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Appearance of the non-indigenous mtDNA haplotypes of *Rhynchocypris lagowskii* in Tohoku, Japan

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ABSTRACT

Classification of fresh water fish cyprinidae is hampered by complexity or lack of morphological diversity. In this study we analyse besed on mtDNA sequences were undertaken to clarify phylogenetic relationship Aburahaya (Rhynchocypris lagowskii) among various site in Tohoku, Japan. Evolutionary rate in cytochrome b gene (Cyt-b) region we examined. To assess genetic structure within these populations we analyzed using statistical parsimony networks and relationships between the populations were examined using a neighbor-joining (NJ) method. Three major geographical groups are found in this study. The most parsimonious network of mtDNA haplotype of aburahaya 19 localities, estimated using the TCS algorithm. In this network also showed three geographical groups. Haplotype 1-32 is one group, haplotype 33-36 is group 2, haplotype 37-53 is group 3 and 54 is out group.

Key Words: *Population genetics, mtDNA, Cyprinide, Aburahaya, Geographic groups and Haplotype network*

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I. Introduction

In most freshwater fishes, it is very difficult to identified phylogenetic structure and relationship by traditional approach. Because of a multitude of stressors, overharvest, alien invasive species, climate change and population, including urbanization and associated habitat alteration and loss, have resulted in freshwater ecosystem and freshwater fish becoming one of the most threatened on Earth (Cooke et al., 2013). On the other hand, both empirical data and evolutionary theory indicate that global genetic variation could be maintained or even increased by a fragmented population, which is occur by allopatric speciation due to genetic isolation of fragmented populations (Carson, 1990; Xu et al., 2010). *Rhynchocypris lagowskii* is a small cyprinid and common freshwater fish that is endemic to Japan also distributed widely in East Japan. We identified three groups (namely Group 1: Tohoku,

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North Kanto, East Chube; Group 2: Ishikawa, Toyama and Group 3: Chube) for same species in East Japan previously (Hassan et al., 2015). Normally the Tohoku populations belong to the group 1. However, there is an opinion for the population in Iwate prefecture (Tohoku region). The population in Iwate prefecture is the non-indigenous fish (Sakai et al., 2007; Suzuki, 2016). In this study we were searched for the distribution of indigenous and non-indigenous *Rhynchocypris lagowskii* in Tohoku region. Concerning this fact, the present investigation focused the origin of the Iwate populations and checks the distribution of indigenous and non-indigenous fish by using mtDNA haplotype.

II. Materials and Methods

Study area and sample collections: Samples were collected different locations from the sea of Japan and Pacific ocean. After catching the fish they were preserved with 99% ethyl alcohol for DNA extraction. Figure 01 shows the location of the sampling sites.

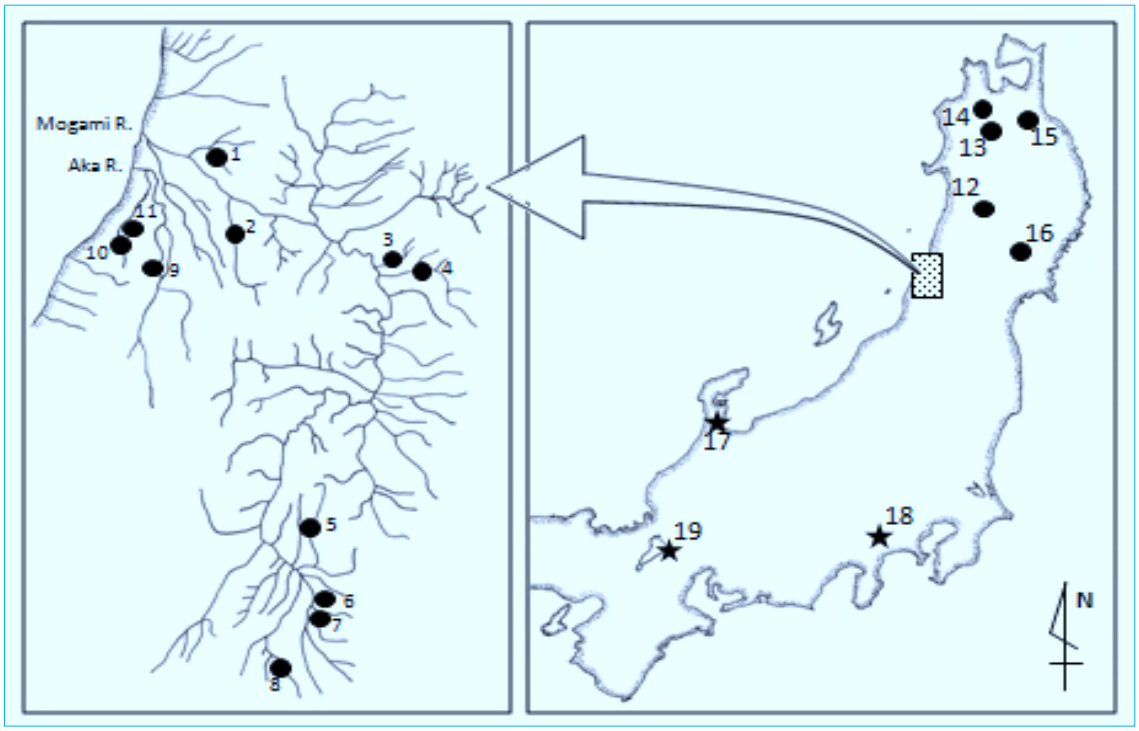

Figure 01. Sampling locations of *Rhynchocypris lagowskii*. Numbers of locations correspond to those in Table 01. [Source: abctech, Japan]

Mitochondrial DNA extraction: One hundred twenty two individuals from all population's samples were used for mtDNA analysis. We were preserved *R. lagowskii* individuals with 99% ethyl alcohol after clipping their fins. Total DNA was isolated from a piece of fin or muscle by standard methods (Asahida et al., 1996).

Polymerase chain reaction amplification: We amplified the mitochondrial DNA cyt-*b* region by polymerase chain reaction (PCR) using a pair of oligonucleotide primers: L14391 (5′-ATGGCAAGCCTACGAAAAAC-3′) and H15551 (5′-GATTACAAGACCGATGCTTT-3′) originated (Sasaki et al., 2007). The PCR amplification were carried out in a thermal cycler 9700 (Applied Biosystems, Foster City CA, USA) under following conditions: initial 1 minute denaturation at 94°C, and followed by 30 cycles, each of 15 s at 94°C, 15 s at 50°C and 30 s at 72°C ending with an additional 5 min at 72 °C for final elongation.

Sequence method: PCR products were purified by filtration with a (EXSO-SAP-IT). These purified products were used as a template DNA for cycle sequencing reactions performed using Big Dye Terminator Cycle Sequencing Kits 3.1 standard protocol in 10-µl volumes consisting of PCR products 0.5 µl. Sequence buffer 1.75µl. Premix 0.5 µl. The forward primer H15551 (5pM) 0.5 µl. and were ran on an ABI 3100 automated DNA Sequencer (Applied Biosystems).

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III. Results and Discussion

Diversity of mtDNA sequences

The nucleotide sequence was established for the 470 base pairs (bp) of the mitochondrial DNA cyt-*b* region were sequenced *R. lagowskii* species. Nucleotide coordinates corresponded to the mammalian mitochondrial cyt-*b* mtDNA gene (Irwin et al., 1991). Variable nucleotide positions found in the 470-bp fragment for the *R. lagowskii* species examined are shown in (Table 03).We constructed phylogenetic tree based on the nucleotide sequence obtained from cyt-*b* region (Figure 02). The sequence of 470 sites of the cyt-*b* region was determined for 122 specimens of *R. lagowskii* with out-group. According to these variable sites, a total of 54 haplotypes were obtained from the species of *R. lagowskii* (Figure 03). Haplotype diversity (h) ranged from (0.2000 ± 0.1541 to 1.0000 ± 0.2722) and nucleotide diversity (π) ranged from (0.000000 ± 0.000000 to 0.017730 ± 0.011063). We found very high nucleotide diversity in NY (0.017730 ± 0.011063), YA (0.015400 ± 0.009413), HI (0.012340 ± 0.008305) and IW (0.016170 ± 0.010159) that notified the high polymorphism (Table 02).

To see their gene genealogy, we constructed a phylogenetic network based on mtDNA cyt-*b* sequences (Figure 03). The most parsimonious network of mtDNA haplotype of *R. lagowskii* 19 locations, estimated using the TCS algorithm (Templeton et al., 1992). Total 54 haplotype are occurs in 19 locations. In this network showed three geographical groups. Haplotype 1-32 is group 1, haplotype 33-36 is group 2, haplotype 37-53 is group 3 and haplotype 54 is out group (Figure 03). The neighbor-joining tree (NJ) of mtDNA haplotypes also showed similar topologies and consistently revealed three deeply diverged groups (Group 1, 2 and 3) of *R. lagowskii*. Group 1 consisted of haplotype from the specimens collected from Mogami river system and Aka river system area. Group 2 haplotypes were Kamishou River all samples and Nyu River one sample. And group 3 haplotypes were Mabuchi River, Kitakami River, Sakawa River and Yodo River system area) (Figure 02).

Within population genetic diversity

To clarify the genetic relationship between the populations examined, phylogenetic trees were constructed using NJ methods. The topology of the NJ tree based on the mtDNA distance among the 19 populations of *R. lagowskii* generally reflected their geographic locations (Figure 02).

Table 01. Sampling locality river name date of collection and sample size of *Rhynchocypris lagowskii* species. Twenty one sequence with asterisks (*) in the previous paper

River no	River system	River name	Abbreviation	Location	Sampling date	N
1	Mogami River	Aizawa River	AI	Japan Sea	2012.08.26	8
2	Mogami River	Tachiyazawa River	TA	Japan Sea	2012.08.26	10
3	Mogami River	Nojiri River	NO	Japan Sea	2012.08.17	7
4	Mogami River	Nyu River	NY	Japan Sea	2012.08.17	6
5	Mogami River	Yoshino River	YO	Japan Sea	2012.09.23	4
6	Mogami River	Suna River	SU	Japan Sea	2012.09.14	3
7	Mogami River	Oguro River	OG	Japan Sea	2012.07.29	7
8	Mogami River	Ootaru River	OS	Japan Sea	2012.08.26	7
9	Aka River	Kakuda River	KA	Japan Sea	2012.09.18	6
10	Aka River	Yazawa River	YA	Japan Sea	2012.09.02	7
11	Aka River	Fujisawa River	FU	Japan Sea	2012.08.12	8
12	Omono River	Maruko River	MA	Japan sea	2013.05.18	10
13	Yonashiro River	Kosaka River	KO	Japan Sea	2013.07.13	1
14	Iwaki River	Hira River	HI	Japan Sea	2013.07.13	5
15	Mabuchi River	Asamizu River	AS	Pacific Ocean	2013.07.13	6
16	Kitakami River	Iwai River	IW	Pacific Ocean	2013.05.19	6
17	Kamishou River	Kamishou River	KM	Japan Sea	2012.09.15	5*
18	Sakawa River	Shakuri River	SH	Pacific Ocean	2013.02.09	8*
19	Yodo River	Amano River	AM	Pacific Ocean	2013.08.16	8*

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Table 02. Halpotype diversity and nucleotide diversity of *Rhynchocypris lagowskii* species

River no	Abbreviation	Halpotype diversiy	Nucleotide diversity
1	AI	0.2500 ± 0.1802	0.000532 ± 0.000754
2	TA	0.2000 ± 0.1541	0.000851 ± 0.000969
3	NO	0.6667 ± 0.1598	0.001621 ± 0.001534
4	NY	0.8000 ± 0.1721	0.017730 ± 0.011063
5	YO	0.5000 ± 0.2652	0.002128 ± 0.002108
6	SU	1.0000 ± 0.2722	0.007092 ± 0.006165
7	OG	0.9524 ± 0.0955	0.005471 ± 0.003810
8	OS	1.000 ± 0.0764	0.006890 ± 0.004619
9	KA	0.7333 ± 0.1552	0.001844 ± 0.001722
10	YA	0.9524 ± 0.0955	0.015400 ± 0.009413
11	FU	0.8571 ± 0.1083	0.003040 ± 0.002350
12	MA	0.7556 ± 0.1295	0.007896 ± 0.004923
13	KO	1.0000 ± 0.0000	0.000000 ± 0.000000
14	HI	1.0000 ± 0.1265	0.012340 ± 0.008305
15	AS	0.3333 ± 0.2152	0.002128 ± 0.001903
16	IW	0.9333 ± 0.2117	0.016170 ± 0.010159

Table 03. Distribution of the 53 haplotypes from 470 base pair position of the mitochondrial DNA cyt-*b* region in *Rhynchocypris lagowskii* species

Hap	AI	TA	NO	NY	YO	SU	OG	OS	KA	YA	FU	MA	KO	HI	AS	IW	KM	SH	AM	
1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
2	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	
3	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
4	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	
5	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
6	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
7	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	
8	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
9	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	
10	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	
11	-	-	2	1	-	-	-	1	-	-	-	-	-	1	-	-	-	-	-	
12	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
13	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	
14	-	-	-	-	-	-	-	-	3	1	-	-	-	-	-	-	-	-	-	
15	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	
16	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**Group 1**
17	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	
18	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
19	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	
20	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
21	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
22	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
23	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
24	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	
25	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
26	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	
27	7	9	4	3	3	-	1	1	-	1	3	5	-	-	-	-	-	-	-	
28	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	
29	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	

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Table 03. Continued.

30	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
31	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	
32	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	
34	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Group 2
35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	
36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	
37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	
38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	-	-	-	-	
39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	
40	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	
41	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	-	-	-	-	
42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	
43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	
44	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	4	Group 3
45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	
46	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	
47	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	
49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	
50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	
51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	
52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	
53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	

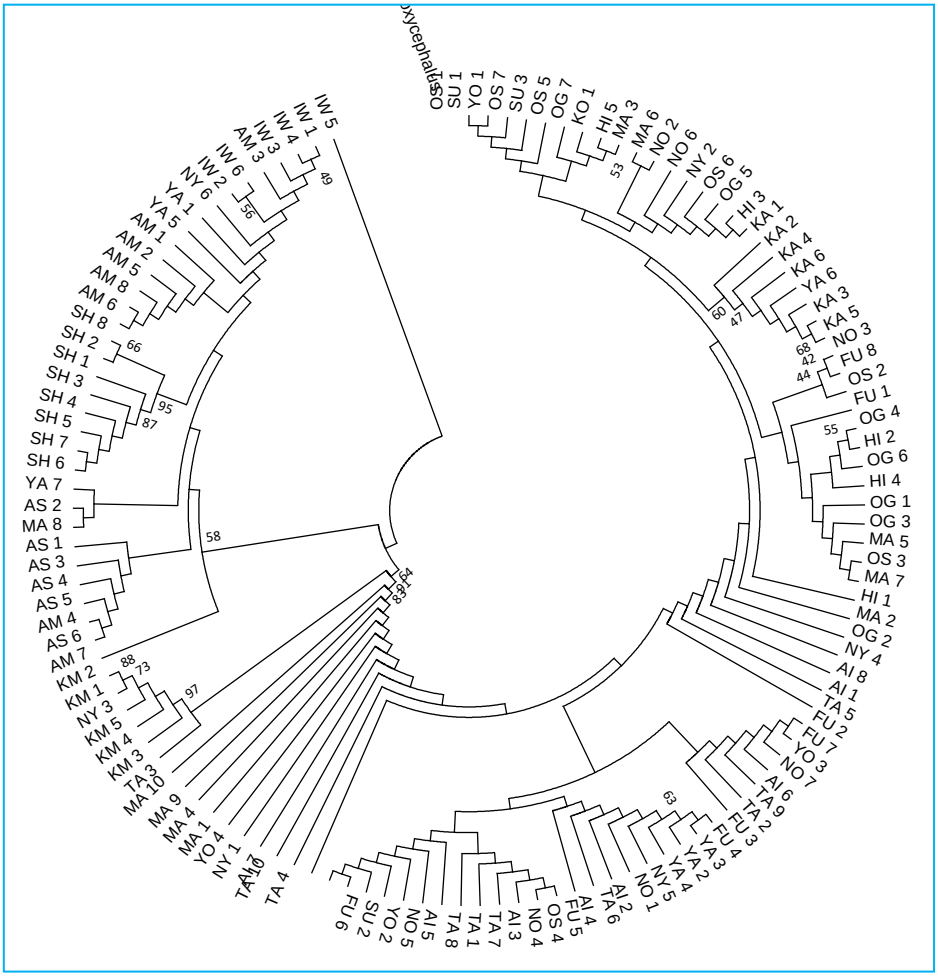

Figure 02. Unrooted neighbor joining tree of *Rhynchocypris lagowskii* species of the cyt *b* region of mtDNA segment without group based on Kimura's two-parameter distances. Numbers above branches are bootstrap values for 1000 replications. TA- Tachiyazawa River, AI- Aizawa River, NO- Nojiri River, NY- Nyu River, OT- Ootaru River, YO- Yoshino River, SU- Suna River, OG- Oguro River, KA- Kakuda River, YA- Yazawa River, FU- Fujisawa River, MA- Maruko River, KO- Kosaka River, HI-Hira River, KM- Kamishou River, AS- Asamizu River, IW- Iwai River, SH- Shakuri River, AM- Amano River.

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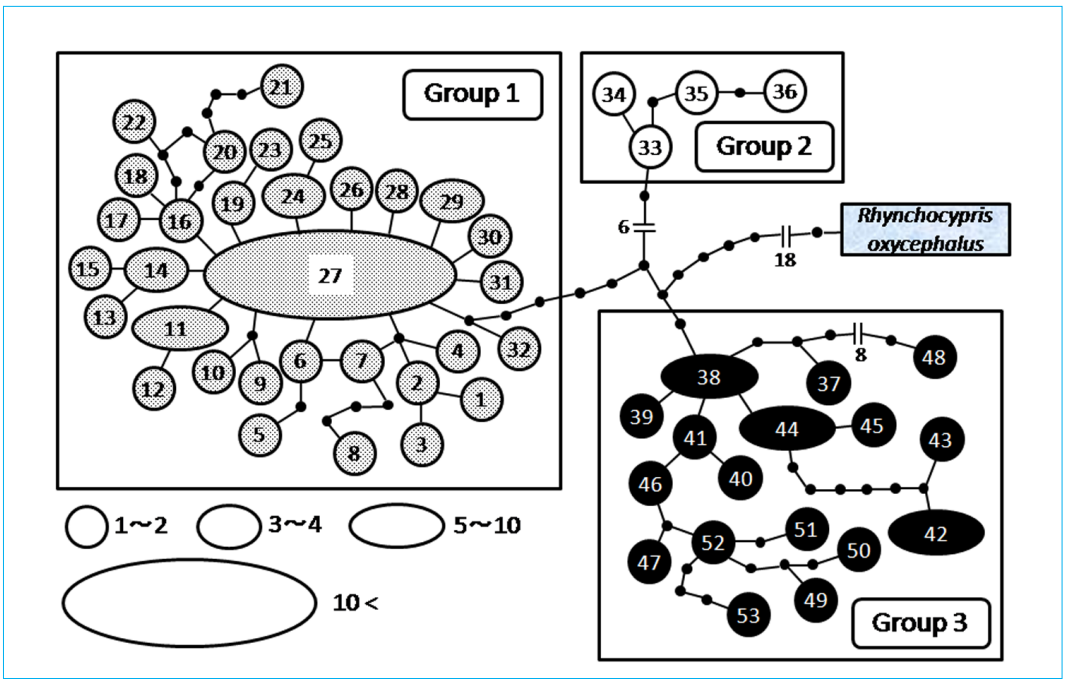

Figure 03. Haplotype network of *Rhynchocypris lagowskii.* The small black-filled circles are missing haplotypes needed to connect observed haplotype. The size of the circle is proportional to the number of individuals.

IV. Discussion

Phylogenetic relationship of *R. lagowskii*

Analysis of the nucleotide sequence of the mitochondrial cyt-*b* reason revealed three group divergence in *R. lagowskii*. Such divergence is characteristic of interspecific differentiation and testifies that some *R. lagowskii* mtDNA types differ from the major mitochondrial gene pool of this species. Genetic diversity is due to specific mtDNA types found in fishes from the NY- Nyu River, YA- Yazawa River and MA- Maruko River. The results of the *R. lagowskii* based on specimens collected from the entire distribution range of the species shows three phylogeographic groups. Specifically, the populations in group 3 showed remarkable differentiation from the group 1 population. The result of this study suggested with the findings of Matsuda et al. (1997) and Takehana et al. (2003). They also found that population of one group was geographically separated from others.

Mogami River System and Aka River System group 1 covers of the most distribution area of R. lagowskii in the Japan Sea area. Mogami and Aka River System (group 1) cover the most distribution area of R. lagowskii in the Japan Sea area. The reasons for this distribution can be explained by the presence of a minimum of one refugium in a part of group 1 due to quaternary climate changes (Lee and Maki, 2013). However the area was not specified in our study. *R. lagowskii* may start to expand from those Refugia to either north-eastern part of Japan at the period of the postglacial climate change. In the Mogami River System all most sampled showed same genetic structures. We used many locations in the Mogami River System the purpose is to investigation the invaded individuals. Only Nyu River shows different genetic variations. As the Mogami River flows close to the Nyu River, the distance between the river mouths the genetic characters of the *R. lagowskii* populations are almost identical between the two rivers. In addition, fish living at different latitudes may exhibit different reproductive properties as they are affected by environmental factors such as photoperiod and water temperature (Shimizu, 2008).

The group 2 collected from KM river all samples and NY river one sample are found in this group (Table 03).The distributions of group 2 (KM river) haplotype (Toyama Prefecture and Ishikawa Prefecture) suggest the phenomenon. KM river is closely located with other river's course resulted river scramble (Hassan et al., 2015).

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The group 3 collected from AM, AS, SH, YA and IW Rivers (Table 03). The distributions of group 3 haplotype suggest the natural movements of gene from the Pacific Ocean via the river system. Similarly it was found that gene flowed from the eastern Seto Island Sea region into the Pacific Ocean through Kii Channel (Kuwashiro, 1959; Ota et al., 2004). However the differentiation of the group 2 Kitakami River systems Iwai River, Iwaki River system Hira River, Yonashiro River system Kosaka River population from the other is much larger than this, indicating that the isolation of the species, has been sustained over a long period during which other lowland fish species maintained gene flowed between those regions. This range distribution might have retained large range harbor haplotypes sharing consistently (Sugahara et al., 2011).

IV. Conclusion

Current study stated that *R. lagowskii* shifted a different range of distribution at last glacial period. Similar opinion was also concluded by Maliouchenko et al. (2007) for European Betula species; Toyama and Yahara (2009) for Viola species and Saeki et al. (2011) for red and silver maple also reported. But our study was also provided an evidence for the variation of genetic structure by the climate changes in different ecological conditions. Further research is suggested to compare various predictive models applied to freshwater fish is warranted to identify the *R. lagowskii* is indigenous or non-indigenous.

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