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Genetic diversity of the regionally endangered Chinese ricefish (*Oryzias sinensis*) in Taiwan, with comments on its conservation status

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Abstract

The Chinese ricefish (*Oryzias sinensis*) is a freshwater fish that is regionally endangered in Taiwan. To evaluate its conservation status and further formulate conservation plans for this species in Taiwan, this study analyzed the fish collected from all known localities, including a newly discovered population. The phylogenetic trees based on D-loop and COI divided the Chinese ricefish into two major clades: Taiyangpi-Shuanglienpi and Taiping-Gongliao. This study showed that both the Shuanglienpi and Taiyangpi wild populations had significantly higher Hd and π values, while the Shuanglienpi restored population only had one haplotype from the wild. However, their genetic diversity may have decreased over the past few decades. The Gongliao and Taiping populations shared the same haplotype as a population from a previous study. We propose that the Gongliao and Taiping populations were founded by a few individuals and might have originated from a single introduction event from southeastern mainland China. Low genetic diversity was clearly observed in the Taiping, Gongliao and Shuanglienpi restored populations. Based on the present investigation and previous studies, the invasive poeciliid fish (such as mosquitofish and green swordtail) contribute the most to the decline of the Chinese ricefish population and genetic diversity in Taiwan.

Key words: Medaka, mitochondrial DNA, genetic uniformity, conservation

Introduction

The Chinese ricefish, *Oryzias sinensis*, is a small freshwater medaka fish that is widely distributed in East Asia and the Indo-China Peninsula (Parenti 2008). In Taiwan, this species was considered to be extinct for over 40 years until it was rediscovered in Shuanglienpi in 1992 (Tzeng *et al.* 2006). The fish are restricted to northeastern Taiwan and have become a regionally endangered species (Tzeng *et al.* 2006).

Only a few molecular phylogeny studies have been conducted on Chinese ricefish in Taiwan. Tzeng *et al.* (2006) analyzed the phylogenetic relationships among Chinese ricefish in Taiwan based on the mitochondrial D-loop and concluded that the fish could be divided into three populations: Gongliao, Taiyangpi (Taiyang Pond) and Shuanglienpi (Shuanglien Pond). However, among these three localities, two had very few samples for studying (three individuals for Gongliao and five for Taiyangpi) and only a single haplotype was detected in the two populations.

Since Tzeng *et al.* (2006), two surveys of Chinese ricefish have been reported in Taiwan (Chen 2009; Chen *et al.* 2011). More populations of Chinese ricefish were found in Shuanglienpi, Gongliao and Ruyi Lake (Chen 2009). Chen (2009) mentioned that the Chinese ricefish is a dominant species in the Ruyi Lake, along with two other species, the paradise fish (*Macropodus opercularis*) and the crucian carp (*Carassius auratus*), which were also found there in 2006–2007. Chen *et al.* (2011) further confirmed a similar composition of fish species in this lake. The two studies revealed that the ricefish populations comprise 16.7% of all captured fish in the studied habitats.

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The conservation statuses of Taiwanese native freshwater fishes, including the studied ricefish, have been modified in recent years. The ricefish was listed as near threatened (NT) in the 2012 Red List of Freshwater Fishes of Taiwan (Chen *et al.* 2012) and later updated to vulnerable status (VU) in 2017 (Yang *et al.* 2017). Today, this ricefish species is still considered to be endangered (Shao 2022). However, ecological and biological data on the species remain insufficient to accurately measure its conservation status. Thus, more information is needed on the genetic diversity and evolutionary relationships among newly discovered Chinese ricefish populations to assess the species' conservation status and recommend effective conservation policies. To achieve this goal, this study analyzed samples from all known Chinese ricefish populations in Taiwan.

Materials and Methods

Sampling materials. All localities where Chinese ricefish had been recorded in recent years in Taiwan were surveyed in 2009–2019 for this study. These localities included Gongliao and Ruyi Lake in New Taipei City, as well as Taiyangpi and Shuanglienpi in Yilan County. Additionally, a Chinese ricefish population recently discovered in Taiping, New Taipei City, was also investigated during an expedition (Figure 1).

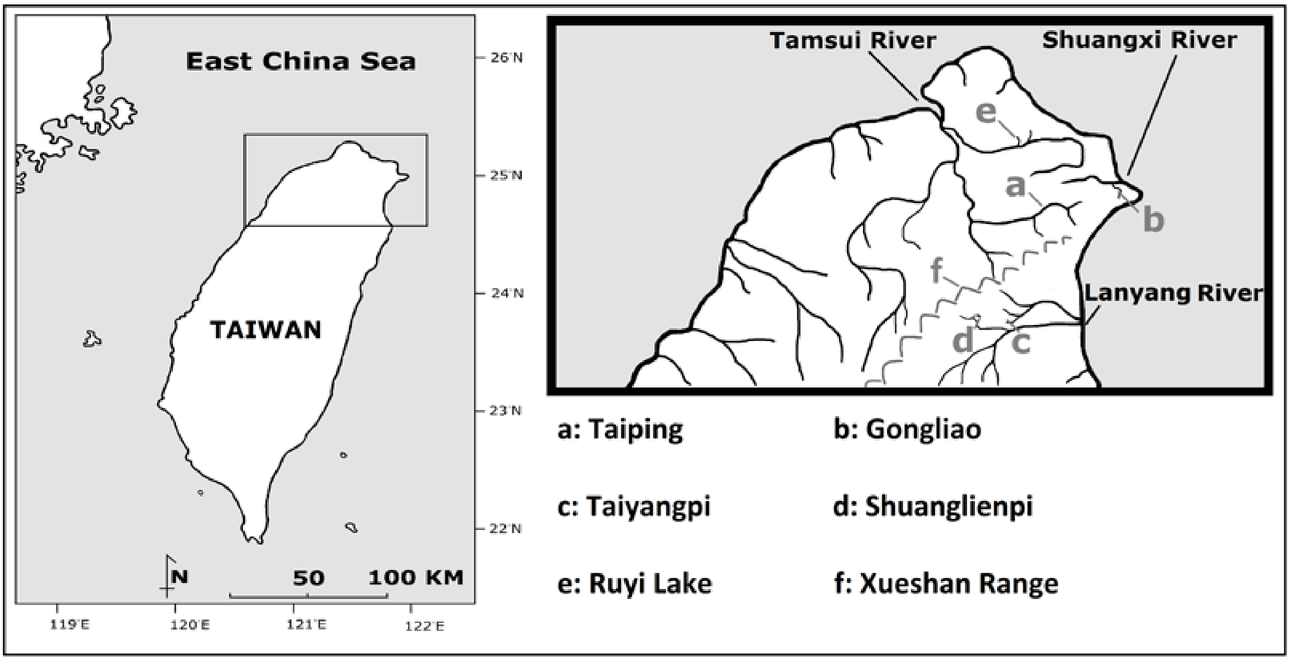


FIGURE 1. Sampling sites in northern Taiwan. Detailed localities and rivers are shown in the upper right image.

During our surveys, the Chinese ricefish were found in four of the five localities: Gongliao, Taiping, Taiyangpi and Shuanglienpi. In the Shuanglienpi area, eight individuals were collected from a creek near Shuanglienpi and 10 individuals were obtained from a manmade pond in the restoration and education center of Shuanglienpi. The Chinese ricefish collected from these two sites were treated as different populations in this study: the Shuanglienpi wild population and the Shuanglienpi restored population.

Five populations of Chinese ricefish were found from these four localities: the Gongliao population (12 individuals), Taiping population (17), Taiyangpi population (8), and the Shuanglienpi wild population and the Shuanglienpi restored population mentioned above. Three of them —Gongliao, Taiyangpi and Shuanglienpi—had their molecular materials studied and reported by Tzeng *et al.* (2006).

All our Chinese ricefish samples were collected using hand nets in different river basins. The Gongliao population was collected from the Shuangxi River, the Taiping population from the Tamsui River, and both the Taiyangpi and Shuanglienpi populations from different tributaries of the Lanyang River. The sampling localities are shown in Figure 1. The tissue samples used for molecular analysis were preserved in 99.5% ethanol.

Molecular studies. DNA of the samples was extracted using a kit (QuickGene DNA tissue kit S, KURABO). The mitochondrial D-loop was amplified by PCR using the nine primers from Tzeng *et al.* (2006) and an additional newly designed primer (forward primer oryzias 15570F: 5' –GCTCAGAGAAAGGGGACTCTAACCC–3' and reverse primer oryzias 42R: 5' –CTTAACATCTTCAGTGTTATGCTTTAGT–3'). A pair of primers—FishF2 and FishR2—were used for COI following Ward *et al.* (2005). All these primers were also used for DNA sequencing. PCR was done in a MODEL 2700 or 9700 thermal cycler (Perkin-Elmer) and 35 cycles were carried out. The annealing step was at 52°C for 60 seconds for the D-loop and 52–55°C for 60 seconds for COI. Double-stranded PCR products were purified using a high pure product purification kit (Roche, USA) before undergoing direct cycle sequencing with dye-labeled terminators (ABI Big-Dye kit). Labeled fragments were analyzed using an ABI PRISM Model 377-64 DNA Automated sequencer (ABI, USA).

BIOEDIT 5.9 (Hall 2001) was used to align unequal length sequences and manual modifications were performed before phylogenetic analysis. The haplotypes diversity (Hd) and nucleotide diversity (π) based on D-loop and COI were calculated using DnaSP 4.00.2 (Rozas *et al.* 2003). The Fst value and Analysis of molecular variance (AMOVA) were conducted for the COI gene using Arlequin 3.5 (Excoffier & Lischer 2010). Nucleotide sequence alignment was visually verified using BIOEDIT version 5.9 (Hall 2001). Sequence analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (Kumar *et al.* 2016).

The maximum likelihood (ML) method was employed for phylogenetic analyses in this study. ML analyses were carried out using MEGA 7.0 (Kumar *et al.* 2016). Branch support for the ML trees was established via bootstrap analyses (with 2000 replications). The best-fit model of the ML analyses was selected using MEGA 7.0. For all the phylogenetic analyses, four congeneric ricefish species obtained from GenBank—*Oryzias latipes*, *Oryzias curvinotus*, *Oryzias celebensis* and *Oryzias sarasinorum* (Table 1)—were used as outgroups (Setiamarga *et al.* 2008; Hirayama *et al.* 2010; Wang *et al.* 2017; Ma *et al.* 2019). The codes for each population are listed in Table 2.

TABLE 1. Sampling localities, codes and accession numbers of the ricefish used in this study.

Sample IDs	Sample size	Locality/river	D-loop		COI	
			Accession	Source	Accession	Source
OSL	8	Shuanglienpi wild population / Lanyang River	ON638903-ON638905	This study	ON619547-ON619548	This study
OSR	10	Shuanglienpi restored population / Lanyang River	ON638906	This study	ON619549	This study
OTY	8	Taiyangpi / Lanyang River	ON638907-ON638909	This study	ON619550-ON619551	This study
OGL	12	Gongliao / Shuangxi River	ON638910	This study	ON619552	This study
OTP	17	Taiping / Tamsui River	ON638911	This study	ON619553	This study
—	5	Shuanglienpi / Lanyang River	AF383179	Tzeng *et al.* 2006	—	—
—	2	Shuanglienpi / Lanyang River	AF383180	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383181	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383182	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383183	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383184	Tzeng *et al.* 2006	—	—

......*Continued on the next page*

TABLE 1. (Continued)

Sample IDs	Sample size	Locality/river	D-loop		COI	
			Accession	Source	Accession	Source
—	1	Shuanglienpi / Lanyang River	AF383186	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383187	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383188	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383189	Tzeng *et al.* 2006	—	—
—	1	Shuanglienpi / Lanyang River	AF383190	Tzeng *et al.* 2006	—	—
—	5	Taiyangpi / Lanyang River	AF383191	Tzeng *et al.* 2006	—	—
—	3	Gungliao / Shuangxi River	AF383195	Tzeng *et al.* 2006	—	—
—	1	South Korea	—	—	HQ536422	Kim *et al.* 2016, unpublished data
—	6	South Korea	—	—	HQ536422-HQ536428	Kim *et al.* 2016, unpublished data
Oryzias latipes (outgroup)	1	Matsuyama, Japan	AP008945	Hirayama *et al.* 2010	AP008945	Hirayama *et al.* 2010
Oryzias curvinotus (outgroup)	1	Hainan, China	KY364884	Wang *et al.* 2017	KY364884	Wang *et al.* 2017
Oryzias celebensis (outgroup)	1	—	MN064715	Ma *et al.* 2019	MN064715	Ma *et al.* 2019
Oryzias sarasinorum (outgroup)	1	—	AB370891	Setiamarga *et al.* 2008	AB370891	Setiamarga *et al.* 2008

Results

Field investigation data. Five populations of Chinese ricefish were found in three river systems (Table 1). However, no Chinese ricefish were found in the Ruyi Lake during our surveys in 2019, even though they had been recorded there in the past (Table 3).

Sample IDs. The codes and GenBank accession numbers of each sample are listed in Table 1. A total of 55 individuals from five populations of Chinses ricefish were collected, and both D-loop and COI were sequenced in this study. Furthermore, 14 D-loop haplotypes from 25 Taiwanese individuals (Tzeng *et al.* 2006) and two COI haplotypes from seven Korean individuals (Kim *et al.* 2016, published sequences in GenBank) were obtained from GenBank for comparison (Table 1). In total, 19 D-loop haplotypes from 80 individuals, seven COI haplotypes from 62 individuals and 12 (COI+D-loop) haplotypes from 55 individuals were included.

The length of the D-loop, COI and concatenated COI and D-loop (COI+D-loop) was 915, 552 and 1467 bp, respectively. The phylogenetic trees were constructed by the maximum likelihood method with the T92+G (Tamura

3-parameter+G), TN93+I (Tamura-Nei+I) and HKY+ G (Hasegawa-Kishino-Yano+G) models for COI, D-loop and COI+D-loop, respectively.

The phylogenetic tree reconstructed with Taiwanese D-loop sequences (Fig. 2) was divided into two major clades with high bootstrap support (95–99). The first clade comprised three haplotypes from 13 individuals from the Taiyangpi population (OTY) (five from Tzeng *et al.* 2006), 25 individuals from the Shuanglienpi wild population (OSL) (17 from Tzeng *et al.* 2006) and 10 individuals from the Shuanglienpi restored population (OSR). The second clade comprised only one haplotype from 32 individuals, 17 of which were from the Taiping population and 15 were from the Gongliao population (three from Tzeng *et al.* 2006). In addition, a subdivision with a medium bootstrap value (63) was found between the wild and restored Shuanglienpi population and the Taiyangpi population.

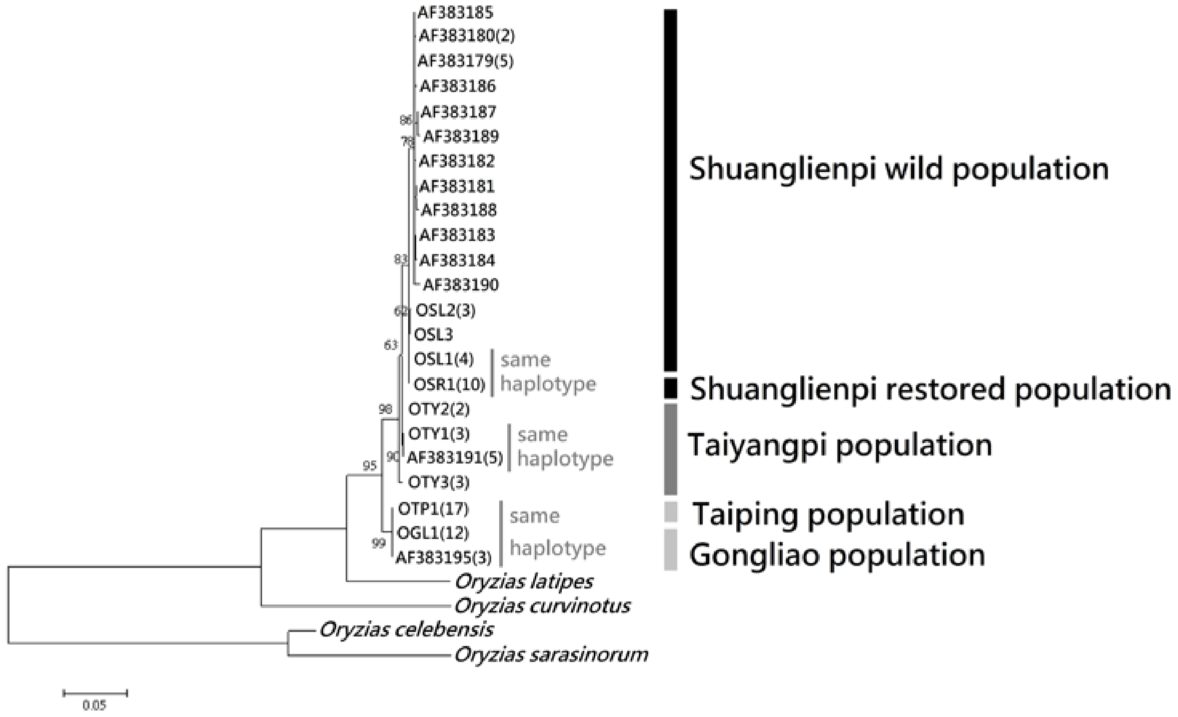


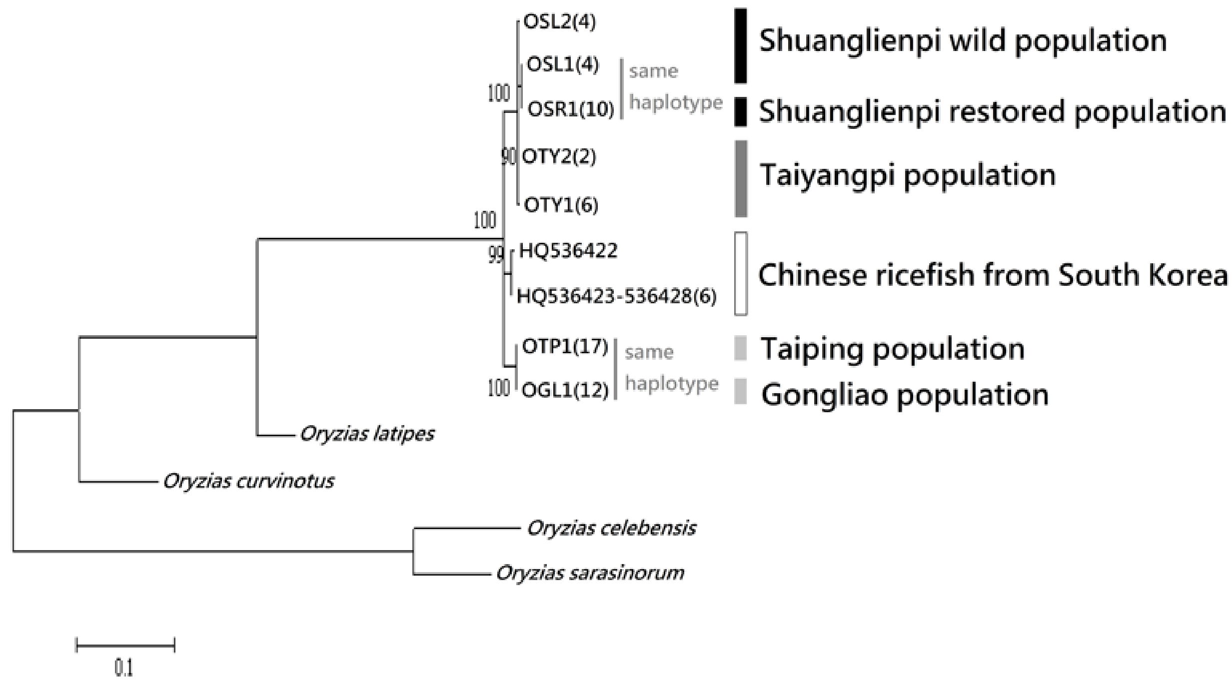
FIGURE 2. Molecular phylogenetic tree of Chinese ricefish in Taiwan based on the D-loop sequence constructed with the maximum likelihood method (bootstrap values less than 50 not shown). The sample size of each haplotype is given in parentheses after the OTU. Haplotypes from Tzeng *et al.* (2006) are shown with GenBank accession numbers.

The phylogenetic tree reconstructed with Taiwanese and Korean COI sequences (Fig. 3) was divided into three major clades with high bootstrap support (100). The first clade comprised most of the Taiwanese individuals from Shuanglienpi wild population + Shuanglienpi restored population + Taiyangpi population (OSL+OSR+ OTY). The second clade comprised two haplotypes from seven Korean individuals. The third clade comprised one haplotype, which is from 29 samples from the Taiping population + Gongliao population (OTP+OGL).

In addition, the concatenated phylogenetic tree (D-loop and COI) showed a very similar topology to the D-loop tree (Fig. 4). The nodes between OSR+OSL+OTY and OTP+OGL had high bootstrap support (94).

The Hd values of Taiping (OTP), Gongliao (OGL), Taiyangpi (OTY), the Shuanglienpi wild population (OSL) and the Shuanglienpi restored population (OSR) were 0.000, 0.000, 0.75, 0.679 and 0.000 for the D-loop; 0.000, 0.000, 0.429, 0.571 and 0.000 for COI, respectively. The π values of OTP, OGL, OTY, OSL and OSR were 0.00000, 0.00000, 0.00398, 0.00121 and 0.00000 for the D-loop, and 0.00000, 0.00000, 0.00078, 0.00311 and 0.00000 for COI, respectively (Table 2).

For comparison, the Hd values of the D-loop for the Gongliao, Taiyangpi and Shuanglienpi populations obtained from Tzeng *et al.* (2006) were also calculated as 0.000, 0.000 and 0.882, while their π values were 0.000, 0.000 and 0.00261, respectively.



FIGRUE 3. Molecular phylogenetic tree of Chinese ricefish in Taiwan based on COI sequences constructed with the maximum likelihood method. Bootstrap values less than 50 are not shown. Two haplotypes of Chinese ricefish from South Korea from Kim *et al.* (unpublished data, 2016) are shown with GenBank accession numbers.

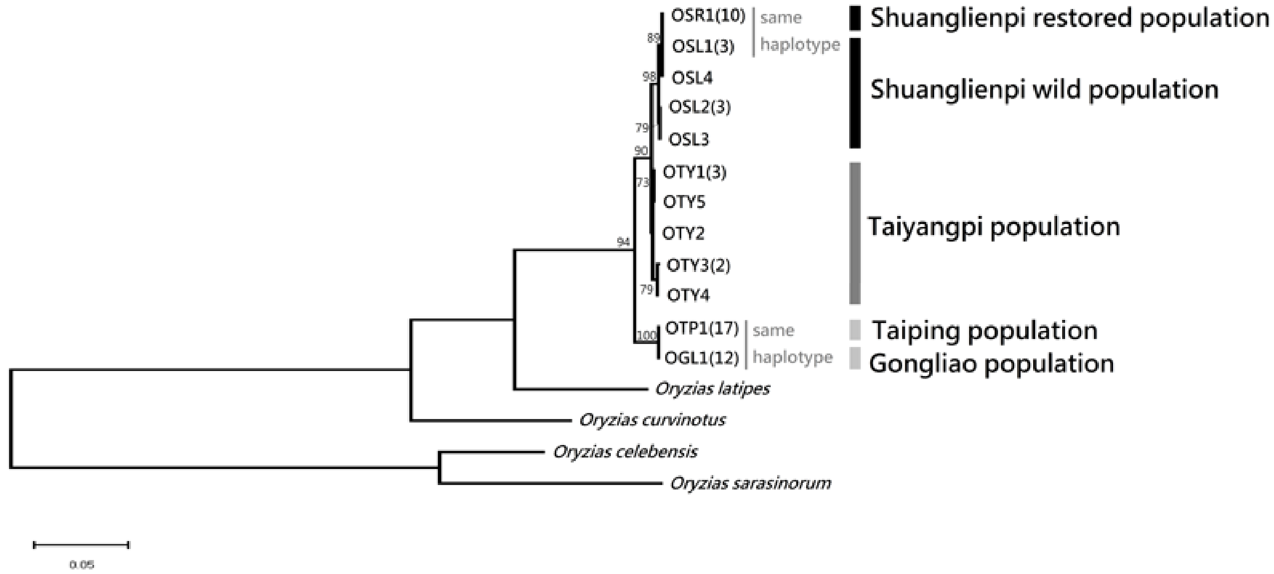


FIGURE 4. Molecular phylogenetic tree of Chinese ricefish in Taiwan based on combined COI and D-loop sequences constructed with the maximum likelihood method. Bootstrap values less than 50 are not shown.

In the Korean population, the Hd and π values of the COI gene were calculated as 0.286 and 0.00104, respectively (Table 2).

The Analysis of molecular variance (AMOVA) results revealed that most of the genetic variation (94.68%) was among the three groups (Table 4). Table 5 revealed a high Fst value with low gene flow among the Taiyangpi-Shuanglienpi, Taiping-Gongliao and Korean populations.

ABLE 2. Genetic variation for each population of Chinese ricefish in Taiwan based on the D-loop and COI.

Sampling locality (Sample IDs)	Sample size	D-loop haplotype (No.)	COI haplotype (No.)	COI+D-loop haplotype (No.)	D-loop haplotype diversity (Hd)	D-loop nucleotide diversity (π)	COI haplotype diversity (Hd)	COI nucleotide diversity (π)	Source
Taiping (OTP)	17	1	1	1	0.000	0.00000	0.000	0.00000	This study
Gongliao (OGL)	12	1	1	1	0.000	0.00000	0.000	0.00000	This study
Taiyangpi (OTY)	8	3	2	5	0.75	0.00398	0.429	0.00078	This study
Shuanglienpi wild population (OSL)	8	3	2	4	0.679	0.00121	0.571	0.00311	This study
Shuanglienpi restored population (OSR)	10	1	1	1	0.000	0.00000	0.000	0.00000	This study
Gongliao	3	1	—	—	0.000	0.00000	—	—	Tzeng *et al.* 2006
Taiyangpi	5	1	—	—	0.000	0.00000	—	—	Tzeng *et al.* 2006
Shuanglienpi	17	12	—	—	0.882	0.00261	—	—	Tzeng *et al.* 2006
South Korea	7	—	2	—	—	—	0.286	0.00104	Kim *et al.* 2016 (unpublished data)

TTABLE 3. Changes in the status of the Chinese ricefish and the invasion timeline of the mosquitofish and green swordtail into its habitats.

Locality	Shuanglienpi						
Year	1992	1995	2000s	2006-2008	2009	2011	2017-2019
Chinese ricefish	first recorded (Tzeng *et al.* 2006)	existed*	—	—	suppose existed, but not recorded (Chen 2009)	suppose existed, but not recorded (Chen 2011)	rare (this study)
mosquitofish	not recorded**	existed*	existed**	existed (Chen 2009)	—	one of three most dominant species***	dominant species (this study)

Locality	Ruyi Lake		
Year	2006-2007	2009	2019
Chinese ricefish	common (Chen 2009)	common (16.7% of all captured fish) (Chen *et al.* 2011)	dissapeared (this study)
green swordtail	not recorded (Chen 2009)	not recorded (Chen *et al.* 2011)	first recorded / common (this study)

* Information from previous field work in 1995 (Tzi-Yuan Wang).

** Information from Chen-Fu Huang, director of the Shuanglienpi Restoration and Education Center (2018).

*** Data from the project for conservation and utilization of the Shuanglienpi wetland, Taiwan (2011).

TABLE 4. AMOVA of three studied groups (OSL-OSR-OTY, OGL-OTP and South Korea).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among group	2	251.669	6.82876	94.68025
Within group	59	22.637	0.38368	5.31975
Total	61	274.306	7.21244	100

TABLE 5. Pairwise Fst value of three studied groups (OSL-OSR-OTY, OGL-OTP and South Korea).

Population pairwise Fst's (Tamura-Nei distance)	OSL-OSR-OTY	OGL-OTP	South Korea
OSL-OSR-OTY	0		
OGL-OTP	0.9518	0	
South Korea	0.9022	0.99144	0

Discussion

Genetic diversity in Taiwan. The phylogenetic trees reconstructed by the D-loop and COI showed that Chinese ricefish collected from New Taipei City and Yilan County can be divided into different clades from the Tamsui and Lanyang Rivers (Figs. 2, 3). The Xueshan (Snow Mountain) Range is known as a natural geographical barrier between the Tamsui River basin and the Lanyang River basin. Previous studies have reported that such a geographical barrier can create differences in fish fauna, as was the case in the eastern and western regions of South Korea (In *et al.* 2013). Since Chinese ricefish mainly inhabit lowland water (Chen & Fang 1999), the Xueshan Range might have isolated the western Tamsui River and eastern Lanyang River populations, which is supported by the high Fst value (0.9518) and low gene flow between the two geo-distinct clades (Table 5). Furthermore, the Taiyangpi and Shuanglienpi populations were divided into different subclades in the ML trees reconstructed by D-loop or COI sequences with high bootstrap values. The two populations were also isolated in different tributaries of the Lanyang River.

Mitochondrial DNA markers can be used as molecular evidence to explore the origin and dispersal of lowland freshwater fish. For example, Kano *et al.* (2018) proposed a persuasive explanation for the possible origin of paradise fish in the Ryukyu Archipelago. In our study, both the Shuanglienpi wild population and the Taiyangpi population showed very high Hd and π values in the D-loop gene (0.679 and 0.00121 for OSL, 0.75 and 0.00398 for OTY) and COI gene (0.571 and 0.00311 for OSL, 0.429 and 0.00078 for OTY). These populations also had more haplotypes in the D-loop (three for OSL and OTY). Tzeng *et al.* (2006) revealed high Hd and π values in these populations. The Shuanglienpi wild population with eight individuals was further compared with the Korean population with seven individuals. The study revealed that the Shuanglienpi wild population had higher Hd and π values for the COI gene (Hd and π as 0.571 and 0.00311) compared to the Korean population (Hd and π as 0.286 and 0.00104). In addition, Table 2 showed similar high Hd and π values for D-loop sequence in the Shuanglienpi wild population when compared to Tzeng *et al.*'s study (2006). It is noteworthy that the Shuanglienpi population had remarkably high genetic diversity. On the other hand, in the Taiyangpi population, our study revealed much higher Hd and π values (Hd and π as 0.75 and 0.00398) in the D-loop when compared to Tzeng *et al.*'s study (Hd and π as low as zero). Our study also found much higher Hd and π values in the COI gene (Hd and π as 0.429 and 0.00078, respectively). Thus, long-term sampling could be useful for monitoring and better estimating the dynamic genetic diversity in populations. The two Taiwanese populations with higher genetic diversity should be more important for species conservation.

In contrast, even though the Gongliao and Taiping populations are located in different rivers (Shuangxi River and Tamsui River; see Fig. 1), only one identical haplotype was discovered in the two distinct populations. Tzeng *et al.* (2006) indicated that the Gongliao population is grouped with a distinct western Chinese population from Yunnan. However, the Gongliao population is also distinct from the other Taiwanese populations. The low genetic diversity and phylogeny in the Gongliao and Taiping populations suggest a probable founder effect. Therefore, we speculate that the Chinese ricefish inhabiting the paddy fields in Gongliao and Taiping might have been introduced from China. It is common to find the same haplotype in discontinuous locations in the populations of introduced species. For examples, In *et al.* (2013) reported that the Japanese ricefish (*O. latipes*) found western Korea probably arrived there by anthropogenic causes. Shih *et al.* (2011) also reported a genetic uniformity (sharing the same mtDNA 16S rRNA and COI haplotype) in two populations of an introduced freshwater crab (*Sayamia germaini*) in Kaohsiung, Taiwan. Thus, a founder effect, with a single haplotype in Gongliao and Taiping populations, probably occurred as a result of individuals from China introducing to one of these two distinct paddy field, and finally introduced to other paddy field.

The habitat type can also provide a clue for the possible origin of Chinese ricefish in Taiwan. Unlike Shuanglienpi, which is classified as a natural wetland and lake, both Gongliao and Taiping habitats are paddy fields built over one hundred years ago to plant aquatic agricultural crops such as rice, taro and lotus. All ditches connecting the paddy fields to adjacent rivers are also manmade. The Chinese ricefish found in locations such as an artificial pond (Ruyi Lake) or an isolated paddy field (Gongliao and Taiping) that lacks creeks or ditches as a possible migration route to adjacent rivers could be treated as an introduced population. In addition, water pollution, habatat destruction and invasion of alien species in the adjacent China has a negative impact on ricefish survival. Regardless of whether these two populations are indigenous or introduced, the unique haplotype of the populations shows the importance for their conservation. Regardless of whether these two populations are indigenous or introduced, the unique haplotype of the populations shows the importance for their conservation. Therefore, preserving these two unique populations will be beneficial for conserving ricefish. Further population sampling from southeastern China will help reveal the details on the species' evolutionary history.

On the other hand, two haplotypes were found in the Shuanglienpi wild population, but only one of the wild haplotypes had proliferated into the Shuanglienpi restored population (Figs. 2, 3). Thus, the genetic diversity in the restored population was also lower than that in the wild population, which indicates that this restored population also originated from a few individuals. Such a small and isolated population of threatened species is prone to result in the loss of genetic diversity (Holt *et al.* 1996). This study and Tzeng *et al.* (2006) demonstrate that haplotype diversity may fluctuate within the Shuanglienpi wild population.

Conservation status in Taiwan. According to our field investigation, the Chinese ricefish can be found in four localities: Gongliao, Taiyangpi, Shuanglienpi and Taiping. Although the fish were recorded as comprising 16.7% of all captured fish in Ruyi Lake (Chen 2009; Chen *et al.* 2011), our survey revealed that the ricefish had disappeared in Ruyi Lake by 2019. Meanwhile, an invasive green swordtail fish (*Xiphophorus hellerii*) became one of the most dominant fish in Ruyi Lake, even though it was not recorded in 2006, 2007 or 2009 (Table 3) (Chen 2009; Chen *et al.* 2011).

Our investigations further revealed that Chinese ricefish in Shuanglienpi have also become extremely rare while the mosquitofishes (*Gambusia affinis*) seem to have become one of the most dominant fish species, with hundreds of them caught during our seven expeditions in 2017–2019. Only eight individuals were collected from a creek near the main pond of Shuanglienpi and only three haplotypes were found during the expeditions. The mosquitofish have been widely introduced to almost all the low altitude waters of Taiwan since 1911 (Sato *et al.* 1972). Wang *et al.* (2014) reported that the mosquitofish can attack and kill the Japanese ricefish when these two species are co-cultured in an experimental environment. The invasive fish have been found in Shuanglienpi since at least 1995 (information from Tzi-Yuan Wang) and became numerous in the 2000s (information from Mr. Chen-Fu Huang, director of the Shuanglienpi Restoration and Education Center, 2018). This could explain why the high genetic diversity, 12 haplotypes of 17 individuals in 1995 (Tzeng *et al.* 2006), decreased to low genetic diversity, 3 haplotypes of 8 individuals in 2019 (this study). Chen (2009) and Chen *et al.* (2010) also suggested that the decline in Taiwanese populations is attributable to the invasive species. In addition, Chen *et al.* (2012) proposed that the decline in the populations is attributable to water pollution. Based on the present cases in Shuanglienpi, we conjectured that the invasive mosquitofish has the largest impact on ricefish survival in Taiwan. A similar situation might be happening in Ruyi Lake, in which the green swordtail could play the same role as the mosquitofish and eventually cause the Chinese ricefish to go extinct in ten years. The changes in the status of Chinese ricefish, along with the timeline of the mosquitofish and green swordtail invading the Chinese ricefish habitats (Shuanglienpi and Ruyi Lake), are summarized in Table 3. In short, according to the time point of invasion of these alien fish, as well as the time point of the decline of the Chinese ricefish population, the green swordtail may have played a role similar to the mosquitofish and eventually resulted in the extinction of Chinese ricefish in Ruyi Lake. We conclude that the invasion of these poeciliid fish was one of the major reasons for the decline in Chinese ricefish populations in Taiwan.

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Appendix I. Multiple-sequences alignment of D-loop sequences used in this study.

#OGL1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.curvinotus ---------- ---------- ---------- ---------- ---------- ---------A TTTTCATACA CCAGTTTT
#O.celebensis ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.latipes CCGAGCGCTG CCATTCATAG CCTCAAACAT GTTTGAGCTG TAAAGACATT CTTCCGGCCC CCAACACGTG CATGCGCG
#O.sarasinorum ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL3 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL4 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSR1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTP1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTY1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTY2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTY3 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTY4 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OTY5 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------

#OGL1 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#O.curvinotus GTAATCCAGA GCGCATCACT TTTGCCACCAACGTTAAATT AACGTTGCAC AAACGTTGCA TCAGCGCCCC ATGGACAC
#O.celebensis ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.latipes TTGCATGCGC GTTGCATGCG CGTTGCATGC GCGTTGCATG CGCGTTGCAT GCGCGTTGCA TGCGCGTTGC ATGCGCGT
#O.sarasinorum ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL1 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OSL2 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OSL3 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OSL4 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OSR1 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTP1 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTY1 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTY2 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTY3 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTY4 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC
#OTY5 ---------- ---------- ---------- ---------- ---------- ---------- -CCGAGGTCC GCCGTACC

#OGL1 TGCATG-CGC GTTG-CATGG CCTAAA-ATG GCCGAGT--A CAAGCATGC- ----ACACAC AC---TGCAC ACACGTGA
#O.curvinotus TAAATGACGC GAGGGCGCTA AATAAACACC CCCTACC--T CTAGCACCCT TTTAACGATT TCACTTTTTT TTTTTTTT
#O.celebensis ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----CCGA
#O.latipes TGCATG-CGC GTTG-CATAA CCCAAA-ACG GCCTAGTGAA CAAGCGTGTG TTTGATGCGC ACGCGTGTGA ACGCGTGT
#O.sarasinorum ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#OSL1 TGCATG-CGC GTTG-CATGC CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC ACACGTGA
#OSL2 TGCATG-CGC GTTG-CATGG CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC ACACGTGA
#OSL3 TGCATG-CGC GTTG-CATGC CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC

ACACGTGA
#OSL4 TGCATG-CGC GTTG-CATGC CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC ACACGTGA
#OSR1 TGCATG-CGC GTTG-CATGC CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC ACACGTGA
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#OTY5 TGCATG-CGC GTTG-CATGC CCTAAA-ATG GTCGAGT--A CGAGCACGC- ----ACACAC AC---TGCAC ACACGTGA

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#O.celebensis GCTCCGCCTC TCCCCAACAA TAGATGTAAA ATAGATGTAA AATTTATACC TTATACTTGT ATGTAATATA ACCATTAA
#O.latipes GCGCAAAACG TTCACA-CAA CACTC-AAAA ATGTGCGTCG CCGGGCTCTG CCAATATAGT GCCGAGTACT TCCAAAAA
#O.sarasinorum ---------- --CCGAGCTC TGCCCGCACA GTC------- AATATGTCCT TTATACTTGT ATGTAATATA ACCATTAA
#OSL1 ACGCAAAACG TTCTCT-CAA CTCTCGAAAA ATAAGCGTCG CCGGACTCTG CCAAAATAGT GCCGAGCACT TCCAAAAA
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#O.celebensis TT--TAATTA AACCATTAAT GCCTAGTACA CTTTA----- ATTAATGATG TTAAA-TAAA AAATGAATAA TAAACTAT

#O.latipes GTCCCAAAAA AGTCCCAAAA TACATATATG TATTATCCCC ATATGTGGT- TTTAA-CCAT TTTTGCCTAG TACACCTC

#O.sarasinorum TT--TAATTA AACCATTAAT GCCTAGTACA TACGG----- -TTAATGATA CTAAACTAAA GAATGAATAA CAAACTGC

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#O.curvinotus GACCATGCAA GTCAATTATA TTTACCCCGC GCTCCAGGCC GCAGTACATA CACCTACAGT TGGTGTATTT AGCACAAG

#O.celebensis AACTTAAATA AATACATATG TATAAACCCC A-TAAAAATA ATGAAATGCT AA---GAGAT TTATATATAA CCTATAAC

#O.latipes GTCCATGCAA GTCAATTATA TTTACCCCGC GCTCCAGGCC GCAGAGCATA CACCTACGAT TGGTGTATTT AGCACAAG

#O.sarasinorum AAGTGATATA AGTGTTTATG CTTATTCCCC A-CTATACTA ACAGAATGCT AA---GAGAT TTATATATAA CCTAAAAC

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#O.celebensis TTGTTTAAAA CTAGTTTCAA GTCACCCACA TCCTTCCTTC AATGATTACT TAATGTAGTA AGAGACCACC ATCAGTTG

#O.latipes TGTGCCTCAG CTAGTTTCAA GTCGTCAGCA TCCTTCCTTC AATTGTTATT TAATGTAGTA AGAGCCCACC ATCAGTTG

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#O.celebensis ATTACTTAAT GCCAACGGTT CTTG-ATGGT CAGGGACAGT TATTTGTGGG GG-TTTCACA GTGTGAACTA TTCCTGGC

#O.latipes ATTTCTTAAT GTTAACGGTT ATTG-AAGGT GAGGGACAAA AATC-GTGGG GGGTTTCACT TCTTGAATTA

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#OTY3 ATTTCTTAAT GTTAACGGTC CTTGTAAGGT CAGGGACAGA AATC-GTGGG GG-TTTCACT TCTTGAACTA TTCCTGGC

#OTY4 ATTTCTTAAT GTTAACGGTC CTTGTAAGGT CAGGGACAGA AATC-GTGGG GG-TTTCACT TCTTGAACTA TTCCTGGC

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#O.curvinotus ATTTGGCTCT ACATCTCAAG GCCATACA-T TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#O.celebensis ATTTGGTTCC -TATTTCAGG GCCATAC-GT TCATTATTAC TCACACGTTC CTTGACGCTT GCATAAGTTA ATGGTGGA

#O.latipes ATTTGGCTCT ACATCTCAAG GCCATATAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#O.sarasinorum ATTTGGTTCC -TATTTCAGG GCCATAC-TT TCATTATTAC TCACACATTT CCTGACGCTT GCATAAGTTA ATGGTGAA

#OSL1 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

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#OSL3 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OSL4 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OSR1 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OTP1 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OTY1 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OTY2 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OTY3 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA
#OTY4 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA
#OTY5 ATTTGGCTCT ACATCTCAAG GCCATTTAGT TTCTCGTCTC TCACACTTTC ACTGGCCCTG ACATTGGTTA ATGGTGGA

#OGL1 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#O.curvinotus GTACATACTC CTCGT-TACC CCCCATGCCG GGCGTTCTTT CTAATGGACA ACGGGTTTTC CTTTTTTTTT CCTTTTCA
#O.celebensis GTACATATGA CGGGAGCACC CCCCATGCCG GGCGTTCTTT CTAATGGGCA ACTGGTATTT TTTTTTTTTT CCTTTTCA
#O.latipes GTACATACTC CTCGT-TACC CACCAAGCCG AGCGTTCTTT CTAATGGGCA GGGGGTTCTC TTTTTTTTTT CCTTTCAA
#O.sarasinorum GTACATATGA CGGGAGCACC CCACATGCCG GGCCTTCTTT CTAAGGGGCA GCTGGTATTT TTTTTTTTT- CCTTTTCA
#OSL1 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OSL2 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OSL3 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OSL4 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OSR1 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OTP1 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC TTTTTTTTTT CCTTTTCA
#OTY1 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC CTTTTTTTTT CCTTTTCA
#OTY2 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC CTTTTTTTTT CCTTTTCA
#OTY3 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC CTTTTTTTTT CCTTTTCA
#OTY4 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC CTTTTTTTTT CCTTTTCA
#OTY5 GTACATACTC CTCGT-TACC CACCAAGCCG GGCGTTCTTT CTAATGGGCA GCTGGTTCCC CTTTTTTTTT CCTTTTCA

#OGL1 TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTTG GCCGCAGGAA ATAT-GGT
#O.curvinotus CTTGG-CAT- TTCACAGTGC ATAC-AGACC TTGATGACAA GGTTGAACAT TTAGAAATCG GCCGCAAAGA ATATTGGT
#O.celebensis CTTGGTCATA TTCACAGTGC ACACTAAAGT TAGCTGACAA GGTTGAACAT TTAGAAATCG GCCGCAAATA ATATTGGT
#O.latipes TTTG--CAT- TTCACAGGGC ATAC-AAACC TTGTTGACAA GGTTGAACAT TTAAAACTCG GCCG------ --------
#O.sarasinorum TTTGG-CAT- TTCACAGTGC ACACTAAAGT CAACTAACAA GGTTGAACAT TTAGAAATCG GCTGGAAG-A ATATTGAT
#OSL1 TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA ATAT-GGT
#OSL2 TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA

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ATAT-GGT
#OSL3           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT
#OSL4           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT
#OSR1           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT
#OTP1           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTTG GCCGCAGGAA
ATAT-GGT
#OTY1           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT
#OTY2           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT
#OTY3           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG GCTGCAAGAA
ATAT-GGT
#OTY4           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG GCTGCAAGAA
ATAT-GGT
#OTY5           TTTG--CAT- TTCACAGTGC ATAC-AGACC TTGTTGACAA GGTTGAACAT TTAGAACTCG ACTGCAAGAA
ATAT-GGT

#OGL1           GAATTATTGT AAGATATTAA CAGATGAATT GCATAACTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#O.curvinotus   GAATTATTCA AAGATATTAA CAGATGAATT GCATAAGTGA TATCAAGAGC ATAAATAACC AAATGAAACT
AGGAACGT
#O.celebensis   GAGTTATTGA AAGATATTAA TAGATAACTT ACATAACTGA TATCAAGAGC ATAAATAACC AAATGAAACT
ACTAAAGT
#O.latipes      ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.sarasinorum GAGTTATTAA AAGACATTAC TAAATAACTT ACATAACTGA TATCAAGAGC ATAATTAACC AAATGAAACT
ACTAAAGT
#OSL1           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OSL2           GAATTATTGT AAGATATTAA CAGATAAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OSL3           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OSL4           GAATTATTGT AAGATATTAA CAGATAAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OSR1           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTP1           GAATTATTGT AAGATATTAA CAGATGAATT GCATAACTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTY1           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTY2           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTY3           GAATTATTGT AAGATATTAA CAGATGAACT GCATAACTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTY4           GAATTATTGT AAGATATTAA CAGATGAACT GCATAACTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
#OTY5           GAATTATTGT AAGATATTAA CAGATGAATT GCATAATTGA TATCAAGAGC ATAA-TAGTC AAAAAAATCT
AGGAACGT
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#OGL1 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG TAAGAGCTCT GTCATTCC
#O.curvinotus TTCTATAAT- ATGCCCCCCG GCTCCCGCGC GTCAAACCCC CCTA--CCCC CCTAAACTAG TAAGAAGTCT ATTATTCC
#O.celebensis TTCTATTATA TTTCCCCCCG GCTTTTGCGG GTCAAACCCC CCCTACCCCC CCAATACTAG TAAGATTTCT ATCATTCC
#O.latipes ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.sarasinorum TTCTATAATA TTTCCCCCCG GCTTTTGCGG GTCAAACCCC CCCTACCCCC CCAATACTAG TGAGATATCT ATTACTCC
#OSL1 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OSL2 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OSL3 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OSL4 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OSR1 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OTP1 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG TAAGAGCTCT GTCATTCC
#OTY1 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OTY2 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OTY3 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OTY4 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC
#OTY5 TCCTATCATT ATGACCCCCG GCTTCTACGC GTCAAACCCC CCTA--CCCC CCTATACTAG AAGGAGCTCT GTCATTCC

#OGL1 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTCCC AAATTTGTGT GTATTTA--- --------
#O.curvinotus TGCAAACCCC CCGGAAACAG GAAACCCCCT ACTAGCATTT TAGCCCGCCC AAATTTGTGT GTATTTATAT TATTTGTA
#O.celebensis TGCAAACCCC CCGGAAACAG GAAAACCCCT ACTAATATTT TTTTTTTCGC TTGTTTGTTC ATATTTACAT TACTTGCA
#O.latipes ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------
#O.sarasinorum TGCAAACCCC CCGGAAACAG GAAAACCCCT ACTAATATTT TTTTTTCCAC TTGCTTGTTC ATATTTACAT TA-TTGCA
#OSL1 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- --------
#OSL2 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- --------
#OSL3 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- --------
#OSL4 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- --------
#OSR1 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- --------
#OTP1 TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTCCC AAATTTGTGT GTATTTA--- ---
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#OTY1          TGCAAACCCC CCGGAAACAG GAAAGCCCCC ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- ---
-----
#OTY2          TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAGTTTGTGT ATATTTA--- ---
-----
#OTY3          TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- ---
-----
#OTY4          TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAATTTGTGT ATATTTA--- ---
-----
#OTY5          TGCAAACCCC CCGGAAACAG GAAAGCCCCT ACTAATATTT TATCCCTTCC AAGTTTGTGT ATATTTA--- ---
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#OGL1         ---------- -
#O.curvinotus  ATATTGCAAA A
#O.celebensis  AAATGTAAAA -
#O.latipes     ---------- -
#O.sarasinorum AAATGCAAAA -
#OSL1         ---------- -
#OSL2         ---------- -
#OSL3         ---------- -
#OSL4         ---------- -
#OSR1         ---------- -
#OTP1         ---------- -
#OTY1         ---------- -
#OTY2         ---------- -
#OTY3         ---------- -
#OTY4         ---------- -
#OTY5         ---------- -
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Appendix II. Multiple-sequence alignment of COI sequences used in this study.

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#OGL1         TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAA ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC
TTC GTA ATA ATT
#O.curvinotus  CTA AGT CAA CCA GGC TCC CTA TTA GGA GAC GAC CAG ATC TAT AAC GTA ATT GTA ACT GCA CAT
GCT TTC GTA ATA ATT
#O.celebensis  CTG AGC CAA CCA GGC TCT CTC CTA GGC GAC GAC CAG ATT TAT AAT GTA ATC GTA ACT GCA CAT
GCC TTT GTT ATA ATC
#O.latipes     TTA AGT CAG CCC GGG TCT CTA TTA GGT GAC GAC CAA ATC TAT AAT GTA ATT GTA ACC GCG CAT GCC
TTC GTA ATA ATT
#O.sarasinorum CTA AGT CAG CCA GGC TCT CTG CTA GGC GAC GAC CAA ATT TAT AAC GTA ATC GTC ACT GCA CAC
GCT TTC GTC ATA ATC
#OSL1         TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC
TTC GTA ATA ATT
#OSL2         TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC
TTC GTA ATA ATT
#OSL3         TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC
TTC GTA ATA ATT
#OSL4         TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC
TTC GTA ATA ATT
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#OSR1 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTP1 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAA ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTY1 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTY2 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTY3 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTY4 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT
#OTY5 TTA AGT CAA CCC GGG TCT CTT TTA GGT GAT GAC CAG ATT TAC AAC GTA ATT GTA ACT GCA CAT GCC TTC GTA ATA ATT

#OGL1 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGG GGT TTT GGT AAC TGA CTT ATC CCC CTA ATG ATT GGG GCC CCA GAT ATA
#O.curvinotus TTC TTT ATA GTA ATG CCA ATC ATA ATT GGG GGG TTT GGC AAC TGA TTA ATC CCT TTA ATG ATT GGA GCC CCC GAC ATG
#O.celebensis TTT TTT ATA GTA ATA CCA ATT ATG ATT GGA GGG TTT GGA AAC TGA CTA GTT CCT CTG ATG CTC GGG GCA CCA GAC ATG
#O.latipes TTC TTT ATA GTA ATA CCA ATC ATG ATT GGG GGC TTT GGC AAC TGA TTA ATT CCT CTA ATG ATC GGA GCC CCT GAT ATG
#O.sarasinorum TTT TTT ATA GTA ATG CCT ATT ATG ATT GGA GGG TTC GGA AAC TGG TTA ATT CCT CTG ATG CTT GGA GCC CCA AAC ATG
#OSL1 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OSL2 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT GTC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OSL3 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT GTC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OSL4 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OSR1 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OTP1 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGG GGT TTT GGT AAC TGA CTT ATC CCC CTA ATG ATT GGG GCC CCA GAT ATA
#OTY1 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OTY2 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OTY3 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OTY4 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA
#OTY5 TTC TTT ATA GTA ATG CCA ATT ATG ATT GGA GGT TTT GGT AAC TGA CTT ATC CCC TTA ATG ATT GGG GCC CCA GAT ATA

#OGL1 GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT TCC TCT GGC GTG
#O.curvinotus GCC TTC CCA CGG ATA AAT AAT ATA AGC TTT TGA CTC CTG CCC CCT TCT TTC CTT CTA TTA TTG GCC TCA TCT GGT GTA

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#O.celebensis  GCT TTC CCA CGA ATA AAC AAT ATA AGT TTC TGA CTT CTA CCC CCT TCT TTT CTT CTT CTT TTA GCC
TCC TCT GGT GTA
#O.latipes     GCC TTT CCT CGA ATA AAC AAC ATG AGC TTC TGA CTA CTT CCC CCC TCC TTC CTT CTA TTG TTA GCT
TCC TCT GGC GTG
#O.sarasinorum GCT TTC CCA CGA ATA AAC AAT ATA AGT TTT TGA CTT CTG CCC CCC TCT TTT CTT CTT CTT CTA GCC
TCC TCG GGC GTA
#OSL1          GCC TTC CCT CGG ATG AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTA CTA GCT
TCC TCT GGC GTG
#OSL2          GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OSL3          GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OSL4          GCC TTC CCT CGG ATG AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTA CTA GCT
TCC TCT GGC GTG
#OSR1          GCC TTC CCT CGG ATG AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTA CTA GCT
TCC TCT GGC GTG
#OTP1          GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OTY1          GCC TTC CCT CGT ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OTY2          GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OTY3          GCC TTC CCT CGT ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OTY4          GCC TTC CCT CGG ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG
#OTY5          GCC TTC CCT CGT ATA AAT AAT ATA AGC TTC TGA TTA CTA CCC CCT TCA TTT CTT CTT CTG CTA GCT
TCC TCT GGC GTG

#OGL1          GAA GCT GGT GCA GGG ACA GGG TGA ACC GTA TAT CCT CCC TTG TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#O.curvinotus  GAA GCT GGT GCC GGA ACA GGA TGA ACC GTA TAT CCC CCC TTG TCA GGT AAT TTG GCA CAC GCA
GGG GCC TCC GTA GAT
#O.celebensis  GAA GCA GGG GCA GGA ACT GGT TGA ACA GTT TAT CCG CCA CTA GCT GGT AAC CTA GCC CAC GCA
GGT GCA TCT GTC GAC
#O.latipes     GAG GCC GGT GCA GGG ACA GGA TGG ACC GTG TAC CCG CCT CTA TCA GGC AAC CTG GCG CAT GCG
GGA GCA TCC GTG GAT
#O.sarasinorum GAG GCT GGG GCT GGG ACG GGG TGA ACA GTT TAT CCG CCA CTA GCA GGA AAT CTG GCC CAC GCA
GGC GCA TCT GTT GAC
#OSL1          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#OSL2          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#OSL3          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#OSL4          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#OSR1          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
GGT GCA TCC GTC GAC
#OTP1        GAA GCT GGT GCA GGG ACA GGG TGA ACC GTA TAT CCT CCC TTG TCC GGG AAT CTA GCA CAT GCT GGT
GCA TCC GTC GAC
#OTY1          GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT
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GGT GCA TCC GTC GAC
#OTY2 GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT GGT GCA TCC GTC GAC
#OTY3 GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT GGT GCA TCC GTC GAC
#OTY4 GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT GGT GCA TCC GTC GAC
#OTY5 GAA GCT GGT GCA GGA ACA GGG TGG ACC GTA TAC CCT CCC CTA TCC GGG AAT CTA GCA CAT GCT GGT GCA TCC GTC GAC

#OGL1 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#O.curvinotus TTA ACC ATT TTC TCT CTT CAC CTG GCC GGA ATT TCT TCT ATC CTA GGG GCC ATT AAT TTC ATC ACA ACT ATT ATT AAT
#O.celebensis CTA ACA ATC TTC TCC CTC CAC CTG GCA GGG ATT TCA TCT ATT TTA GGT GCT ATT AAT TTT ATT ACC ACC ATT ATT AAT
#O.latipes TTA ACC ATC TTT TCC CTC CAT CTG GCA GGT ATC TCC TCT ATC TTA GGG GCT ATT AAT TTT ATT ACA ACA ATT ATT AAT
#O.sarasinorum CTT ACT ATT TTC TCC CTT CAC CTC GCA GGA ATT TCT TCT ATC TTA GGT GCC ATC AAT TTT ATT ACC ACT ATT ATT AAC
#OSL1 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#OSL2 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#OSL3 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#OSL4 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#OSR1 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
#OTP1 CTA ACC ATC TTC TCC CTT CAC CTA GCA GGT ATT TCT TCC ATT TTA GGC GCT ATC AAC TTT ATC ACA ACA ATT ATT AAC
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#OGL1 ATG AAA CCC CCA GCT ATC TCT CAA TAT CAG ACC CCC CTA TTT GTA TGA GCT GTA CTG ATT ACT GCT GTT CTA CTT CTA
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#O.celebensis ATA AAA CCT CCA GCT ATT TCT CAA TAT CAA ACA CCA TTA TTT GTA TGA GCA GTA CTT ATT ACT GCT GTA TTA CTC CTT
#O.latipes ATA AAA CCC CCG GCC ATC TCT CAG TAT CAG ACC CCT TTA TTC GTG TGA GCT GTA CTA ATT ACC GCA GTA TTA CTT CTA
#O.sarasinorum ATG AAA CCC CCA GCT ATC TCC CAA TAT CAG ACA CCA TTA TTT GTA TGA GCA GTG CTC ATT ACT

GCT GTA TTA CTC CTT
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GTT CTA CTT CTA
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TTC TTC GAC CCT
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TTT TTC GAC CCC
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TTC TTT GAT CCA
#O.latipes CTC TCC CTC CCT GTG CTC GCT GCA GGT ATC ACT ATG CTC CTA ACG GAC CGA AAC CTA AAT ACA ACA
TTC TTT GAC CCT
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ACG TTC TTC GAC CCA
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TTC TTC GAC CCT
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#OTY4 CTC TCT CTA CCT GTT CTT GCT GCA GGA ATT ACT ATA CTT CTA ACA GAC CGT AAC CTA AAT ACA ACC TTC TTC GAC CCT
#OTY5 CTC TCT CTA CCT GTT CTT GCT GCA GGA ATT ACT ATA CTT CTA ACA GAC CGT AAC CTA AAT ACA ACC TTC TTC GAC CCT

#OGL1 GCA GGA
#O.curvinotus GCA GGA
#O.celebensis GCC GGT
#O.latipes GCA GGG
#O.sarasinorum GCT GGG
#OSL1 GCA GGA
#OSL2 GCA GGA
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#OTY1 GCA GGA
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