



New Records of the Alien Chinese Ricefish (*Oryzias sinensis*) and Its Dispersal History across Eurasia

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Abstract: The diversity of biota in different parts of the planet has demonstrated dramatic changes within the last several decades due to the extinction of native taxa and the appearance of invasive taxa. The correct taxonomic identification of non-native species is important for understanding their dispersal abilities, especially when potential invaders may be of closely related species. Information on the species identity of ricefish (*Oryzias* spp.), which have formed self-sustainable populations in some parts of Eurasia, is contradictory. In this paper, we studied samples from non-native populations of *Oryzias* from several Eurasian regions. The results of our mtDNA COI partial sequence study confirm that the individuals we studied belong to the species Chinese ricefish, *O. sinensis*. Analyses of the literature and our own data suggest that all known alien populations of *Oryzias* in continental Eurasia belong to the same species, *O. sinensis*. A recent finding of *O. sinensis* in the Don delta suggests that one of the species' secondary dispersal pathways could run from the Kuban region through the lower part of the Don basin to the Cis-Azov area.

Keywords: alien species; fish; Russia; China; diversity; biological invasions; genetic identification



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1. Introduction

The diversity of biota in different parts of the planet has demonstrated dramatic changes within the last several decades due to the extinction of native taxa and the appearance of invasive taxa [1–3]. The expansion of invasive organisms outside of their native ranges is a major challenge, as they may affect both the structure and the functioning of native ecosystems, as well as the efficiency of human economic activities [4–6]. However, the correct taxonomic identification of alien species is an important prerequisite for assessing the potential damage they may cause, and for tracking the directions and dynamics of their subsequent spread [7–9].

At the same time, determining the taxonomic identity of alien species can be difficult if two (or more) closely related, morphologically similar species are potentially involved in the colonization of new territories. In this case, taxonomic identification may be successfully performed by using a molecular genetic approach, which has been applied many times in recent years in order to identify alien freshwater species in different Eurasian regions (e.g., river crayfishes [10–12], mollusks [13–15], frogs [16], and pathogens to hydrobionts [17,18]).

Presently, in different parts of Eurasia, members of the genus *Oryzias*, the ricefish, have formed non-native populations. These small fishes, which do not exceed 60 mm in length, belong to the family Adrianichthyidae, of order Beloniformes. The native range of the genus *Oryzias* extends across areas of continental Asia, from the Korean peninsula to India, and on a number of islands: Japan, Hainan, Taiwan, and the Indo-Malay-Philippines Archipelago [19]. However, these fishes have declined in most of their native range in Asia

due to habitat destruction, pesticide use, etc. *Oryzias* fishes commonly inhabit shallow areas of freshwater bodies overgrown with abundant macrophytes. Their high resilience, small size, and rapid turnover of generations have allowed them to become a model subject for genetic, physiological, embryological, toxicological, carcinogenesis, and behavioral studies [20].

The same characteristics have also allowed *Oryzias* fishes to become active invaders, and they have significantly expanded their range in recent decades. Representatives of the genus *Oryzias* have formed numerous self-sustainable populations in water bodies in Kazakhstan [21–23], Uzbekistan [24], Turkmenistan [25], the Krasnodar Territory of Russia [26], southeastern Ukraine [27], Japan [28], and two regions of China: the Tibetan Autonomous Region [29] and the Xinjiang Uyghur Autonomous Region [30]. However, the actual species identification of *Oryzias* from some of the above-mentioned populations has been determined without any deep examination of external morphological and genetic diagnostic features.

To date, 24 species of the genus *Oryzias* are known, but the taxonomy of the genus is still not well established and remains poorly understood, even in its natural range [19]. Until recently, only one species of *Oryzias*, i.e., the Japanese ricefish (*O. latipes*), was assumed to inhabit Japan, the Korean peninsula, and the eastern and southern regions of China [31]. However, in recent years, it has become clear that the *O. latipes* is restricted to the Japanese islands and the southern part of the Korean peninsula [32], whereas a different species, the Chinese ricefish, *O. sinensis*, inhabits the western part of the Korean peninsula, as well as eastern and southern China [21,32,33]. More recently, the population of *Oryzias* from the north-eastern part of Honshu Island was described as a separate endemic species, *Oryzias sakaizumii* [34]. The fish *O. sinensis* was originally described, on the basis of morphological features, as the subspecies *O. latipes sinensis* [35]. Indeed, the morphological differences between the two forms are relatively small and considerably overlap [35,36].

However, the species independence of *O. sinensis* has now been well proven using genetic methods. For example, the survival of second-generation *O. latipes* × *O. sinensis* hybrids has been shown to be reduced [37] and, in addition, different alleles of some protein-coding genes have been detected in these forms [38,39]. More recently, it has been shown that each form is also characterized by its own well-defined cluster of mtDNA haplotypes, both for the complete mitochondrial genome and for the COI gene, which is commonly used to separate closely related species [40,41]. The genetic distance between *O. latipes* and *O. sinensis* for the mitochondrial COI gene has been estimated at 14.0% [41], whereas a 2.0% difference is generally accepted as an interspecific difference [42]. This implies that both species can be diagnosed unambiguously, without any overlap, by this characteristic.

A number of papers indicate that populations of *Oryzias* outside their native range are represented exclusively by the species *O. latipes* [21,25,28,29], but, at least in some cases [21], this may be due to the use of an outdated view on the taxonomy of this genus which does not consider *O. sinensis* a separate species. So far, only in Japan have genetic analyses been used to prove that non-native populations of *Oryzias* belong to the species *O. latipes* [43]. Other papers credit non-native populations to the species *O. sinensis* [24,25,27,30,44], but only in one case have the authors confirmed their species identification by morphological analysis [27]; genetic analysis was not performed in any of the cited works to confirm the identity of *O. sinensis* amongst the studied individuals.

Recent dispersal pathways of representatives of the genus *Oryzias* across Eurasia are not fully understood. In particular, it is unknown how they have come to occur in the south of Ukraine. Although populations of *Oryzias* have been described many years ago in the Krasnodar Territory, no *Oryzias* individuals have been found in the Rostov Region of the Russian Federation, between southern Ukraine and the Krasnodar Territory [45,46]. The occurrence of *Oryzias*, defined by the authors as *O. latipes*, in Tibet also seems rather unexpected [29].

Importantly, various species of the genus *Oryzias* have different behavior [47], and exact species identification is important for their use as model subjects in laboratory experiments, as well as for the correct assessment of their invasive potential in new geographic regions. The aim of this work was to clarify the species identity of representatives of the genus *Oryzias* from populations that have recently emerged in different regions of continental Eurasia on the basis of their genetic features, as well as to analyze their possible means of dispersal within Eurasia.

2. Materials and Methods

The study was conducted in the water bodies of three distant regions of Eurasia: a pond in the Kagalnik village, in the Azov district of the Rostov Region, Russia; an irrigation canal in the vicinity of the Anastasievskaya village, in the Slavyansk district of the Krasnodar Territory, Russia; and a channel inflowing to the Yarlung Tsangpo (Brahmaputra) river in the Tibetan Autonomous Region, China (Figure 1). Fish were caught using deep nets with 1 mm or 5 mm mesh, anaesthetized, and preserved in 96% ethanol. Detailed information on the localities of the fish samples is presented in Table 1.

To determine the species identity using genetic methods, an analysis of a partial nucleotide sequence of the mitochondrial COI gene was performed, following the procedure described in Artamonova et al. [48]. Total cellular DNA was isolated with the DNA-EXTRAN-2 reagent kit (Synthol, Russia) in accordance with the producer's instructions.

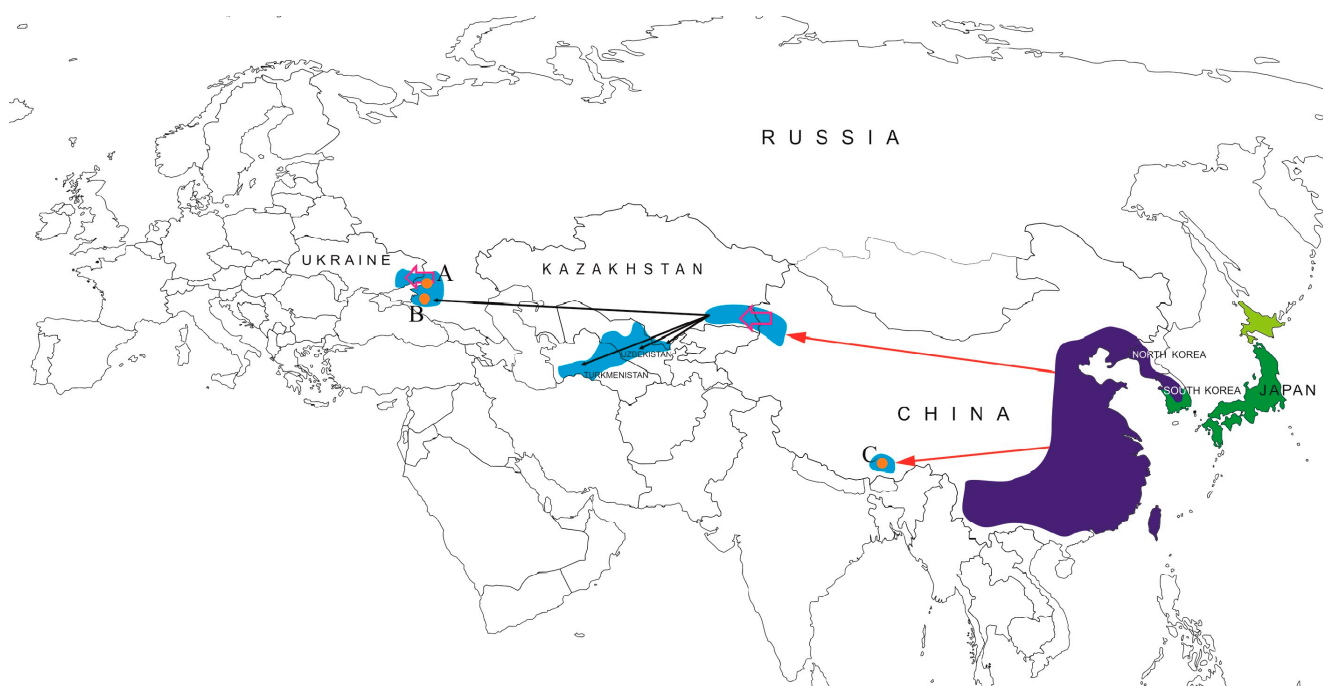


Figure 1. Map of Eurasia indicating the localities of ricefish (*Oryzias*) samples in the Rostov Region (A) and the Krasnodar Territory of the Russian Federation (B), as well as the Tibet Autonomous Region of China (C). Dark blue and light blue colors show the native and invaded ranges of the Chinese ricefish, *Oryzias sinensis*, respectively. Dark green and light green colors show the native and invaded ranges of the Japanese ricefish, *O. latipes*, respectively. Red arrows show probable translocations outside of the native range; black arrows show documented intentional secondary transportations. Wide open pink arrows show local short-distance translocations and self-distribution within river basins.

We obtained a PCR product containing a partial sequence of the mitochondrial COI gene using the Tertsik thermal cycler (DNA Technology, Russia). FishF2 and FishR2 primers were used for the amplification of the corresponding mtDNA region and the sequencing of the resulting PCR product [49]. Amplification was carried out in 25 µL of buffer containing

75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 0.1% Tween 20, and 2 mM MgCl₂ (Fermentas, Lithuania). The amplification mixture contained approximately 300 ng of total cell DNA, 10 pmol of forward and reverse primers, 200 nmol each of four deoxyribonucleotides, and 0.5–0.7 units of polymerase (Bionem, Russia).

Table 1. Sampling localities of fish (*Oryzias* sp.) with regards to information on regions: their coordinates (in geographical degrees); the number of measured fish individuals, N (the number of genotyped individuals is in parentheses); and their standard length SL, mm (means are in parentheses).

Locality	Coordinates	Date	N	SL, mm
Rostov Region, Russia (Don River basin)	N 47°5′43.2″; E 39°19′1.5″	1.05.2019	10 (2)	17.0–23.3 (19.8)
Krasnodar Territory, Russia (Kuban River basin)	N 45°14′7.4″; E 37°59′52″	11.05.2019	8 (2)	18.5–25.5 (22.13)
Tibetan Autonomous Region, China (Yarlung Tsangpo (Brahmaputra) basin)	N 29°21′24″; E 90°42′55″	27.09.2012	1 (2)	26

Each sample was sequenced twice, from the forward and the reverse primer. The sequencing reaction was carried out by taking 20 ng of the PCR product and 3.2 pmol of the corresponding primer using ABI PRISM[®] BigDye[™] Terminator v. 3.1 (Applied Biosystems[™], Waltham, MA, USA) reagents, with subsequent analysis of the reaction products on the ABI PRISM 3730 Applied Biosystems sequencer of the Collective Use Center “Genome” from the Institute of Molecular Biology, the Russian Academy of Sciences.

We analyzed the sequencing results using a specialized BioEdit v. 7.0.5. editor (bioedit.software.informer.com>7.0/ accessed on 12 April 2022) and constructed the haplotype networks using Network 5.0.0.1 software (<http://www.fluxus-engineering.com> accessed on 12 April 2022). Sequences of *O. latipes* and *O. sinensis* available from the GenBank International Database were used for comparison (Table 2). Previously, the mitochondrial COI gene was successfully used by Yoon et al. for the precise identification of these two species [40].

Moreover, morphological analysis was carried out on the sampled fish. For this purpose, the number of pectoral fin rays was used, which is accepted as the most contrast morphological feature differentiating *O. sinensis* and *O. latipes*. According to the first description of *O. sinensis* [35], individuals of this species typically have 9 rays in the pectoral fin, compared to 10 rays in *O. latipes*, and 8 rays in *O. minutillus*, a species also found in southern China.

Table 2. Summary of the COI gene sequences for the *Oryzias latipes* and *O. sinensis* fishes available in the NCBI GenBank.

Designation on Figure 2	GenBank ID	Species	Locality	Source
OS3F62	HQ536423.1	<i>O. sinensis</i>	Korea	[50]
OS1F128	HQ536422.1	<i>O. sinensis</i>	Korea	[50]
OSTaeon	GU013788.1	<i>O. sinensis</i>	Korea: the Dongjin River	[40]
OLSangh	AP008948.1	<i>O. latipes</i> (<i>O. sinensis</i> following Yoon et al., 2011)	China: Shanghai	[51]
OSLC09	LC153109.1	<i>O. sinensis</i>	Japan: Aichi, Higashiyama Zoo	Suzuki-Matsubara M., Murase Y., Moriyama A., unpublished

Table 2. Cont.

Designation on Figure 2	GenBank ID	Species	Locality	Source
OLFJ80	FJ197680.1	<i>O. latipes</i>	not specified	Park J.-Y., An H.-S., Cho Y.-A., Kim K.-K., unpublished
OLSOK	AP008947.1	<i>O. latipes</i> (<i>O. sinensis</i> following Yoon et al., 2011)	inbred strain, derived from eastern Korea	[51]
OLKaga	AP008940.1	<i>O. latipes</i>	Japan: Kaga	[51]
OLKO94	LC335803.1	<i>O. latipes</i>	Japan: Kanagava	[52]
OLAmino	AP008944.1	<i>O. latipes</i>	Japan: Amino	[51]
OLHN1	AB498066.1	<i>O. latipes</i>	Japan: HNI	[53]
RO1	MW695407	<i>O. sinensis</i>	Russia: Rostov province	This paper
RO1	MW695408	<i>O. sinensis</i>	Russia: Krasnodar Territory	This paper
MBS2	MW695409	<i>O. sinensis</i>	China, TibetanAutonomous Region	This paper

3. Results

According to our results, all sampled individuals of *Oryzias* from the Krasnodar Territory and the Rostov Region of Russia bear the same haplotype (Table 2), while *Oryzias* individuals collected in the Tibet Autonomous Region have another haplotype, differing by three nucleotide substitutions (Table 2; Figure 2). The median haplotype network (Figure 2) shows at least three well-defined groups of haplotypes. The haplotypes identified in the fish sampled in our study are part of a cluster formed by *O. sinensis* sequences. It should be specifically mentioned here that, although one of the haplotypes in this group, OLSangh, was listed in the GenBank (Genbank ID AP008948) as belonging to *O. latipes*, another study indicated that it belongs to *O. sinensis* [40]. Possibly, the haplotypes OLKaga and OLHN1 belong to the recently described species *O. sakaizumii*.

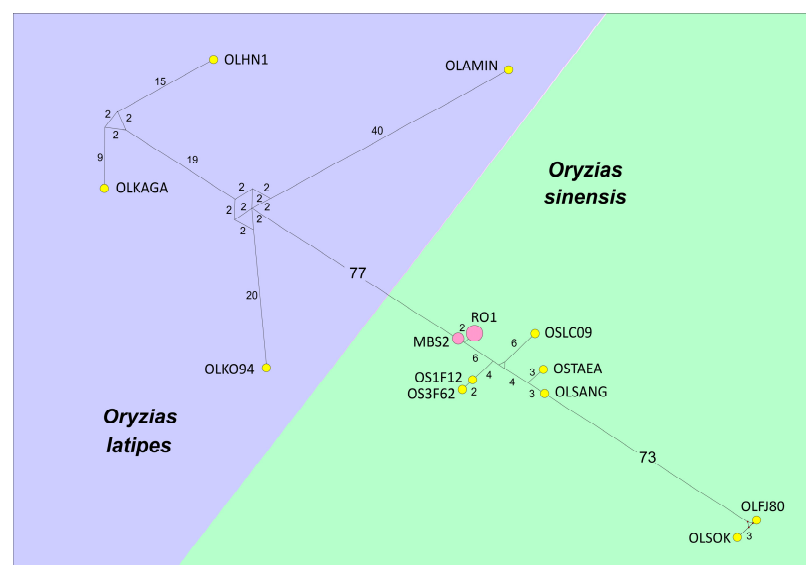


Figure 2. Median haplotype network of the COI gene sequence for fishes belonging to the genus *Oryzias*. See Table 2 for sample designations and descriptions. The numbers on the lines reflect the numbers of nucleotide substitutions.

The morphological analysis revealed that the fish from the delta of the Don River (Rostov Region) have 8–10 (on average 8.5) rays in the pectoral fin. The fish from the Kuban River basin (Krasnodar Territory) have 8–9 (on average 8.75) rays, and those from the Yarlung Tsangpo basin (Tibetan Autonomous Region) have 9 rays. According to our results, the number of pectoral fin rays does not allow for the unambiguous taxonomic verification of *O. sinensis*. Within the examined fish samples, the highest variability of ray numbers was observed in the populations sampled from Russia, which reflects a previous report [35].

4. Discussion

According to genetic data, the fish that formed self-sustainable populations in two regions of the Russian Federation (the Rostov Region and the Krasnodar Territory), as well as in the Tibetan Autonomous Region of China, belong to the species *Oryzias sinensis*. According to earlier morphological studies, *Oryzias* fishes from Kazakhstan [21] and Ukraine [27] belong to the same species, but confirmation with molecular genetic methods is desirable.

The acquired data allow us to reconstruct the dispersal routes of *O. sinensis* across Eurasia with a high degree of certainty. It can be proposed that *O. sinensis* was transported to the Tibetan and Xinjiang Uyghur Autonomous Regions from eastern China. The exact timing of these introductions is unknown, but the genus *Oryzias* was not reported in Tibetan water bodies until the very end of the 20th century [54,55]. Its appearance in the mentioned region may have the same human-mediated vector as the appearance of another alien fish species, stone moroko (*Pseudorasbora parva*), in the same water body. The introduction of *P. parva* to the region is considered to be a result of the Buddhist religious practice of releasing live fish into water bodies [56]. However, an aquaculture-mediated cause cannot be ruled out. The appearance of *O. sinensis* in the Xinjiang Uyghur Autonomous Region is most likely explained by its unintentional introduction due to aquaculture vector, i.e., via the fishery supplementation of open waters by unverified stocking material.

From the Xinjiang Uyghur Autonomous Region, *O. sinensis* migrated to Kazakhstan via the Ili River, where numerous specimens of this species were recorded in October 1970 [57]. Subsequently, a total of 6200 *Oryzias* individuals were transported from this river to the Krasnodar Territory of Russia, and were released into six water bodies on 4 July 1974 for mosquito larval control [58]. It is proposed that the new population of *Oryzias* in Krasnodar expanded to water bodies in the Rostov Region (due to self-distribution along the hydrological network, and secondary unintentional translocations together with aquaculture stocking material), and further penetrated into southeastern Ukraine, where it was recorded in the Cis-Azov region on 15 June 2003 [27]. *Oryzias* fishes from Kazakhstan were also imported to Uzbekistan and Turkmenistan for mosquito control [24,59].

Thus, analysis of the *Oryzias* dispersal routes in continental Eurasia shows that *O. sinensis*, specifically, is expanding its range. An additional argument may be the fact that all reliably known routes of *Oryzias* dispersal in continental Eurasia began in China where *O. sinensis* is present, but *O. latipes* does not occur [32]. Non-native populations of *O. latipes* are only known to exist on the islands of the Japanese archipelago [28,43]. It is additionally noteworthy that the haplotype of the stone moroko found in Tibet is also present in native populations of this species inhabiting southeastern China [60]; so, it seems very likely that both the stone moroko and *O. sinensis* arrived to Tibet from southeastern China by the same vector and route.

In conclusion, it should be noted that we cannot exclude that the taxonomy of *Oryzias* is much more complex than presently assumed, and that OLSOK haplotype carriers, together with individuals with the OLFJ80 haplotype (GenBank ID FJ197680), belong to another, as-yet-undescribed species, since this group is separated from the compact cluster formed by *O. sinensis* by at least 77 nucleotide substitutions, which is about 12% of the sequence length studied (Figure 2). This markedly exceeds the typical level of interspecific variation in fishes (approximately 2% for the COI sequence, as suggested by Hebert et al. [42]).

The correct verification of species membership is not only important for identifying their dispersal routes, but also for determining the possible consequences of introducing an alien species into new ecosystems. For example, the invasiveness risk, an indicator that assesses the ability of a species to naturalize in a new region and change the native ecosystems, may be accurately calculated for certain species. The invasiveness risk of *O. sinensis* for the Brahmaputra River basin in the Tibetan Autonomous Region is assessed as moderately high [61].

A study of the expansion of *O. sinensis* suggests that it appears to be sufficiently tolerant to the cold, as it is able to naturalize in the water bodies of the Tibetan Plateau and the Rostov Region of Russia. Therefore, we cannot exclude the further expansion of new subranges of this species under global warming conditions in the Tibetan Plateau, southern Russia, and Ukraine, as well as penetration into India, downstream of the Brahmaputra River.

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