International Journal for Parasitology: Parasites and Wildlife 25 (2024) 100985

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Genetic variability of *Myxobolus nagaraensis* (Bivalvulida: Myxobolidae) infecting freshwater gobies *Rhinogobius* Gill 1859 (Gobiiformes: Oxudercidae) from rivers in Japan

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ARTICLE INFO

Keywords:
Myxobolus nagaraensis
Rhinogobius spp.
ITS region
LSU rDNA

ABSTRACT

Myxobolus nagaraensis is a myxozoan parasite first reported in freshwater gobies (*Rhinogobius* spp.) from the Nagara River, Gifu Prefecture, Japan. Myxospores of *M. nagaraensis* form plasmodia in the visceral cavities of gobies, commonly presenting as distended abdomens. Although *Rhinogobius* is a common fish genus in Japan, details of *M. nagaraensis*, including genetic information, remain unknown. We compared the nucleotide sequences of the ribosomal RNA gene (rDNA) of *M. nagaraensis* from three different host species (*R. fluviatilis*, *R. nagoyae*, and *R. similis*) caught in three different rivers in Japan (Sakai, Sagami, and Kaname). The ITS region (ITS-1, 5.8S rDNA, and ITS-2) and large subunit (LSU) rDNA exhibited 49 and 55 variable sites, respectively. The highest nucleotide diversity was observed in the ITS region (0.00962), whereas that of the LSU rDNA was 0.00187. Differences in host species, rather than rivers, were a significant factor for genetic variation in both the ITS region (62.58%; $P < 0.001$) and LSU rDNA (55.22%; $P < 0.01$). Significant genetic variation was observed in *M. nagaraensis* from *R. similis* compared to *R. fluviatilis* ($P < 0.001$) or *R. nagoyae* ($P < 0.001$) from the same river. Such details are valuable for understanding parasite dispersal and its ecological impact on *Rhinogobius* hosts.

1. Introduction

Rhinogobius species (Gobiiformes: Oxudercidae) is a genus of small gobies distributed in the freshwater rivers of East and Southeast Asia (Fujita, 2019). In Japan, *Rhinogobius* gobies are called under the Japanese name of "yoshinobori", except for *Rhinogobius similis* (syn. *R. giurinus*) which is referred to as "gokuraku-haze", a type species for the genus (Suzuki et al., 2016). Successful adaptation to freshwater environments has led *Rhinogobius* species to have a distinct ecology, including amphidromous, fluvial, lake-river migrating, and lentic life histories (Yamasaki et al., 2015). The taxonomy of *Rhinogobius* gobies in Japan has long been controversial, and as a result, the scientific names of several "yoshinobori" species are yet to be determined. Instead of giving a formal specific name, such species are commonly described using the two-capital-letter alphabet (e.g. *Rhinogobius* sp. BB, DL, KZ, MO, OM, and YB; Suzuki et al., 2020). Recently, detailed morphological descriptions have been made for several of these informal species, granting them formal scientific names, as described by Takahashi and Okazaki (2017) and Suzuki et al. (2019, 2020).

Myxobolus nagaraensis, a myxozoan parasite, was first reported in 2007 in *Rhinogobius* spp. From the Nagara River in Gifu Prefecture, Japan (Yokoyama et al., 2007). As observed in other *Myxobolus* species, the life cycle of *M. nagaraensis* is suggested to alternate between *Rhinogobius* hosts and unidentified invertebrate hosts, possibly oligochaeta worms, but the full life cycle remains to be elucidated. In *Rhinogobius* hosts, myxospores of *M. nagaraensis* form cysts in the host visceral cavity, which commonly present as a distended abdomen. Although its distinct gross pathology, reports on *M. nagaraensis* infection in *Rhinogobius* fishes has been scarce (Hanasaki, 2018; Hioki et al., 2019) and its detailed impact on *Rhinogobius* populations remains unknown.

As demonstrated in many other eukaryotic taxa, sequences of the small subunit (SSU) ribosomal RNA gene (rDNA) have proven useful in myxozoan species for constructing phylogenetic relationships (Fiala et al., 2015). Although 2–3 times longer than SSU rDNA, large subunit

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https://doi.org/10.1016/j.ijppaw.2024.100985
Received 9 July 2024; Received in revised form 5 September 2024; Accepted 5 September 2024
Available online 7 September 2024

(LSU) rDNA offers taxonomic information similar to that of SSU rDNA (Bartošová et al., 2009). The internal transcribed spacer region 1 (ITS-1) is more variable than SSU or LSU rDNA because of faster evolutionary changes in the region, and has been used to distinguish myxozoan populations from different hosts or geographical regions (Fiala et al., 2015). For example, variations in ITS-1 sequences have been used to understand the dispersal and evolutionary history of *Myxobolus cerebralis*, a causative parasite of whirling disease in salmonid fish (Whipps et al., 2004a; Lodh et al., 2012). ITS-1 sequences have also revealed the presence of different genotypes associated with host species and virulence levels in *Ceratonova shasta*, a myxozoan parasite that affects salmonid fishes in North America (Hurst and Bartholomew, 2012).

A certain degree of intraspecific variation is also expected for *M. nagaraensis*, considering the broad habitat range and species variety of *Rhinogobius* species. Here, we compared the nucleotide sequences of the entire ITS region (including ITS-1, 5.8S rDNA, and ITS-2) and the LSU rDNA of *M. nagaraensis* from three different host species caught in five different rivers in Japan. This study aimed to provide novel genetic information regarding the largely unknown *M. nagaraensis*. Such details of intraspecific variations in myxozoan parasites are crucial for understanding parasites dispersal and its ecological impacts on *Rhinogobius* hosts, particularly for "yoshinobori" species that are endemic to specific regions in Japan.

2. Materials and methods

2.1. Sample collection

Twenty-four diseased fish with enlarged abdomens were caught from three rivers (Sakai, Sagami, and Kaname) running through Kanagawa Prefecture, Japan. Additionally, two diseased fish were collected, one from the Nishina River in Shizuoka Prefecture and the other from the Chikugo River in Fukuoka Prefecture. The fish were identified immediately after being caught and euthanized in 10 ml of 99% ethanol in 1 L of water and then preserved in 70% ethanol. When identification was difficult at the spot, the identification procedure was done in a laboratory. *Rhinogobius* species were classified and identified based on Nelson et al. (2016), Jeon et al. (2021), and "Fishes of Japan with Pictorial Keys to the Species, 3rd Edition" (Nakabo, 2013). The scientific name for *Rhinogobius similis* follows Suzuki et al. (2016). The *Rhinogobius* species examined in this study and their locations are listed in Table 1. Following the identification of the fish species, cysts were excised from the diseased fish for spore measurements and DNA extraction.

2.2. Myxospore morphology

Twenty myxospores from each host were examined using an Olympus BX53 microscope equipped with a DP22 camera (Olympus, Tokyo, Japan). Measurements were made under a stereomicroscope using cellSens® Standard software (Olympus) according to the metrics of Yokoyama et al. (2007). The length and width of the spores and polar capsules were measured from the front of the spores. Spore thickness was also measured from the sutural view of the spores. The measurements for this experiment are described as the mean in μm, with the range in parentheses.

2.3. DNA extraction and amplification

DNA was extracted from the excised cysts using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The following primers were used for PCR amplification of the SSU rDNA and ITS region: 18e (5′-CTGGTTGATTCTGCCAGT-3′; Hillis and Dixon, 1991) and Myxo28S1F-V (5′-CACTTCACTCGCAGTTACT-3′; Whipps et al., 2004b). Primers used for LSU rDNA were NLF160 (5′-ACCTCCACTCAGGCAAGATTA-3′) and NLR3284 (5′-TTCTGAC TTAGAGGCGTTCAG-3′; Van der Auwera et al., 1994). The reaction mixture included 50 ng of DNA template, 1 × PrimeSTAR® MAX Premix (Takara Bio Inc., Shiga, Japan), and 0.2 μM primers in a total reaction volume of 50 μL. The thermal profile for PCR was 35 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 15 s, and extension at 72 °C for 10–20 s. Amplified products were visualised on 1.0% agarose gel and determined to be positive when a specific band was visible in the gel. Positive PCR products were directly purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel, Düren, Germany) according

Table 1
Sampling locations and species of *Rhinogobius* fish host examined in this study.

Prefecture	River	Branch	Coordinates	Host species	Collection Year/Month	Host ID
Kanagawa	Sagami	Nakatsugawa branch	35.458 N 139.365 E	*Rhinogobius fluviatilis*	2018–Dec	mx18002
		Nakatsugawa branch	35.458 N 139.365 E	*Rhinogobius fluviatilis*	2018–Dec	mx18003
		Nakatsugawa branch	35.458 N 139.365 E	*Rhinogobius fluviatilis*	2018–Dec	mx18004
		Onsogawa branch	35.429 N 139.355 E	*Rhinogobius fluviatilis*	2018–Dec	mx18007
		Onsogawa branch	35.429 N 139.355 E	*Rhinogobius fluviatilis*	2018–Dec	mx18008
		Nagaikegawa branch	35.402 N 139.378 E	*Rhinogobius fluviatilis*	2018–Dec	mx18010
		Tamagawa branch	35.430 N 139.329 E	*Rhinogobius nagoyae*	2018–Dec	mx18005
		Tamagawa branch	35.430 N 139.329 E	*Rhinogobius nagoyae*	2018–Dec	mx18006
		Nagaikegawa branch	35.402 N 139.378 E	*Rhinogobius nagoyae*	2018–Dec	mx18009
	Sakai	Sakaigawa branch	35.392 N 139.480 E	*Rhinogobius fluviatilis*	2018–Oct	mx18015
		Sakaigawa branch	35.392 N 139.480 E	*Rhinogobius fluviatilis*	2018–Dec	mx18034
		Sakaigawa branch	35.392 N 139.480 E	*Rhinogobius nagoyae*	2018–Oct	mx18011
		Sakaigawa branch	35.404 N 139.476 E	*Rhinogobius nagoyae*	2018–Oct	mx18024
		Sakaigawa branch	35.388 N 139.484 E	*Rhinogobius nagoyae*	2019–Dec	mx19004
		Kashiogawa branch	35.336 N 139.496 E	*Rhinogobius similis*	2020–Jul	mxsa20001
		Kashiogawa branch	35.336 N 139.496 E	*Rhinogobius similis*	2020–Jul	mxsa20002
		Kashiogawa branch	35.408 N 139.536 E	*Rhinogobius similis*	2020–Jul	mxsa20006
		Kashiogawa branch	35.378 N 139.532 E	*Rhinogobius similis*	2020–Jul	mxsa20007
		Kashiogawa branch	35.378 N 139.532 E	*Rhinogobius similis*	2020–Nov	mxsa20013
		Kashiogawa branch	35.378 N 139.532 E	*Rhinogobius similis*	2020–Nov	mxsa20014
	Kaname	Shibutagawa branch	35.361 N 139.344 E	*Rhinogobius nagoyae*	2020–Dec	mx20001
		Shibutagawa branch	35.361 N 139.344 E	*Rhinogobius nagoyae*	2020–Dec	mx20002
		Miname-Kaname irrigation canal	35.356 N 139.275 E	*Rhinogobius nagoyae*	2020–Dec	mx20003
		Miname-Kaname irrigation canal	35.356 N 139.275 E	*Rhinogobius nagoyae*	2020–Dec	mx20004
Shizuoka	Nishina		34.775 N 138.789 E	*Rhinogobius similis*	2021–Oct	mxtky22001
Fukuoka	Chikugo	Tachiaraigawa branch	33.375 N 130.592 E	*Rhinogobius flumineus*	2022–Nov	mxtky22002

to the manufacturer's protocol. Amplified products of LSU rDNA were extracted from the gel using the same kit, as multiple bands were observed.

2.4. Cloning and sequencing

Purified PCR products for the SSU rDNA, ITS region and LSU rDNA were cloned into the pCR™2.1-TOPO®vector (Invitrogen, Waltham MA, USA), which was used to transform One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen). Transformants were selected by blue-white screening on LB agar plates containing X-gal and ampicillin. Plasmid DNA was extracted using NucleoSpin® Plasmid QuickPure (Macherey Nagel) and five clones per infected fish were sequenced in a sequencing facility using primers listed in Table 2. For species identification, sequences obtained here were compared with a 1624 bp partial SSU rDNA sequence of *M. nagaraensis* (Accession No. AB274267; Yokoyama et al., 2007), a region amplified by primers MX5 (5′-CTGCGGACGGCTCAGTAAATCAGT-3′) and MX3 (5′-CCAGGACA TCTTAGGGCATCACAGA-3′; Andree et al., 1999).

2.5. Sequence alignment, overview of genetic diversity, population structure, and phylogenetic analysis

The sequences were edited and aligned using MUSCLE (Edgar, 2004) implemented in MEGA7 (Kumar et al., 2016) with the available sequences for myxozoan parasites and verified manually. The number of variable sites and nucleotide diversity (π) for each region was calculated using DnaSP v6 (Rozas et al., 2017). Hierarchical genetic variation for the ITS region and LSU rDNA of samples from Kanagawa Prefecture was analyzed by analysis of molecular variance (AMOVA; Excoffier et al., 1992). Three datasets were created: i) three host populations, *R. fluviatilis*, *R. nagoyae*, and *R. similis*, from the Sakai River with 55 clones; ii) three geographical populations, Sakai River, Sagami River, and Kaname River, for *R. nagoyae* with 50 clones; and iii) two geographical populations, Sakai River and Sagami River, for *R. fluviatilis* with 40 clones. AMOVA was performed using Arlequin v3.2.1 (Excoffier and Lischer, 2010) with partitioning the variation among host populations or geographical populations, among hosts within a population, and within individual fish hosts. The fixation index (Fst) was calculated using Arlequin v3.2.1 (Excoffier and Lischer, 2010) to measure the levels of genetic differences between different populations.

The phylogenetic relationship for the ITS region and the LSU rDNA was inferred using the maximum likelihood method using MEGA7 (Kumar et al., 2016). A Tamura-3-parameter model and a Tamura-Nei model were selected as the substitution model for the ITS region and LSU rDNA respectively based on the Bayesian information criterion. The reliability of the tree was assessed by bootstrap analysis with 1000 replicates. A TCS network (Clement et al., 2002) was inferred using PopART (Leigh and Bryant, 2015) with sampling rivers and fish host species as traits for both the ITS region and LSU rDNA. Sequences obtained in this study were submitted to the DNA Data Bank of Japan (DDBJ) database under the accession numbers LC764501–LC764583.

Table 2
Primers used for rDNA sequencing of *Myxobolus nagaraensis*.

	Primer	Sequence	Reference
SSU rDNA, ITS region	MC5	5′-CCTGAGAAACGGCTACCACATCCA-3′	Molnár et al. (2002)
	MC3	5′-GATTAGCCTGACAGATCACTCCACGA-3′	Molnár et al. (2002)
	18R	5′-CTACGGAAACCTTGTTACG-3′	Whipps et al. (2003)
	Mc5S1R	5′-ATGACTCACTAGGCTTGC-3′	Whipps et al. (2004a)
LSU rDNA	NLR1694	5′-TCTYAGGAYCGACTNAC-3′	Van der Auwera et al. (1994)
	Ma28-310f	5′-TGGGAATGCAGTCTGAAGTG-3′	Urawa et al. (2011)
	NLR1126	5′-ATCCTGAGGGATATTTCG-3′	Van der Auwera et al. (1994)
	NLF1260	5′-TGGTAAGCAGAACTGGC-3′	Bartošová et al. (2009)
	NLR3422	5′-CTCTACYCGTGGTTTCTGTCC-3′	Bartošová et al. (2009)
	28S3R	5′-GbAGCACTGGGCAGAAATC-3′	Whipps et al. (2004b)
Vector cloning site	M13F	5′-GTAAAACGACGGCCAGT-3′	Messing (1983)
	M13R	5′-CAGGAAACAGCTATGAC-3′	Messing (1983)

3. Results

3.1. Parasite identification by spore morphology and partial SSU rDNA sequence

Plasmodia were encapsulated by the host's connective tissue and formed large cysts occupying the visceral cavity, and occasionally putting pressure on the internal organs, including the stomach, intestine, kidney, and ovary. In some cases, cysts filled with myxospores was formed subcutaneously, either on the side of the caudal penduncle or behind the head. Myxospore morphology from different rivers and hosts in the present study followed the description by Yokoyama et al. (2007), with an ovoid frontal view and two pyriform polar capsules. Although most of the measurements were in the range of the reference values by Yokoyama et al. (2007), the mean spore length and thickness were slightly longer and wider for *M. nagaraensis* from *R. similis* from the Nishina River (mean spore length 13.4 [11.3–15.6] μm, width 7.2 [5.4–8.9] μm) and *Rhinogobius flumineus* from the Chikugo River (mean spore length 13.0 [10.2–14.2] μm, width 7.2 [6.0–9.0] μm) compared to the reference values (mean spore length 11.9 [10.5–13.5] μm, width 6.5 [6.0–7.0] μm; Yokoyama et al., 2007, Table 3). Partial SSU rDNA sequences of ~1625 bp in length from 26 samples showed 99.57–99.94% identity with the only deposited sequence of *M. nagaraensis* (Accession No. AB274267). Host specificity (*Rhinogobius* sp.), spore development site, morphological measurements of myxospores, and molecular data were in agreement with the descriptions of Yokoyama et al. (2007), which confirmed that the parasite in this study was *M. nagaraensis*.

3.2. Overview of genetic diversity, phylogenetic analysis and population structure analysis

In the present study, 130 clones were sequenced for each rDNA region. The lengths of the SSU rDNA, ITS region, and LSU rDNA were approximately ~2139 bp, ~972 bp, and ~3628 bp, respectively, with each region exhibiting 24, 49, and 55 variable sites, respectively (Supplementary Tables 1–3). 17, 25, and 41 distinct nucleotide sequences were obtained for the SSU rDNA, ITS region, and LSU rDNA, respectively (Supplementary Tables 1–3). Sequence ID, described as SSU1–SSU17, ITS1–ITS25, and LSU1–LSU41, were assigned for each region with the corresponding accession numbers, LC764501–LC764517, LC764518–LC764542, and LC764543–LC764583, respectively (Supplementary Table 4). The highest nucleotide diversity was observed in the ITS region (0.00962), whereas the values for the LSU rDNA and SSU rDNA were 0.00187 and 0.00125, respectively.

Hierarchical genetic variation in the ITS region and LSU rDNA of *M. nagaraensis* samples from Kanagawa Prefecture was analyzed using AMOVA (Table 4). The highest variation of *M. nagaraensis* was observed among host populations for the Sakai River in both the ITS region (62.58%) and the LSU rDNA (55.22%), with significant variance components. Genetic variation in the ITS region and LSU rDNA of

Table 3
Morphological measurements of myxospores of *Myxobolus nagaraensis* with reference measurements by Yokoyama et al. (2007). Measurements were based on 20 spores from each sample and described as mean with the range in parentheses (all measurements are provided in μm).

River	Sakai			Sagami		Kaname	Nishina	Chikugo	Nagara
Host	*R. similis*	*R. nagoyae*	*R. fluviatilis*	*R. nagoyae*	*R. fluviatilis*	*R. nagoyae*	*R. similis*	*R. flumineus*	*Rhinogobius* sp. OR (Yokoyama et al., 2007)
Spore length	12.4 (10.5–15.0)	11.6 (8.6–13.2)	12.4 (10.5–13.5)	12.2 (10.8–13.7)	12.7 (10.9–14.4)	12.4 (10.5–14.0)	13.4 (11.3–15.6)	13.0 (10.2–14.2)	11.9 (10.5–13.5)
Spore width	8.8 (7.2–10.4)	8.4 (6.2–9.5)	8.6 (6.8–9.7)	9.2 (8.2–10.6)	9.5 (8.3–10.6)	9.0 (7.0–10.5)	9.0 (7.9–10.4)	10.3 (9.0–11.4)	9.0 (8.0–11.0)
Spore thickness	6.3 (5.1–7.5)	5.8 (4.6–7.0)	5.7 (4.3–6.8)	6.1 (5.2–7.1)	5.8 (4.8–7.0)	6.5 (5.2–7.6)	7.2 (5.4–8.9)	7.2 (6.0–9.0)	6.5 (6.0–7.0)
Polar capsule length	5.3 (4.2–6.7)	5.5 (4.7–6.1)	5.6 (4.6–6.7)	5.5 (2.6–6.4)	5.5 (4.3–7.1)	5.5 (3.9–7.1)	4.8 (3.8–5.7)	5.5 (4.4–6.3)	5.5 (4.5–6.0)
Polar capsule width	2.6 (1.8–3.4)	2.6 (2.0–3.1)	2.7 (2.1–3.2)	2.8 (2.2–3.4)	2.8 (1.6–3.7)	2.6 (2.0–3.7)	2.4 (1.7–3.1)	3.0 (2.2–3.9)	3.0 (2.5–4.0)

Table 4
Analysis of molecular variance (AMOVA) of *Myxobolus nagaraensis* based on ITS region and LSU rDNA.

Source of variation		ITS region				LSU rDNA			
		d. f.	Sum of squares	Variance components	Percentage of variation	d. f.	Sum of squares	Variance components	Percentage of variation
Hosts for Sakai River (*R. fluviatilis, R. nagoyae, R. similis*)	Among populations	2	197.406	5.56231	62.58[a]	2	111.967	3.04817	55.22[b]
	Among hosts within population	8	61.467	1.08939	12.26[a]	8	48.833	0.90811	16.45[a]
	Within hosts	44	98.400	2.23636	25.16[a]	44	68.800	1.56364	28.33[a]
	Total	54	357.273	8.88807		54	229.6	5.51991	
Rivers for *R. nagoyae* (Sakai, Sagami, Kaname)	Among populations	2	11.037	−0.16065	−5.04	2	13.500	0.12511	3.72
	Among hosts within population	7	57.183	1.20581	37.86[a]	7	32.800	0.36114	10.73
	Within hosts	40	85.600	2.14000	67.19[a]	40	115.200	2.88000	85.56
	Total	49	153.820	3.18516		49	161.500	3.36625	
Rivers for *R. fluviatilis* (Sakai River, Sagami River)	Among populations	1	2.217	−0.10481	−7.42	1	2.217	−0.54259	−19.6
	Among hosts within population	6	22.733	0.56778	40.18[a]	6	62.133	1.76111	63.61[a]
	Within hosts	32	30.400	0.95000	67.23[a]	32	49.600	1.55000	55.99[a]
	Total	39	55.350	1.41296		39	113.950	2.76852	

[a] $P < 0.001$.
[b] $P < 0.01$.

M. nagaraensis among different rivers was the lowest in both *R. nagoyae* and *R. fluviatilis* populations, with non-significant variance components ($P > 0.05$). The highest variation for *M. nagaraensis* from *R. nagoyae* was observed within the individual host species for both the ITS region (67.19%) and LSU rDNA (85.56%). Similarly, the highest variation for *M. nagaraensis* from *R. fluviatilis* was observed within individual host species for the ITS region (67.19%), whereas variation was the highest among hosts within the same river for the LSU rDNA (63.61%).

Significant genetic differentiation for *M. nagaraensis* by pairwise comparison ($P < 0.001$) was observed between the *R. similis* and *R. fluviatilis* populations for both the ITS region ($Fst = 0.74821$) and LSU rDNA ($Fst = 0.67580$; Table 5). Similarly, the difference between *R. similis* and *R. nagoyae* populations for both the ITS region ($Fst = 0.63608$) and LSU rDNA ($Fst = 0.63172$) were highly significant ($P < 0.001$). However, the differences between *M. nagaraensis* isolates from *R. fluviatilis* and *R. nagoyae* populations for both the ITS region ($Fst = 0.17212$) and LSU rDNA ($Fst = 0.04470$) were not significant ($P > 0.05$).

Table 5
Genetic differentiation (Fst) among three *Rhinogobius* species from Sakai River. Below and above diagonal shows pairwise Fst value for ITS region and pairwise Fst value for LSU rDNA respectively.

Pairwise Fst	*R. fluviatilis*	*R. nagoyae*	*R. similis*
R. fluviatilis		0.04470	0.67580[a]
R. nagoyae	0.17212		0.63172[a]
R. similis	0.74821[a]	0.63608[a]	

[a] $P < 0.001$.

Phylogenetic analysis was conducted using 130 sequences each of the ITS region (Fig. 1) and the LSU rDNA (Supplementary Fig. 1) obtained here with registered *Myxobolus cerebralis* sequences as an outgroup. The analysis revealed that the sequences of *M. nagaraensis* isolated from *R. similis*, including samples from both Kanagawa and Shizuoka prefectures, clustered together in one clade. Additionally, sequences of *M. nagaraensis* isolated from *R. flumineus* from the Chikugo River clustered together. No phylogenetic trends regarding host or geographical locations were observed for *M. nagaraensis* isolates from *R. fluviatilis* and *R. nagoyae* from the Sagami, Sakai and Kaname Rivers. The TCS network analysis (Supplementary Fig. 2) showed that the isolates of *M. nagaraensis* from *R. smilis* and *R. flumineus* in the Chikugo River were separated from other host species for both the ITS region and LSU rDNA. Whereas the separation among the isolates for *R. fluviatilis* and *R. nagoyae* was not evident.

4. Discussions

The present study confirms the presence of *M. nagaraensis* in multiple rivers in Japan. Genetic variations were observed in both the ITS region and LSU rDNA of *M. nagaraensis*. The nucleotide sequences of *M. nagaraensis* collected from *R. similis* were distinct from those of other *Rhinogobius* species, highlighting the importance of host specificity rather than geographical location in the parasite population structure.

The genetic variations observed here in *M. nagaraensis* isolated from

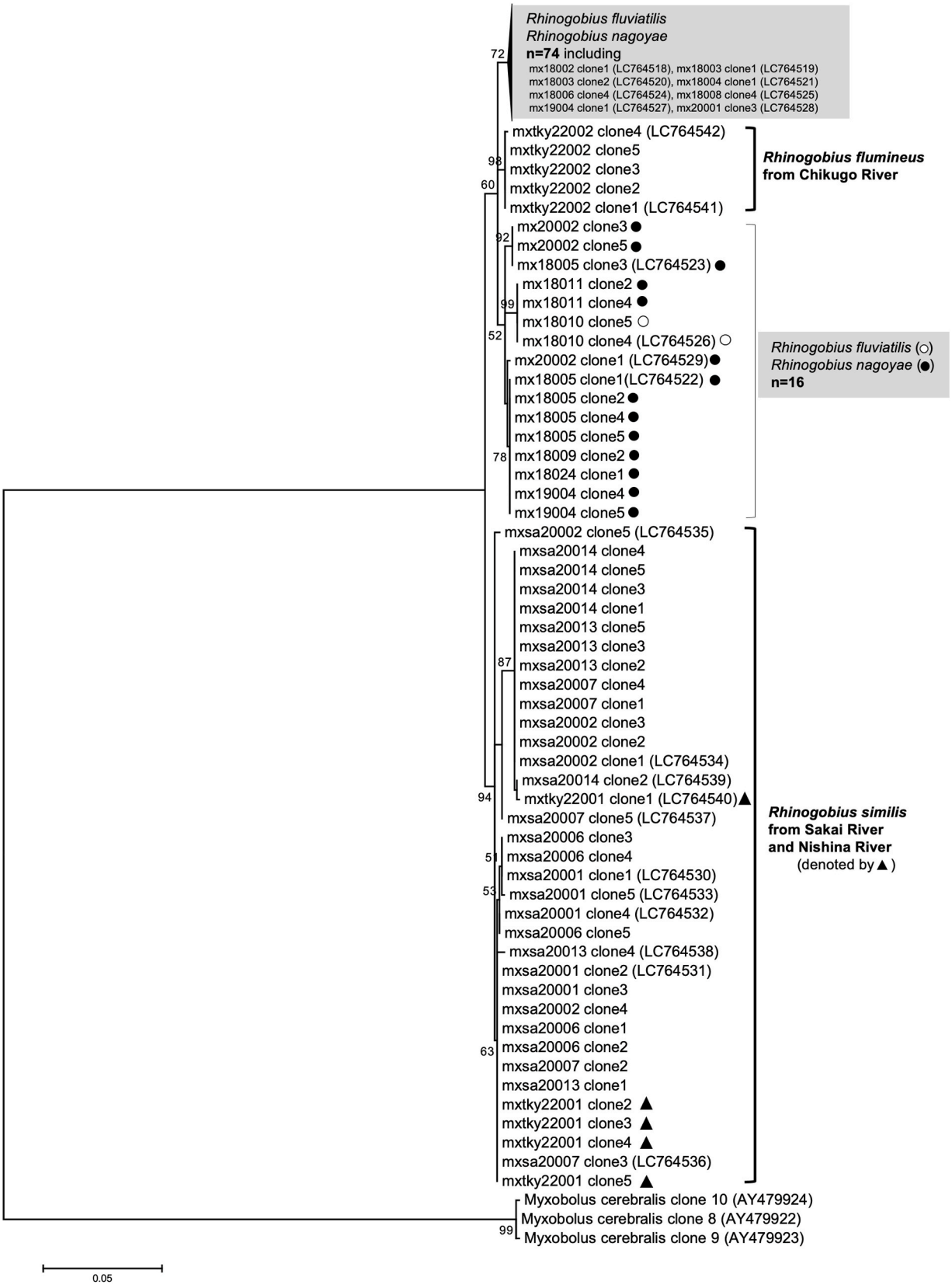

Fig. 1. Phylogenic relationship for ITS region was inferred using the maximum likelihood method using MEGA7 (Kumar et al., 2016). A Tamura-3-parameter model was selected as the substitution model based on the Bayesian information criterion. The consensus tree was obtained after bootstrap analysis with 1000 replications, with percentage bootstrap values more than 50% shown in the tree. The tree is drawn to scale, with branch lengths measured in the number of base substitutions per site.

different host species resemble those of *Ceratonova shasta*, a freshwater myxozoan parasite that infects *Oncorhynchus* species (Pacific salmonids) in the Pacific Northwest (Atkinson et al., 2018). Three primary genotypes have been reported for this parasite: genotype O, I, and II (Atkinson et al., 2018). Strong host-parasite associations have been observed for genotype O with *O. mykiss* (steelhead and rainbow trout) and genotype I with *O. tschawytscha* (Chinook salmon), whereas genotype II has been found in multiple salmonid species (Stinson et al., 2018). Differences in maturation age, migration patterns, and spawning sites have been observed in different species of *Oncorhynchus* (Waples et al., 2001), which have supposedly resulted in *C. shasta* adapting to its host species and evolving into certain genotypes (Stinson et al., 2018). *Rhinogobius* species are also known for their diverse life histories depending on the species, including amphidromous, fluvial, lake-river migrating, and lentic (Yamasaki et al., 2015), which may explain the genetic variability of *M. nagaraensis* in this study.

R. similis Gill, 1859 (syn. *R. giurinus* Rutter, 1897) is a type species of the genus *Rhinogobius* that is distributed throughout Japan (excluding Hokkaido Prefecture), Korea, Taiwan, China, and Northern Vietnam (Suzuki et al., 2016). Phylogenetic analysis of the mitochondrial DNA sequence demonstrated that *R. similis* is genetically divergent from other *Rhinogobius* species (Mukai et al., 2005; Yamasaki et al., 2015), and distinguishably called as "gokuraku-haze" in Japanese while other *Rhinogobius* species are referred to as "yoshinobori". Their life history is characterised as amphidromous; adult fish inhabit and spawn in the lower stream of a river from July to October, whereas hatched larvae migrate to and stay in the sea until they reach a certain size (25 mm–37 mm; Dôtu, 1961; Ishizaki et al., 2016). Although *R. fluviatilis* and *R. nagoyae* both have amphidromous life histories, they spawn earlier during the spring season, as early as May and ending in July (Dôtu, 1961; Tamada, 2000). In addition to the difference in the spawning season, *R. similis* tends to distribute and spawn in the lower stream of the river, whereas *R. fluviatilis* and *R. nagoyae* prefer the middle and upper streams (Tamada, 2000; Saitou et al., 2012). Such differences between *R. similis* and other species suggest that interspecific competition is rare, and that reproductive isolation is maintained in *R. similis*. Host-associated phylogenetic patterns are reported for *Myxobolus* parasites, indicating that the parasite species has been adapting to and evolved with their respective fish host species (Holzer et al., 2018; Liu et al., 2019). It is plausible that the distinct ecological and genetic features of *R. similis* have resulted in the population divergence of *M. nagaraensis*. A sample isolated from *R. similis* in Shizuoka Prefecture also supports the importance of host species, rather than geographical differences. Interestingly, the sequences of an isolate from *R. flumineus* from Fukuoka Prefecture clustered together, differing from the rest of the sequences, which may indicate the presence of both host and geographic variability, although further sampling is required to confirm this.

This genetic variance in *M. nagaraensis* may pose a challenge to the conservation of *Rhinogobius* species. In *C. shasta*, genotype II has a broader host range and infects multiple salmonid species (Stinson et al., 2018). Although strong host specificity has been suggested for *M. nagaraensis* isolated from *R. similis*, the overlap of *M. nagaraensis* sequences isolated from *R. fluviatilis* and *R. nagoyae* indicates low host specificity for these variants. It is possible that *M. nagaraensis* with low host specificity, the so-called generalist, can easily infect and spread to other *Rhinogobius* species, including *R. biwaensis* and *R. ogasawaraenesis*, which are listed on the IUCN Red List (2021). Moreover, differences in virulence and host immune responses have been reported among different genotypes of *C. shasta* (Hurst et al., 2019; Taggart-Murphy et al., 2021). *M. nagaraensis* forms cysts in the body cavity of *Rhinogobius* hosts, which interfere with their vital organs (Yokoyama et al., 2007; Hanasaki, 2018). However, the detailed pathogenesis in *Rhinogobius* hosts, including disease progression and mortality rates, remains poorly understood. Further investigation into the relationship between the genetic variation of *M. nagaraensis* and its impact on disease outcomes in *Rhinogobius* hosts is crucial for effective conservation strategies.

In the present study, multiple nucleotide sequences of *M. nagaraensis* were observed within the same host. Since myxozoan parasites undergo clonal reproduction in vertebrate hosts (Feist et al., 2015), genetic variations in the parasite observed within the same host indicate that the *Rhinogobius* hosts were infected by multiple actinospores of different genotypes in the environment. These multiple infections may have resulted in myxospores of different genotypes within the host fish. However, genetic differences among ITS-1 copies within individual spores have been reported in both *M. cerebralis* and *C. shasta*, with variations ranging from 0.6% in *C. shasta* myxospores to 1.7% in *M. cerebralis* actinospores (Whipps et al., 2004a; Atkinson et al., 2018). Genotype III was once considered a distinct genotype in *C. shasta*, but it was later discovered that both genotypes II and III exist in a single spore, invalidating the distinction between these two genotypes (Atkinson et al., 2018). We did not perform single-spore genotyping in the present study because we were uncertain of the extent of genetic variability in *M. nagaraensis*. It has been clarified that obvious genetic variability exists in *M. nagaraenis*. There is a need to elucidate whether this genetic variability within the same host results from multiple infections of *M. nagaraensis* of different genotypes, or from the presence of multiple sequences of rDNA copies within individual parasite spores, for an accurate understanding of the population genetics of *M. nagaraensis*.

5. Conclusion

In summary, the present study revealed genetic variability in *M. nagaraensis* and that host species play an important role in population structure. Although considered a ubiquitous fish species in Japan, much of the details of *M. nagaraensis*, including its actinospore stage and invertebrate host, remain unknown. Characterising such factors, as well as collecting additional genetic data on *M. nagaraensis*, is crucial to further our understanding of parasite dispersal and predict its ecological impact on *Rhinogobius* species.

Financial support

This study did not receive any specific grants from funding agencies in the public, commercial, or non-profit sectors.

CRediT authorship contribution statement

Kana Kurusu: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Naoyuki Hioki:** Writing – review & editing, Resources, Methodology, Investigation. **Mizuho Shima:** Writing – review & editing, Resources, Methodology, Investigation. **Sunao Kawakami:** Writing – review & editing, Validation, Investigation. **Yuta Hasebe:** Writing – review & editing, Resources, Methodology, Investigation. **Noriyuki Takai:** Writing – review & editing, Resources, Methodology. **Jun Matsumoto:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Aya Masuda:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that there are no known conflicts of interest associated with this publication.

Acknowledgements

Samples from Shizuoka and Fukuoka Prefectures were kindly provided by Ryota Nakamura and Taro Shiogami, respectively. The authors express special gratitude to the staff at the Kanagawa Environmental Research Center, and Shinichiro Shibata, Ryota Enami and Ren Horiuchi

from the Tokyo Metropolitan Tama High School of Science and Technology, for their insightful discussions on the biology and ecology of *Rhinogobius* species. We are grateful to the anonymous reviewers for their critical comments which have greatly improved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100985.

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