. Both the nucleus DNA (18S rRNA) and the mitochondrial DNA (COI) of them were almost completely uniform, which was in contrast to the relative genetic diversity found in specimens from North American populations.

From these results, the following explanation of the introduction, establishment and expansion of these amphipods could reasonably be assumed: A limited number of individuals from a North American native population were probably inadvertently introduced and established somewhere within the Kanto region. The pathway of their introduction was probably *via* a single route, rather than *via* multiple routes. Initially the population is established starting with a few limited individuals, and as they repeat reproduction cycles within their small population, they spontaneously increase their population size, and also gradually expand their distribution area. Their strong capability to adapt to widely varying environments (*i.e.*, wide temperature adaptability; riffles, pools and riverside pools; utilize a wide variety of microhabitats such as submerged plants, patchy accumulations of plant litter on streambeds, gravel river beds, artificial river dikes and the piers of bridges) along with their high rate of fertility was examined and reported in our previous study (Tojo et al. 2010). These characteristics must have contributed greatly to the success and rate of the expansion process.

Now we have a clear understanding of the invasive nature and the expansion pattern of the spread of *C. floridanus*, it is important that we use these findings to consider measures that can be taken to prevent further distribution of this alien species.

We found some *C. floridanus* specimens adhering to foliage of water plants sold in a 'pet shop' in Tokyo (*e.g.*, specimen no. 12 in table 1 and figures 2-3). Therein we observed the risk of unintentional human-caused dispersion within Japan. We must consider all possible modes of their dispersal, and effective measures suitable to prevent their further dispersion.

Taxonomical problems of *Crangonyx* species

In the process of our molecular analyses of the relationships between several crangonyctid amphipod species including *C. floridanus*, we obtained the following unexpected results. The related species, *C. pseudogracilis* inhabiting North America was revealed to be an in-group of *C. floridanus*, according to the results of several analyses based on both the nuclear DNA (18S rRNA) and the mitochondrial DNA (COI) (Figs. 2, 3). As a result, this may suggest the possibility that the species of recently introduced and established amphipods, we have thought to be *C. floridanus* may be in fact the species *C. pseudogracilis*? Incidentally, the species *C. pseudogracilis* also immigrated to Western Europe from North America, and they already have a broad distribution across Western Europe (Crawford 1937; Zhang and Holsinger 2003; Slothouber Galbreath et al. 2009), and also immigrate to the non-native region within North America (from Florida to California; Toft et al. 2002, 2003), *i.e.*, this is very similar to the situation in Japan.

Perhaps, we should consider whether the crangonyctid amphipod group has been incorrectly speciated beyond the ‘actual’ species level (*i.e.*, these amphipods may have been divided into too many species). This group of amphipods throughout the world includes 46 described species, of which 42 species are those found in North America (Zhang and Holsinger 2003). Furthermore, as the distribution of *C. floridanus* and *C. pseudogracilis* overlap, and as they are very similar morphologically, some taxonomical re-evaluation seems be appropriate. As such hitherto several researchers have also pointed out that identification down to the species level within the genus *Crangonyx* has been difficult. Because the native range is so large and potentially overlaps with so many other species of *Crangonyx*, misidentification has occurred repeatedly (Zhang 1997; Hynes 1955; Zhang and Holsinger 2003; Slothouber Galbreath et al. 2009).

In any case, it is clear that amphipods of the genus *Crangonyx* already have established invasion into Western Europe and Japan, and are rapidly expanding their distribution in the both regions. Therefore we consider that measures to contain these alien amphipods are urgently required, in parallel with further study into the classification of these crangonyctid amphipod species.

ACKNOWLEDGMENTS

We acknowledge the valuable suggestions and support of Drs. T. Ito (Eniwa, Hokkaido), H. Kusano (Tama, Kawasaki), S. Ishiwata (Kanagawa Environmental Research Center), T. Tsutsumi (Fukushima University), T. Yoshida (NPO Research Office of Sustainable Riversystems for Natural Cultural Features), K. Miyazaki (Kyoto University) and K. Satake (National Institute for Environmental Studies). We

are also indebted to Mr/s T. Tanizawa, K. Suzuki, K. Miyairi, K. Oka, M. Sueyoshi, T. Kano and M. Mochizuki, Y. Morii (Shinshu University) for their cooperation in field research. This study was supported by grants from River Fund (Foundation of River & Watershed Environment) to K. T. and Research Institute of Marine Invertebrates to K. T.

REFERENCES

- Crawford GI (1937) An amphipod, *Eucrangonyx gracilis* S. I. Smith, new to Britain. *Nature* 139:327
- Englisch U, Koenemann S (2001) Preliminary phylogenetic analysis of selected subterranean amphipod crustacean, using small subunit rDNA sequences. *Org Divers Evol* 1:139-145
- Felsenstein J (1995) Confidence limits on phylogenies: An approach using the bootstrap. *Evol* 39:783-791
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294-299
- Hynes HBN (1955) The reproductive cycle of some British freshwater Gammaridae. *J Ecol* 21:352-387
- Hou Z, Fu J, Li S (2007) A molecular phylogeny of the genus *Gammarus* (Crustacea: Amphipoda) based on mitochondrial and nuclear gene sequences. *Mol Phyl Evol* 45:596-611
- Huelsenbeck JP and Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754-755
- Kanada S, Kuranishi RB, Ishiwata S, Tojo K, Shimizu T, Taira H, Satake K (2007) Distribution of an alien species, *Crangonyx floridanus* Bousfield (Crustacea: Amphipoda: Crangonyctidae) in Japan. *Jpn J Limnol* 68:449-460
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111-120
- Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 9:299-306
- Morino H, Kusano H, Holsinger JR (2004) Description and distribution of *Crangonyx floridanus* (Crustacea: Amphipoda: Crangonyctidae) in Japan, an introduced freshwater amphipod from North America. *Contr Biol Lab Kyoto Univ* 29:371-381
- Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. *Syst Biol* 53:47-67

- Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425
- Slothouber Galbreath JGM, Smith JE, Becnel JJ, Butlin RK, Dunn AM (2009) Reduction in post-invasion genetic diversity in *Crangonyx pseudogracilis* (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites? *Biol Invasion* DOI 10.1007/s10530-009-9442-3
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596-1599
- Tanaka Y, Tanizawa T, Suzuki K, Sekiné K, Tojo K (2007) Distribution of the introduced freshwater amphipod *Crangonyx floridanus* in the Matsumoto basin (Crustacea: Amphipoda, Crangonyctidae). *Bull Shiojiri City Mus Natura History* 9:40-45
- Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl Acid Res* 22:4673-4680
- Toft JD, Cordell JR and Fields WC (2002) New records of crustaceans (Amphipoda, Isopoda) in the Sacramento/San Joaquin Delta, California, and application of criteria for introduced species. *J Crustacean Biol* 22: 190-200
- Toft JD, Simenstad CA, Cordell JR, Grimaldo LF (2003) The effects of introduced water hyacinth on habitat structure, invertebrate assemblages, and fish diets. *Estuar* 26:746-758
- Tojo K, Tanizawa T, Sekiné K, Suzuki K, Miyairi K, Tanaka Y (2007) Distribution of the introduced freshwater amphipod *Crangonyx floridanus* in the Chikuma River (Crustacea: Amphipoda, Crangonyctidae). *Bulletin Shiojiri City Mus Natur Hist* 9:35-39
- Tojo K, Tanaka Y, Kuranishi RB, Kanada S (2010) Reproductive biology and adaptability of the invasive alien freshwater amphipod *Crangonyx floridanus* (Crustacea: Amphipoda, Crangonyctidae). *Zool Sci* (in press)
- Zhang J (1997) Systematics of the freshwater amphipod genus *Crangonyx* (Crangonyctidae) in North America. Dissertation, Old Dominion University, Virginia, U.S.A.
- Zhang J, Holsinger JR (2003) Systematics of the freshwater amphipod genus *Crangonyx* (Crangonyctidae) in North America. *Virginia Mus Nat Hist Mem*, 6:1-274, 183-199

Figure legends

Fig. 1 Geographic distribution of *Crangonyx floridanus* in Japan (the lines are prefectural bandaries). This invading amphipod was first found in 1989, in the Furutone-numa oxbow lake of the Tone-gawa River. Solid circles (●) indicate the habitats, in which this amphipod was newly found up until 1999. Open squares (□) indicate the habitats, in which it was found from 2000 to 2004. And, gray diamonds (◆) and solid stars (★) indicate the newly found habitats, from 2005-2007 and 2008-2009, respectively.

Fig. 2 Phylogenetic relationship between native Floridan and introduced Japanese populations of *Crangonyx floridanus*, including closely related *Crangonyx* species, based on the 369-bp nuclear 18S sequences. The Bayesian dendrogram was constructed based on HKY substitution model, using three *Bactrurus* amphipods as outgroups. The scale bar indicates substitutions per site. The topologies presented by NJ and MP trees are essentially identical to that presented by the Bayesian tree. Baysian posterior probabilities (left), and NJ (center) and MP (right) bootstrap values are specified, when they exceed 50%, respectively.

Fig. 3 Phylogenetic relationship between native Floridan and invaded Japanese populations of *Crangonyx floridanus*, including closely related *Crangonyx* species, based on the 624-bp mitochondrial COI sequences. The Bayesian dendrogram was constructed based on GTR+G substitution model, using two *Synurella* amphipods as outgroup. The scale bar indicates substitutions per site. The topologies presented by NJ and MP trees are essentially identical to that presented by the Bayesian tree. Baysian posterior probabilities (left), and NJ (center) and MP (right) bootstrap values are specified, when they exceed 50%, respectively.

Table 1. Specimens of *Crangonyx* and related amphipods used nuclear 18S rRNA and mitochondrial COI analysis

No. of speci	Family	Species	Location				Accession no.	
			Locality	Prefecture / State	County	Latitude/Longitude	18S	COI
Crangonyctidae								
1		<i>Crangonyx floridanus</i>	Chitose (Chitose-gawa River)	Hokkaido	JPN	42°49' N 141°38' E	EAB512457	AB513827
2		<i>Crangonyx floridanus</i>	Iwaki (Iwaki-gawa River)	Aomori	JPN	40°38' N 140°28' E	EAB512451	AB513820
3		<i>Crangonyx floridanus</i>	Iwaki (Iwaki-gawa River)	Aomori	JPN	40°38' N 140°28' E	EAB512453	AB513821
4		<i>Crangonyx floridanus</i>	Abukuma (Abukuma-gawa R	Miyagi	JPN	37°55' N 140°60' E	-	AB513822
5		<i>Crangonyx floridanus</i>	Abukuma (Abukuma-gawa R	Miyagi	JPN	37°55' N 140°60' E	-	AB513823
6		<i>Crangonyx floridanus</i>	Yoneshiro (Yoneshiro-gawa R	Akita	JPN	40°16' N 140°29' E	EAB512458	AB513817
7		<i>Crangonyx floridanus</i>	Abukuma (Abukuma-gawa R	Fukushima	JPN	37°44' N 140°28' E	-	AB513824
8		<i>Crangonyx floridanus</i>	Aga (Aga-gawa River)	Fukushima	JPN	37°54' N 139°52' E	EAB512459	AB513816
9		<i>Crangonyx floridanus</i>	Furutone (Furutone-numa Po	Chiba	JPN	35°54' N 140°04' E	EAB512455	AB513825
10		<i>Crangonyx floridanus</i>	Furutone (Furutone-numa Po	Chiba	JPN	35°54' N 140°04' E	EAB512456	AB513826
11		<i>Crangonyx floridanus</i>	Musashino (Percolation Pond	Tokyo	JPN	35°43' N 139°33' E	EAB512449	-
12		<i>Crangonyx floridanus</i>	Pet shop (Aquarium of a Pet	Tokyo	JPN	35°34' N 139°43' E	EAB512450	AB513819
13		<i>Crangonyx floridanus</i>	Tama (Tama-gawa River)	Tokyo	JPN	35°37' N 139°38' E	-	AB513818
14		<i>Crangonyx floridanus</i>	Sagami (Sagami-gawa River)	Kanagawa	JPN	35°31' N 139°22' E	EAB512446	AB513814
15		<i>Crangonyx floridanus</i>	Sagami (Sagami-gawa River)	Kanagawa	JPN	35°31' N 139°22' E	EAB512448	-
16		<i>Crangonyx floridanus</i>	Sakai (Sakai-gawa River)	Kanagawa	JPN	35°25' N 139°32' E	-	AB513808
17		<i>Crangonyx floridanus</i>	Sakai (Sakai-gawa River)	Kanagawa	JPN	35°25' N 139°32' E	-	AB513809
18		<i>Crangonyx floridanus</i>	Sakai (Sakai-gawa River)	Kanagawa	JPN	35°25' N 139°32' E	EAB512442	AB513810
19		<i>Crangonyx floridanus</i>	Sakai (Sakai-gawa River)	Kanagawa	JPN	35°25' N 139°32' E	EAB512443	AB513811
20		<i>Crangonyx floridanus</i>	Sakai (Sakai-gawa River)	Kanagawa	JPN	35°25' N 139°32' E	EAB512444	AB513812
21		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512431	AB513830
22		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512432	AB513829
23		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512435	AB513804
24		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512436	AB513805
25		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512437	AB513831
26		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	EAB512438	AB513806
27		<i>Crangonyx floridanus</i>	Genchi (Genchi-ido Well)	Nagano	JPN	36°14' N 137°58' E	-	AB513834
28		<i>Crangonyx floridanus</i>	Sai (Sai-gawa River)	Nagano	JPN	36°18' N 137°56' E	EAB512439	-
29		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512428	AB513800
30		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512429	AB513801
31		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512454	AB513833
32		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512430	AB513832
33		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512441	AB513807
34		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	-	AB513835
35		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512447	AB513815
36		<i>Crangonyx floridanus</i>	Tate (Tate-gawa River)	Nagano	JPN	36°20' N 137°54' E	EAB512440	-
37		<i>Crangonyx floridanus</i>	Izumi (Izumi-gawa River)	Shizuoka	JPN	34°47' N 138°17' E	EAB512445	AB513813
38		<i>Crangonyx floridanus</i>	Izumi (Izumi-gawa River)	Shizuoka	JPN	34°47' N 138°17' E	EAB512452	-
39		<i>Crangonyx floridanus</i>	Nunome (Nunome-gawa Riv	Nara	JPN	34°40' N 135°58' E	EAB512433	AB513802
40		<i>Crangonyx floridanus</i>	Nunome (Nunome-gawa Riv	Nara	JPN	34°40' N 135°58' E	EAB512434	AB513803
41		<i>Crangonyx floridanus</i>	Nunome (Nunome-gawa Riv	Nara	JPN	34°40' N 135°58' E	-	AB513828
42		<i>Crangonyx floridanus</i>	Styx (Styx River)	Florida	USA	n n	-	AJ968909
43		<i>Crangonyx floridanus</i>	Styx (Styx River)	Florida	USA	n n	-	AJ968910
44		<i>Crangonyx floridanus</i>	Styx (Styx River)	Florida	USA	n n	-	AJ968911
45		<i>Crangonyx floridanus</i>	Cross (Cross Creek)	Florida	USA	n n	AJ966707	-
46		<i>Crangonyx floridanus</i>	Lab (Lab Pond)	Florida	USA	n n	AJ966708	-
47		<i>Crangonyx floridanus</i>	Lab (Lab Pond)	Florida	USA	n n	AJ966709	-
48		<i>Crangonyx pseudograkil</i>	Middleton (Middleton Park P	Leeds	GBR	n n	AJ966698	AJ968893
49		<i>Crangonyx pseudograkil</i>	Matsloot	Boerakker	NLD	n n	AJ966699	AJ968894
50		<i>Crangonyx pseudograkil</i>	Loing (LoingRiver)	Souppes-s.-Loing	FRA	n n	AJ966701	AJ968898
51		<i>Crangonyx pseudograkil</i>	Calcasieu (Calcasieu River)	Louisiana	USA	n n	AJ966702	¹ AJ968901
52		<i>Crangonyx pseudograkil</i>	Calcasieu (Calcasieu River)	Louisiana	USA	n n	AJ966703	¹ AJ968902
53		<i>Crangonyx pseudograkil</i>	Calcasieu (Calcasieu River)	Louisiana	USA	n n	AJ966704	¹ AJ968903
54		<i>Crangonyx pseudograkil</i>	Calcasieu (Calcasieu River)	Louisiana	USA	n n	-	¹ AJ968904
55		<i>Crangonyx pseudograkil</i>	Ira Breaux (Ira Breaux Roa	Louisiana	USA	n n	AJ966705	AJ968905
56		<i>Crangonyx pseudograkil</i>	Guelph	Ontario	CAN	43°21' N 80°12' W	EF582897	EF570296
57		<i>Crangonyx pseudograkil</i>	Eem (Eem River)	Eembrugge	NLD	n n	AJ966700	AJ968895
58		<i>Crangonyx pseudograkil</i>	Aa (Aa River)	Rijsbergen	NLD	n n	-	AJ968896
59		<i>Crangonyx pseudograkil</i>	Bourgogne (Canal de Bourgo	Dijion	FRA	n n	-	AJ968897
60		<i>Crangonyx pseudograkil</i>	Bouvron (Le Bouvron)	Cellettes	FRA	n n	-	AJ968899
61		<i>Crangonyx pseudograkil</i>	Loire (Loire River)	gnieres-de-Tourai	FRA	n n	-	AJ968900
62		<i>Crangonyx forbesi</i>	St. Louis (Saint Louis County	Missouri	USA	n n	AF202980	-
63		<i>Crangonyx</i> sp. ³⁾	Barnishee (Barnishee Slough	Tennessee	USA	n n	AJ966706	AJ968906
64		<i>Bactrurus brachycaudus</i>	St. Louis (Saint Louis County	Missouri	USA	n n	AF202979	-
65		<i>Bactrurus brachycaudus</i>	Montgomery (Montgomery C	Illinois	USA	n n	AF202984	-
66		<i>Bactrurus mucronatus</i>	⁴⁾ Saline (Saline County)	Illinois	USA	n n	AF202978	-
67		<i>Bactrurus pseudomucronatus</i>	Oregon (Oregon County)	Missouri	USA	n n	AF202985	-
68		<i>Synurella</i> sp.	Calcasieu (Calcasieu River)	Louisiana	USA	n n	-	AJ968912
69		<i>Synurella</i> sp.	Grada (Lake Grada)	Mississippi	USA	n n	-	AJ968913
70		<i>Synurella</i> sp.	Grada (Lake Grada)	Mississippi	USA	n n	-	AJ968914

n: Exact reading not taken

*: *Crangonyx floridanus* found here for the first time in Japan.

***: Found from water plant in an aquarium of a petshop

¹⁾ Exact correspondence relationships between nuclear DNA data and mitochondrial DNA data are unknown²⁾ Locality date from Hou et al. (2007)³⁾ Locality date from Slothouber Galbreath et al. (2009)⁴⁾ Locality date from English and Koenemann (2001)

Table 2 Primers used in this study

Gene	Primer names	Primer direction	Primer sequence (5' to 3')	References
18S	18S700F	Foward	GTCTGGTGCCAGCAGCCGCG	Englisch and Koenemann (2001)
18S	18S1015R	Reverse	TTTGAGCACTCTGATTTACTCAAGG	This study
COI	LC01490	Foward	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> (1994)
COI	HC02198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> (1994)

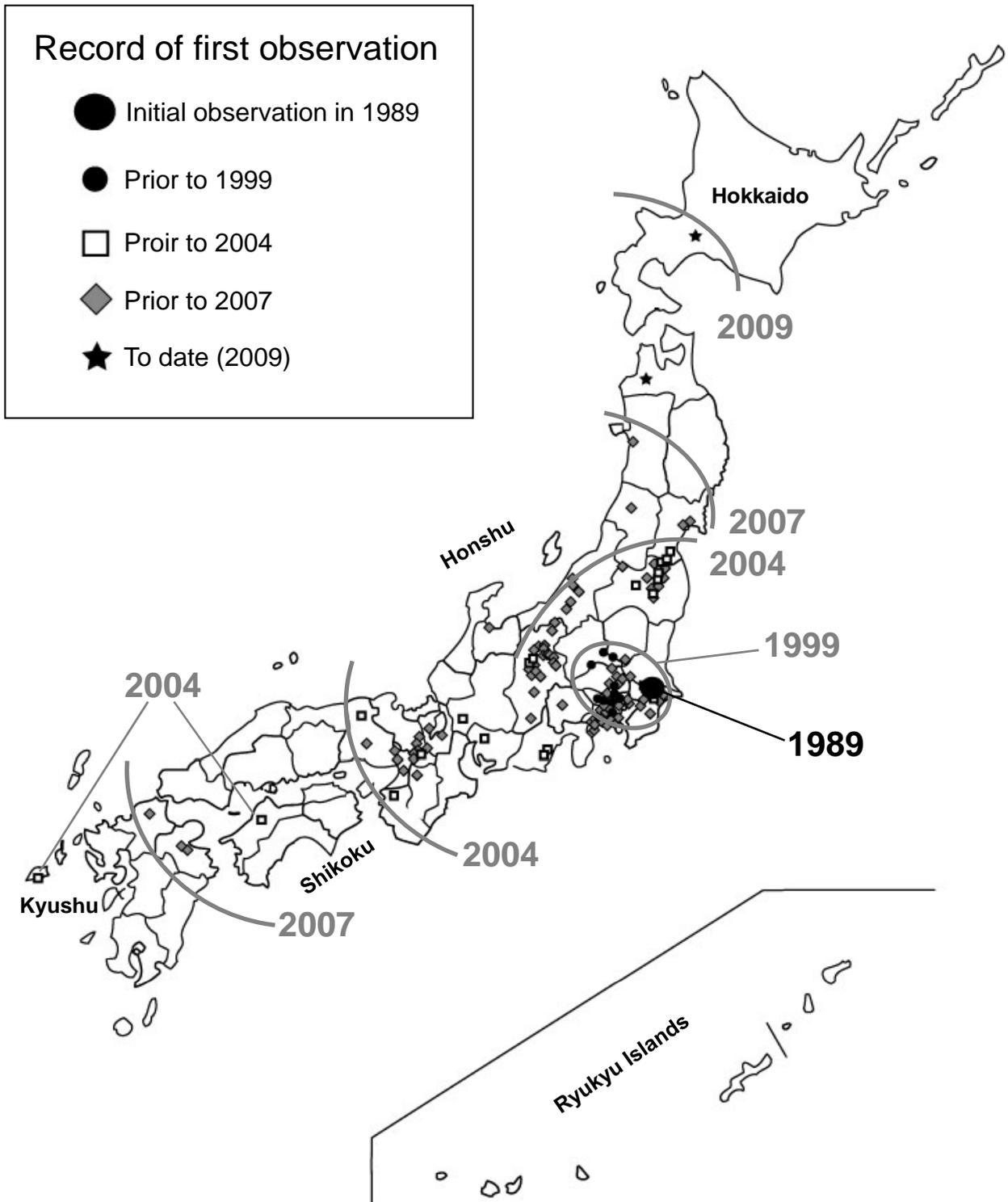


Fig. 1

nDNA: 18S

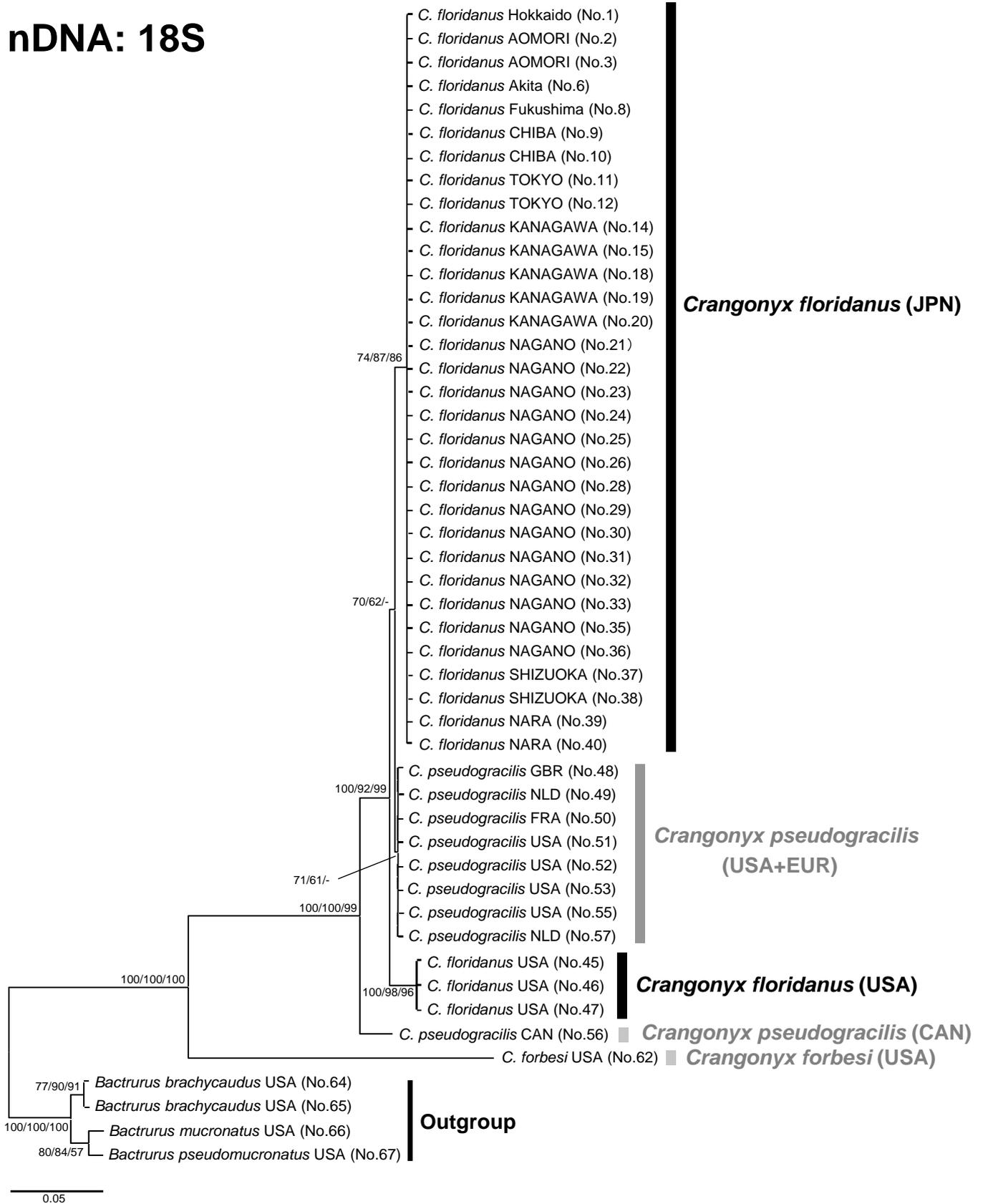


Fig. 2

mtDNA: COI

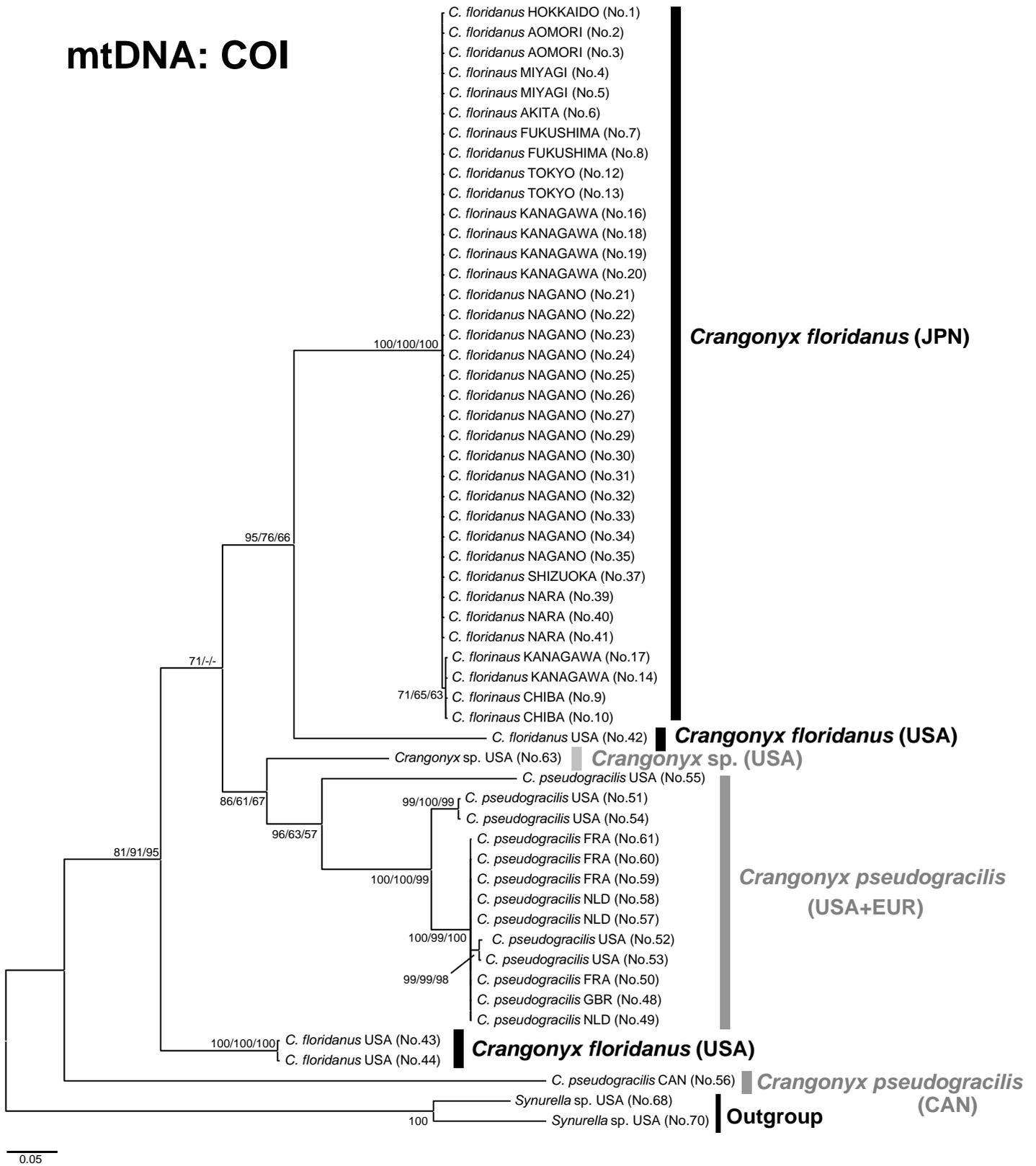


Fig. 3