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Molecular phylogenetic analysis and its impact on the conservation of *Ischnura rubilio* Selys, 1876 (Odonata: Coenagrionidae) in Taiwan

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Abstract. Although *Ischnura aurora* (Brauer, 1865) was traditionally considered to be widely distributed in Asia, the populations west of continental China have recently been identified as equivalent to *Ischnura rubilio* Selys, 1876. While the Taiwanese population has long been regarded as *I. aurora* as well, Taiwan in fact represents the distribution boundary between *I. aurora* and *I. rubilio*. Based on molecular and morphological analyses, we confirm that the "*I. aurora*-like" damselfly in Taiwan corresponds to *I. rubilio*. It is noteworthy that the abdominal blue spots of males in the Taiwan population have a unique phenotype compared to those found in specimens from India and continental China. According to past references and current surveys, the *I. rubilio* population in Taiwan has critically declined, with only one confirmed locality currently remaining. To maintain the Taiwanese population of *I. rubilio*, we recommend that prompt conservation measures of the habitat be implemented, focusing on the concept of the Satoyama Initiative.

Key words. Damselfly, identification, integrative taxonomy, Satoyama Initiative, water terrace

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Data Availability Statement:
All relevant data are within the paper.

Introduction

Many studies have demonstrated that biodiversity declines as agriculture expands (e.g., Butler et al., 2007; Donald et al., 2006; Foley et al., 2011; Gill et al., 2012; Stoate et al., 2009). This is particularly true for species, including insects, the most diverse group of animals, that inhabit boundaries between human agricultural activity and natural habitats, but their targeted conservation has received rather little attention (Dunn, 2005). Satoyama landscapes, where nature and culture intersect (Knight, 2010), may provide refuges for species inhabiting rapidly changing lowlands.

The "*Ischnura aurora*-like" damselfly of Taiwan was first reported as *I. aurora* by Ris (1916), and this name has since been used for the Taiwanese population (e.g., Asahina, 1966, Chujo, 1930, 1931; Ishida et al., 1973; Lieftinck et al., 1984; Lin & Yang, 2016; Matsuki & Lien, 1985, 1989; Nishimura et al., 1984; Tsou, 2005). It was previously considered a common species, widely distributed in the lowland wetlands of Taiwan (Lieftinck et al., 1984). However, in recent years, both its population and habitat have been drastically reduced, possibly due to rapid industrialization and changes in farming methods. The distribution of this species has by now

become so restricted that it must be regarded as rare (Lin & Yang, 2016; Tsou, 2005). The critical situation of the Taiwanese population calls for prompt conservation measures.

Ischnura aurora (Brauer, 1865) was long thought to be a species with a wide distribution from Asia to Oceania, however, Lorenzo-Carballa et al. (2022) reported that *I. aurora* comprised at least two species, based on morphological and DNA sequence differences between populations in Asia and Oceania. Populations from Oceania, including the one on Iwo Island, Japan, correspond to genuine *I. aurora*, whereas the Asian populations, including those in Yunnan, continental China, have been re-identified as *I. rubilio* Selys, 1876, originally described from India (Lorenzo-Carballa et al., 2022). The population on Taiwan, at the distribution boundary between these two species, has not been discussed thus far, though.

In this study, we analyze both nuclear and mitochondrial DNA of the Taiwanese "*Ischnura aurora*-like" damselfly and compare its external morphology with that of Japanese *I. aurora*, confirming that the Taiwanese species in fact represents *I. rubilio*. We also summarize the current and past distribution of *I. rubilio* in Taiwan based on a citizen science-based database and literature records. We discuss the conservation efforts in Taiwan that focus on the Satoyama landscape, and call for government conservation policies that are aimed at maintaining the current population of this species in Taiwan.

Methods

For morphological comparison, eight males and five females of Taiwanese *I. rubilio* and five males and six females of Japanese *I. aurora* were used (Futahashi and Kiyoshi, 2022). Specimens were photographed using a stereoscopic microscope S8APO (Leica Microsystems) with a digital high-definition microscope camera MC120HD (Leica), or CCD scanner GT-X830 (Epson). For molecular phylogenetic analysis, Taiwanese samples of *I. rubilio* were collected at Jianshanhu (25.2438, 121.5638), New Taipei City, and Japanese specimens of *I. aurora* were collected at Iwo Island (24.7975, 141.3209). DNA was extracted from one leg of each specimen. DNA fragments of nuclear DNA (ITS1, 5.8S rRNA, and ITS2) and mitochondrial DNA (mt16SrRNA and mtCOI) sequences were analyzed applying the same methods and using the same primers as Futahashi & Sasamoto (2012) have reported. The obtained sequence data were deposited in the DDBJ/EMBL/GenBank databases (accession numbers LC743962-LC743997). Molecular phylogenetic analyses were conducted using the neighbor-joining (NJ) method with MEGA11 (Tamura et al., 2021), maximum likelihood (ML) with MEGA11 (Tamura et al., 2021), and Bayesian (BA) with MrBayes v3.2 (Ronquist et al., 2012). Bootstrap values for neighbor-joining and maximum likelihood phylogenies were obtained by 1000 bootstrap replications. For Bayesian analysis, a Markov chain Monte Carlo (MCMC) analysis was performed under the general time reversible + invariable sites (GTR + I) model. In total, 3750 trees were generated for each Bayesian analysis (ngen = 500,000, samplefreq = 100, burn-in = 1250).

The distribution map was generated following the method described by Ma et al. (2022). Coordinates were extracted from the Observation Database of Taiwanese Odonata (Chi-Ming Lou, pers. comm.), literature records, and from the collection in the National Museum of Nature and Science, Tsukuba, Japan. Distributional data were categorized into records before 2000, between 2000–2015, and remaining localities. All extracted data are summarized in Table 1. The distribution map was created in SimpleMappr (Shorthouse, 2010).

Results

Molecular phylogenetic analysis

In order to identify the species of the "*Ischnura aurora*-like" damselfly in Taiwan, we analyzed nuclear ITS1 and mitochondrial COI sequences using five individuals from Taiwan and four individuals from Iwo Jima, Japan. In both analyses, individuals from Taiwan were identified as corresponding to *I. rubilio* (Figs 1, 2) while those from Iwo Jima, Japan, were confirmed to correspond to *I. aurora*, as has previously been reported (Lorenzo-Carballa et al., 2022).

Comparative analysis of external morphology

Next, we compared the external morphology of Taiwanese individuals with that of Japanese *I. aurora* (Fig. 3A). In both males and females, *I. rubilio* and *I. aurora* have been reported to be distinguishable by the very narrow black stripe on the second lateral suture of the thorax, and the wider (more than one-third of the width of the prothorax) protrusion of the posterior lobe of the prothorax in *I. rubilio* (Lorenzo-Carballa et al., 2022). Our findings confirmed that the black stripe on the second lateral suture was thinner in the Taiwanese specimens, consistent with the characteristics of *I. rubilio* (Fig. 3B, arrowheads). The protrusion of the posterior lobe of the prothorax was wider in Taiwanese specimens than in Japanese ones (Fig. 4), which is also consistent with the characteristics of *I. rubilio* (Lorenzo-Carballa et al., 2022).

In males of *I. rubilio* from other regions, it has been reported that the entire 8th dorsal segment of the abdomen is blue, the black stripe of the 2nd thoracic segment is less developed and mostly limited to the first half of the segment, and that the dorsal tubercles of the 10th abdominal segment are more distinctly developed (Lorenzo-Carballa et al., 2022). Taiwanese individuals have a less developed black stripe on the 2nd abdominal segment (Fig. 5A) and a more distinct dorsal tubercle

Table 1. Distributional data for *Ischnura rubilio* in Taiwan, including literature records.

Category	County	Locality	Latitude	Longitude	Present year	Sources	Remarks
Records before 2000	Chiayi County	Dalin Township (大林鎮)	23.5935	120.4753	1911	Ris (1916)	The coordinate is approximately the center of the township.
Records before 2000	Taipei City	no data	no precise coordinate	no precise coordinate	1930	Chûjô (1931)	
Records before 2000	Hsinchu City	Hukou (湖口)	24.8825	121.0493	1930	Chûjô (1931)	
Records before 2000	Nantou County	Puli (埔里)	no precise coordinate	no precise coordinate	1936, 1938	Futahashi & Kiyoshi (2022)	Collection in the National Museum of Nature and Science, Tsukuba, Japan.
Records before 2000	New Taipei City	Wulai (烏來)	24.7859	121.5407	1965	Asahina (1966)	The coordinate is approximately the center of the township.
Records before 2000	Taipei City	Songshan (松山)	25.0552	121.5608	1971–1972	Ishida et al. (1973)	The coordinate is approximately the center of the township.
Records before 2000	Pingtung County	Kenting Park (墾丁公園)	21.9364	120.8350	1972	Matsuki & Lien (1985), Matsuki & Lien (1989)	The coordinate approximately extracts from the map of Matsuki & Lien (1989), there are three localities that have been plotted on the map in Pingtung County in Matsuki & Lien (1989). However, only two localities have been listed in the reference, hence the localities of this area were concentrated into two.
Records before 2000	Taipei City	Neihu Jinlong temple (內湖金龍寺)	25.0922	121.5862	1974	Matsuki & Lien (1985)	The record was not included on the map because there was a locality nearby.
Records before 2000	Taipei City	Waishuangxi (外雙溪)	25.1086	121.5598	1974–1975	Nishimura et al. (1984), Matsuki & Lien (1985)	The record was not included on the map because there was a locality nearby.
Records before 2000	New Taipei City	Pinglin (坪林)	24.9121	121.7304	1975	Matsuki & Lien (1985)	The coordinate is approximately the center of the township.
Records before 2000	Nantou County	Puli Shizitou (埔里獅子頭)	23.9958	121.0383	1975	Matsuki & Lien (1985)	
Records before 2000	Taipei City	Neishuangxi (內雙溪)	25.1219	121.5782	1975, 1977–1978	Matsuki & Lien (1985)	The coordinate is approximately the center of the area.
Records before 2000	Taipei City	Nangang (南港)	25.0346	121.6116	1975, 1977, 1980	Matsuki & Lien (1985), Matsuki & Lien (1989)	The coordinate is approximately the center of the township.
Records before 2000	Pingtung County	Changle Village (長樂村)	22.0823	120.8435	1976	Matsuki & Lien (1985), Matsuki & Lien (1989)	The coordinate approximately extracts from the map of Matsuki & Lien (1989).
Records before 2000	Pingtung County	Shimen (石門)	22.1270	120.7725	1981	Nishimura et al. (1984)	
Records before 2000	New Taipei City	Sanxia Baiji (三峽白雞)	24.9053	121.3991	1977	Matsuki & Lien (1985)	
Records before 2000	New Taipei City	Bali (八里)	25.1429	121.4119	1980	Matsuki & Lien (1989)	The coordinate is approximately the center of the township.
Records from 2000–2015	New Taipei City	Liaojiaokeng (料角坑)	24.9706	121.8239	2003–2004, 2008–2010	Observation Database of Taiwanese Odonata	
Records from 2000–2015	New Taipei City	Wangyuankeng (望遠坑)	25.0020	121.9289	2008	Observation Database of Taiwanese Odonata	
Records from 2000–2015	New Taipei City	Tudigongling (土地公嶺)	25.0601	121.8866	2010, 2013–2015	Observation Database of Taiwanese Odonata	
Records from 2000–2015	New Taipei City	Jilin Village (吉林里)	24.9904	121.8872	2012–2013	Observation Database of Taiwanese Odonata	
Remaining locality	New Taipei City	Jianshanhu (尖山湖)	25.2438	121.5638	2000, 2014–2022	Observation Database of Taiwanese Odonata	

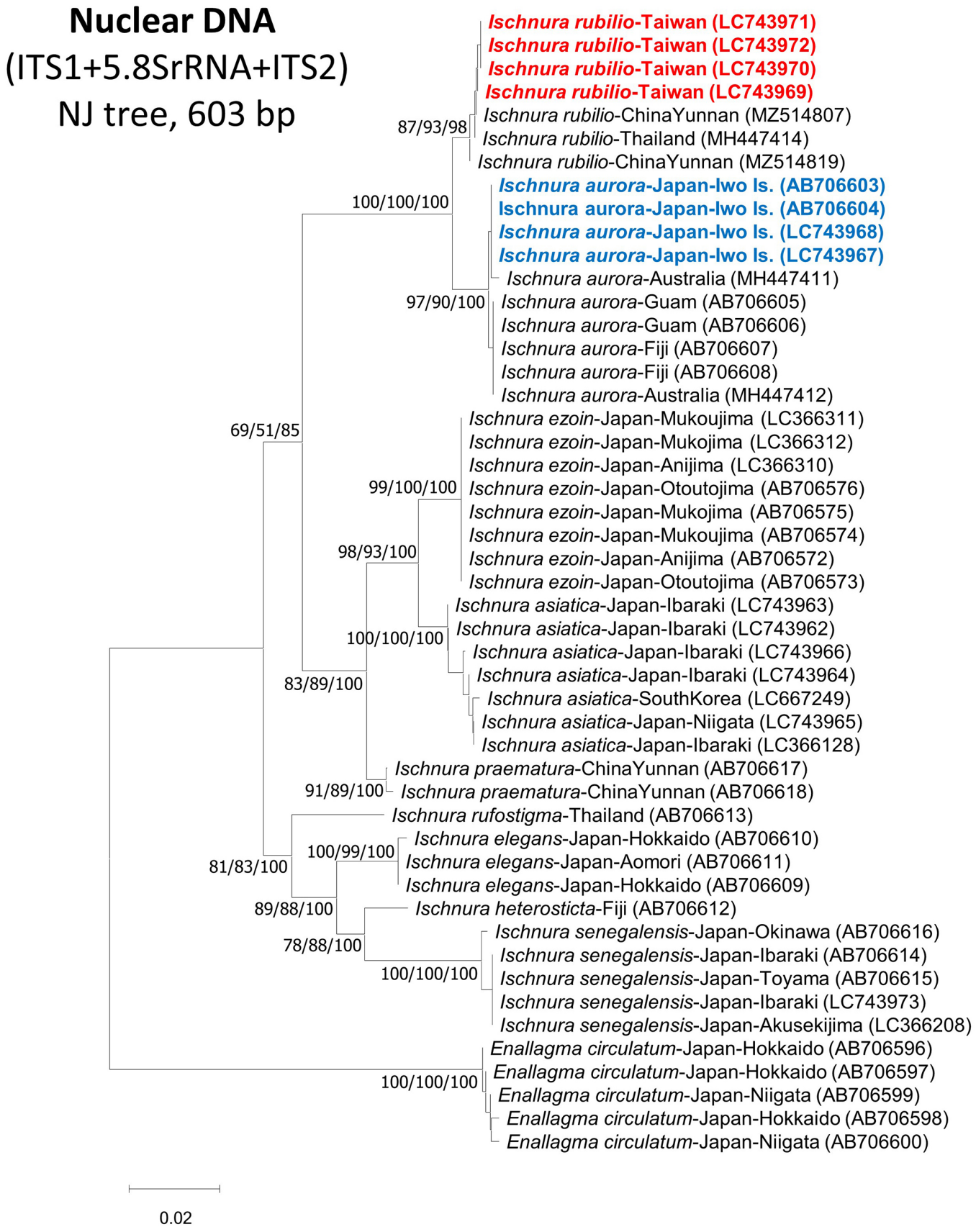

Figure 1. A neighbor-joining phylogeny of *Ischnura* species based on the nuclear ITS region (ITS1 5.8S ITS2, 603 bp). Maximum likelihood and Bayesian phylogenies yielded similar topologies. On each node, statistical support values are indicated in the order of [bootstrap value of neighbor-joining]/[bootstrap value of maximum likelihood]/[posterior probability (percentages) of Bayesian]. Accession numbers are given in parentheses.

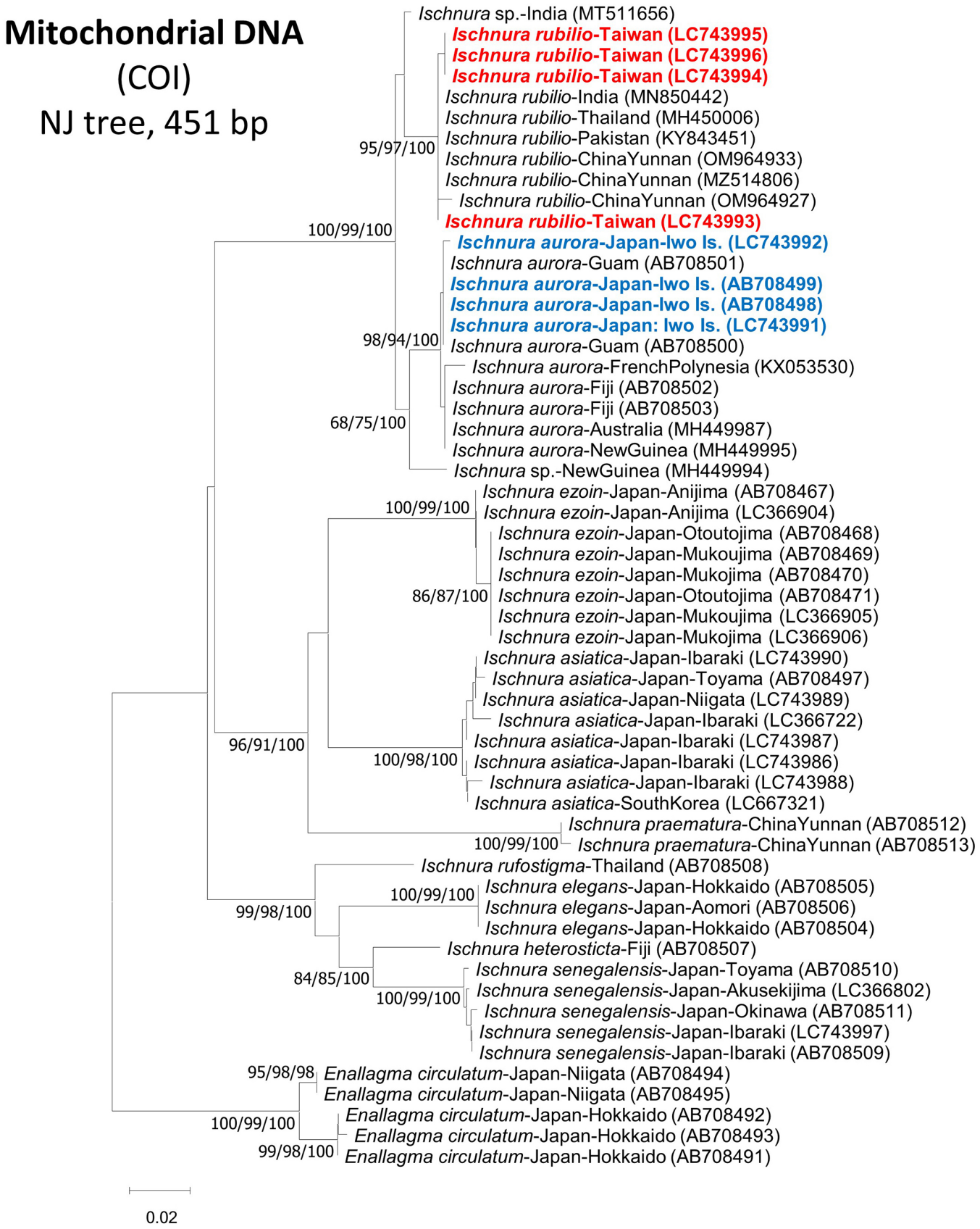

Figure 2. A neighbor-joining phylogeny of *Ischnura* species based on the mitochondrial COI gene (451 bp). Maximum likelihood and Bayesian phylogenies yielded similar topologies. On each node, statistical support values are indicated in the order of [bootstrap value of neighbor-joining]/[bootstrap value of maximum likelihood]/[posterior probability (percentages) of Bayesian]. Accession numbers are given in parentheses.

on the 10th abdominal segment (Fig. 5B), which again is consistent with the characteristics of *I. rubilio*. However, it should be noted that the blue spot on the 8th abdominal segment is limited to the posterior half, which is rather similar to *I. aurora* (Fig. 5B). These findings indicate that Taiwanese individuals are morphologically representative of *I. rubilio*, even though the blue spots on the 8th abdominal segment are different in appearance from those of individuals from India to continental China. In addition, the yellow color of the abdomen in males is lighter, and the thorax of immature females is more reddish in Taiwanese *I. rubilio* (see also Fig. 7).

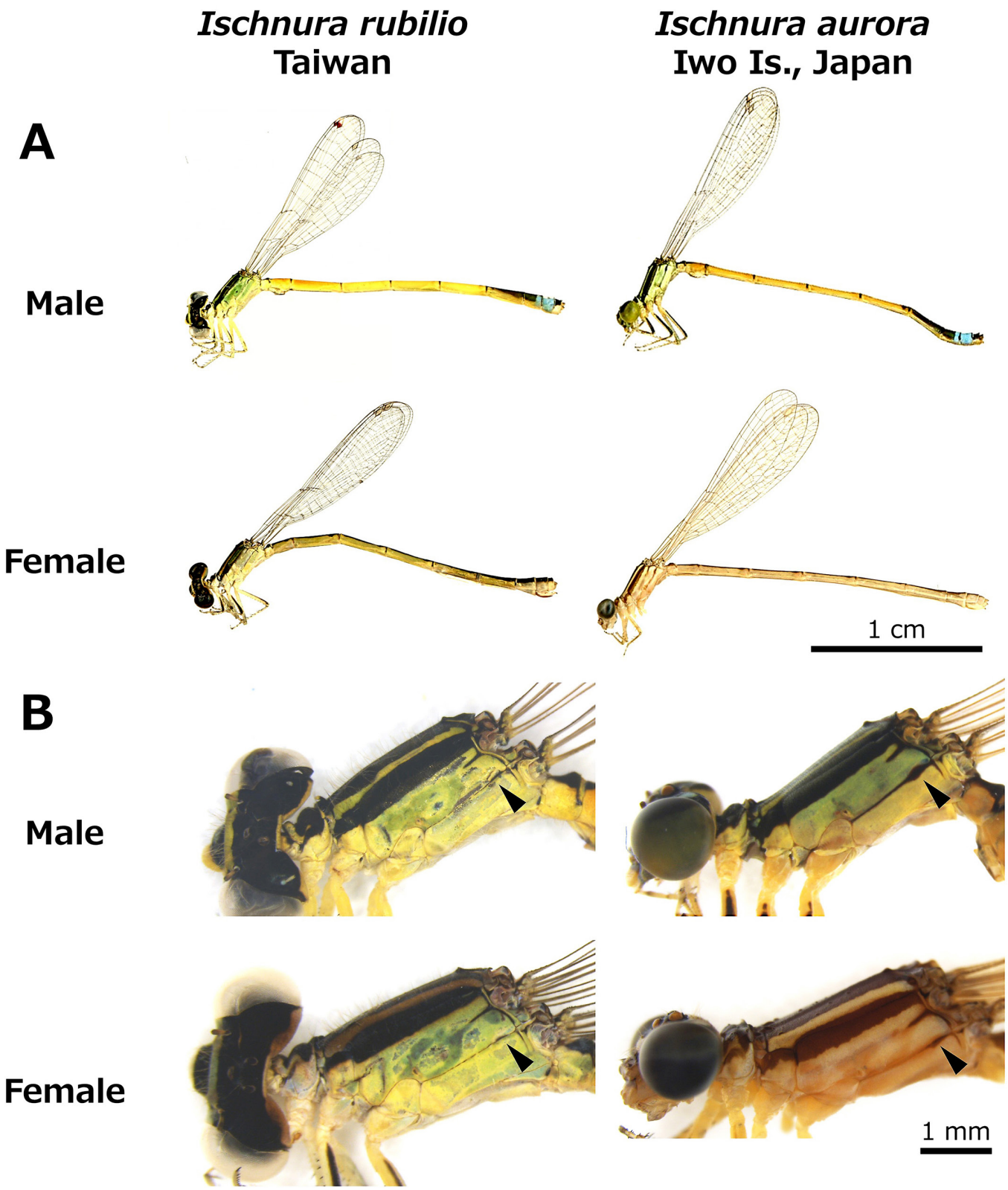

Figure 3. Lateral views of *Ischnura rubilio* from Taiwan (left) and *I. aurora* from Japan (right): A – whole body, B – head and thorax. Arrowheads point out the black stripe on the 2nd lateral suture.

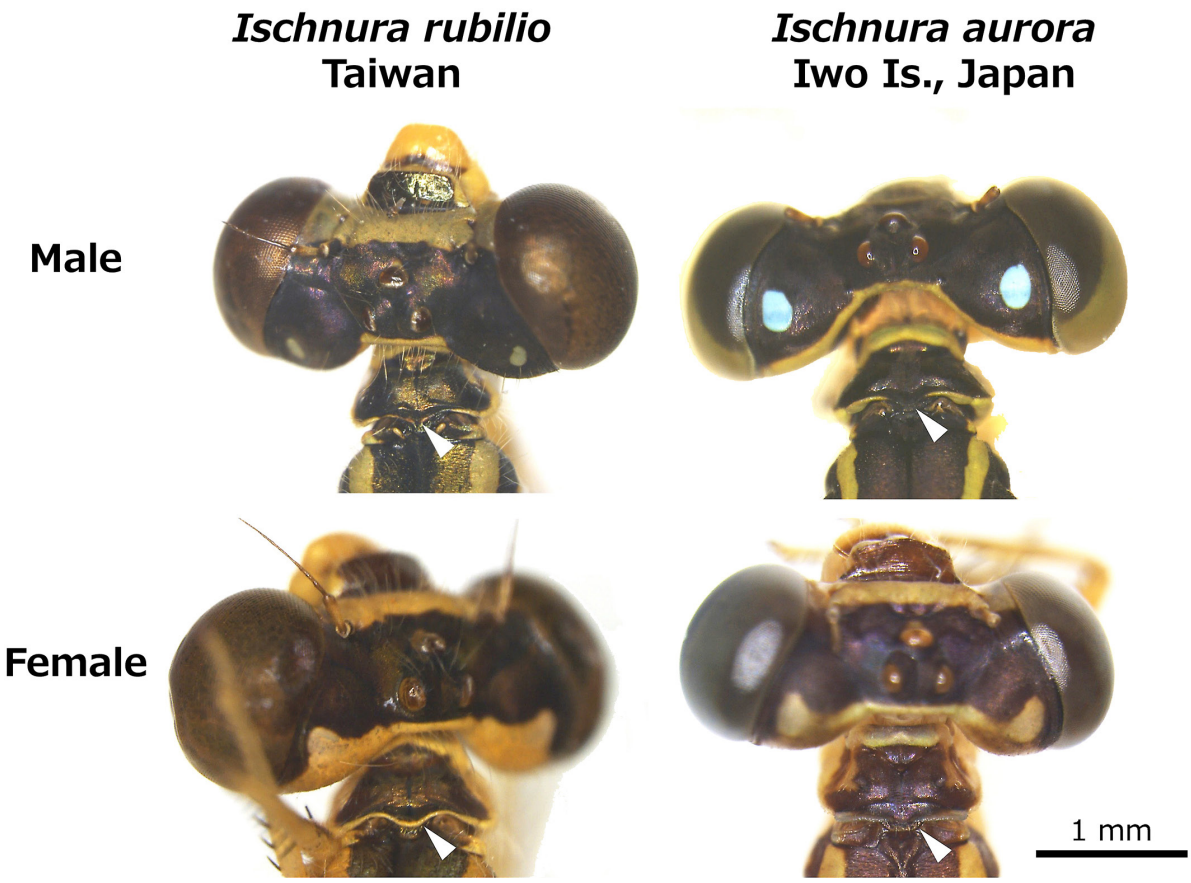

Figure 4. Dorsal views of the head and prothorax of *Ischnura rubilio* from Taiwan and *I. aurora* from Japan. Arrowheads point out the posterior lobe of the prothorax.

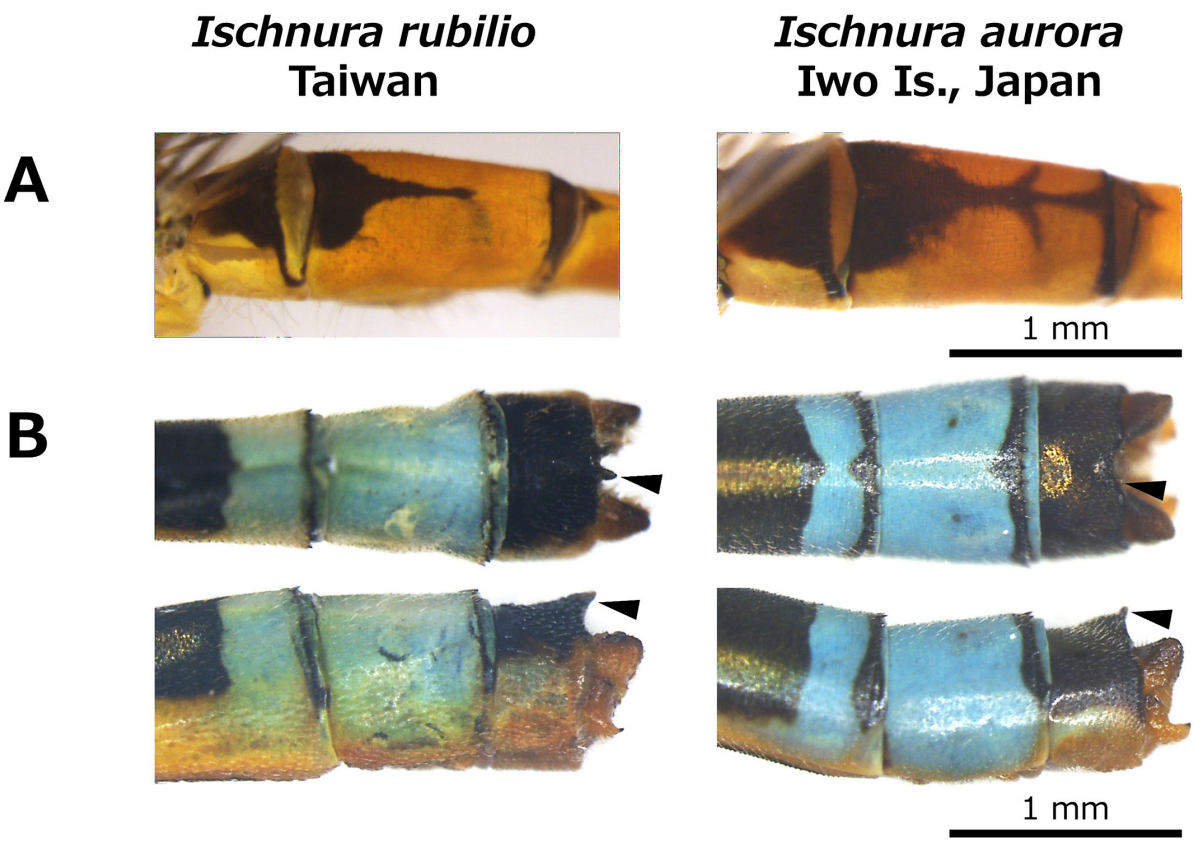

Figure 5. A – Oblique dorsal views of the first and second abdominal segments of *Ischnura rubilio* from Taiwan and *I. aurora* from Japan. B – Dorsal and lateral views of the abdominal appendages of *I. rubilio* from Taiwan and *I. aurora* from Japan. Arrowheads point out the dorsal tubercle.

Historical distribution and current situation of *I. rubilio* in Taiwan

Before the beginning of the current century, *I. rubilio* was distributed in lowlands across Taiwan, including Taipei, New Taipei, Hsinchu, Nantou, Chiayi, and Pingtung (Table 1; Fig. 6). After 2000, this species could only be found in some abandoned or little-disturbed water rice terraces near the coast of New Taipei City. However, the abandoned water terraces were subsequently destroyed by typhoons that left them dry, or farmers changed their planting strategies, which in turn changed plant composition and microhabitats after 2015. From 2015 to the present, the only confirmed remaining habitat is at Jianshanhu in New Taipei City (Fig. 6).

The only remaining locality Jianshanhu, situated in the northernmost part of Taiwan, contains both fallow and farmed water rice terraces that preserve the water in the fields throughout the year (Figs 7A, B). Farmers who leave terraces fallow but filled with water may receive a government allowance in Taiwan, as a means of mitigating rice overproduction and preserving the unique habitat formed by watered fallow terraces. To prevent terrestrialization, farmers in the area use low-

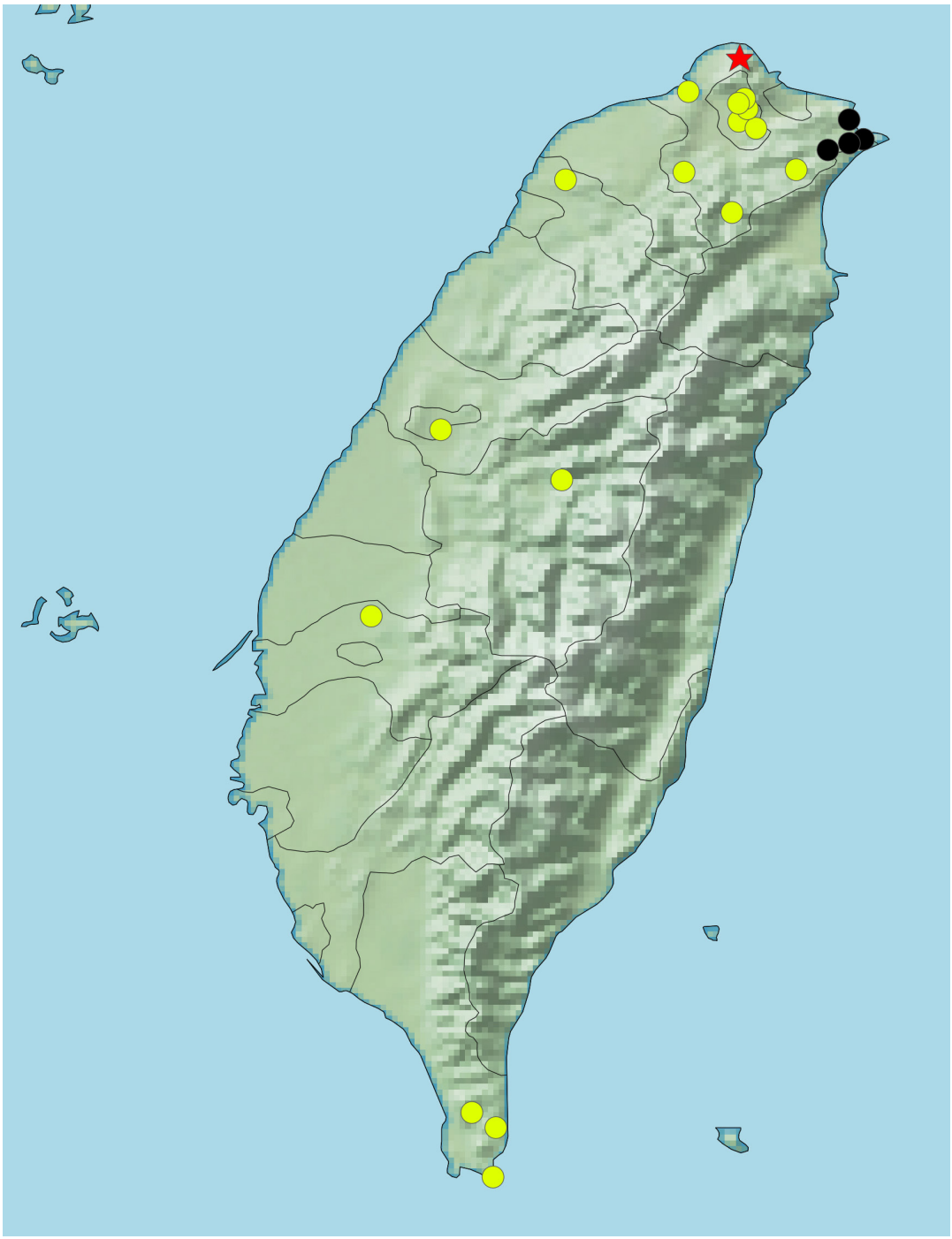

Figure 6. Distribution map of *Ischnura rubilio* Selys, 1876 in Taiwan. The yellow circles represent records from before 2000, the black circles those from between 2000 and 2015; the red star represents the remaining locality.

dose herbicides in the fields to control the growth of long grass, with a low frequency of application (Bo-Wen Shiue, pers. comms.). Most individuals of *I. rubilio* were observed in fallow terraces with lush, submerged vegetation and sparse emergent vegetation (Fig. 7B). Adults were often seen perching on *Eriocaulon* spp. (Figs 7B, C, D), and their larvae inhabit the same type of fields (Fig. 7E). Additionally, rare aquatic plants, *Deinostema adenocaulon* (Fig. 7F) and *Dopatrium junceum* (Fig. 7G), listed in the Red List of Taiwan Plant as VU and NT, respectively, have also been found in the water terraces in the same region (Editorial Committee of the Red List of Taiwan Plant, 2017).

Discussion

Based on our molecular phylogeny and morphological comparisons, the "*I. aurora*-like" damselfly in Taiwan evidently represents *I. rubilio*. In our molecular phylogenetic analysis, *I. rubilio* from Taiwan did not genetically differ from other populations, however, it is unique in terms of the size of the abdominal blue spots of its males compared to those from other regions.

Currently, with only one reliable locality remaining, *I. rubilio* is critically endangered in Taiwan and urgently requires conservation measures. For promoting conservation of the species, the example of *Ceriagrion melanurum* Selys, 1876 in a comparative situation is informative. The distribution of *C. melanurum* is currently restricted to the water terraces at Gongliao in northern Taiwan, where aquatic plant species that have drastically declined and become very rare in other areas of Taiwan still exist (Hsieh et al., 2015), and *C. melanurum* has been enabled to maintain its population (Environmental Ethics Foundation of Taiwan, 2013; Fang & Shiue, 2014).

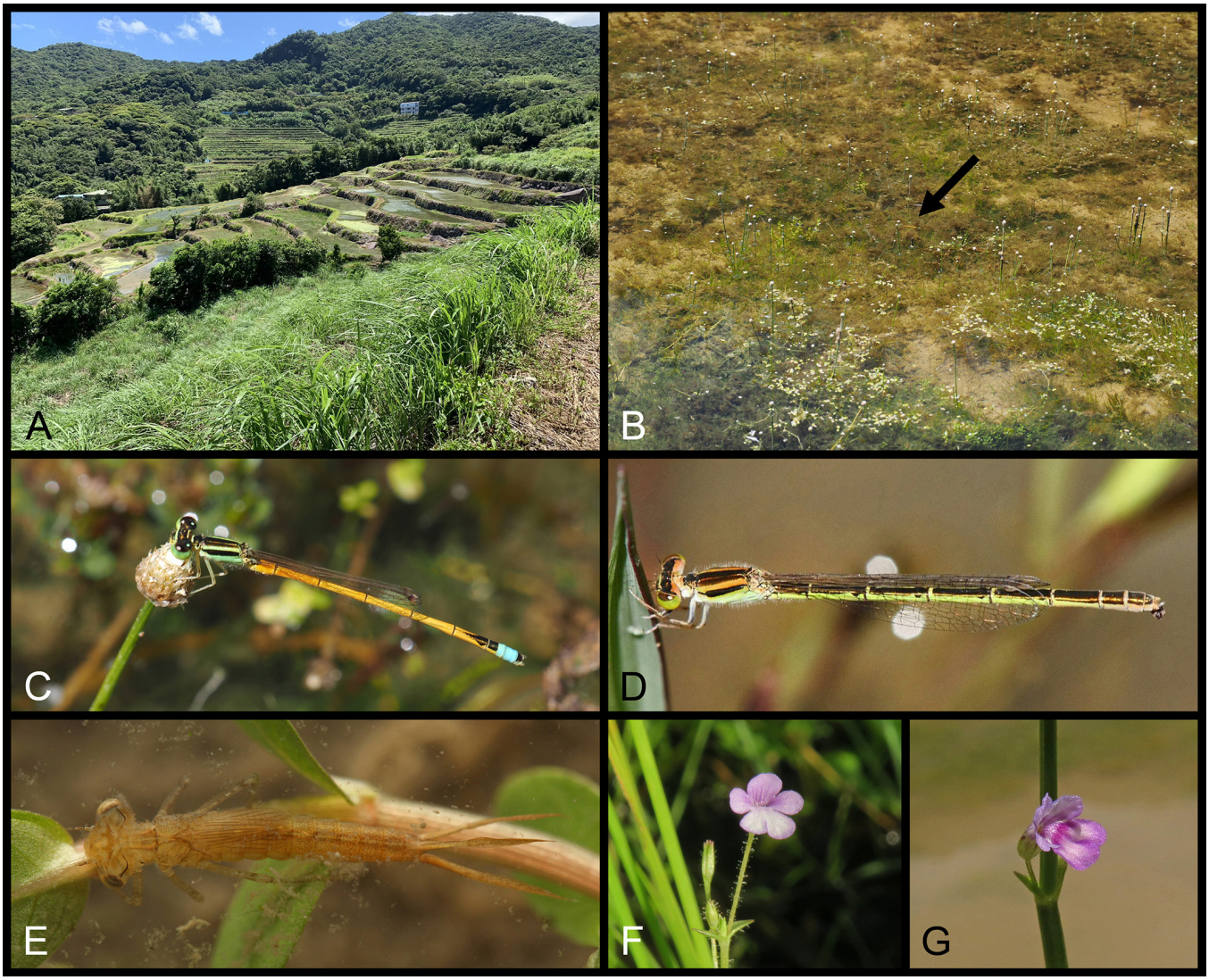

Figure 7. A–B Water terraces at Jianshanhu (尖山湖) in New Taipei City, which represents the only remaining locality of *Ischnura rubilio* in Taiwan; C – adult male of *I. rubilio*; D – adult immature female of *I. rubilio* (Photo by I.-L. Lee); E – larva of *I. rubilio* (Photo by C.-H. Ma); F – co-occurring rare aquatic plant *Deinostema adenocaulon* (Photo by Y.-C. Chou); G – co-occurring rare aquatic plant *Dopatrium junceum* (Photo by Y.-C. Chou).

The concept of "neorefugia" has been defined as constituting artificial habitats that replace lost primary habitats (Nekola, 1999). The water terraces in Gongliao fit this concept as they are the only remaining habitat for multiple animal and plant species (Hsieh et al., 2015), including *C. melanurum*. In collaboration with the Taiwanese government, the Environmental Ethics Foundation of Taiwan has been working with the local farmers in Gongliao who practice eco-friendly farming to protect not only *C. melanurum* but also other rare species and their habitats. The conservation practice of *C. melanurum* is a collaboration between local farmers, NGOs, and the government in the suburban hills to protect the landscape, which is a typical example of the Satoyama Initiative.

Recommendations for conservation of Taiwanese *I. rubilio*

It is conceivable that the water terraces at Jianshanhu could become neorefugia for *I. rubilio*, similar to the situation for *C. melanurum* at Gongliao, and *I. rubilio* makes for a good candidate as a flagship species for Satoyama landscape conservation in the Jianshanhu region. This conservation effort would not only protect the damselfly, but also the rare aquatic plants that have similar microhabitat requirements. Building consensus towards conservation practices in the Satoyama landscape (Uchiyama et al., 2022) is therefore crucial. It should be noted that the low-intensity farming, i.e., environmentally friendly management, may contribute significantly to maintaining the populations of surviving organisms and local biodiversity in general (Donald, 2004; Marja et al., 2014; Natuhara, 2022; Perfecto & Vandermeer, 2008). The low-frequency and low-dose application of herbicides in the fields of Jianshanhu accidentally helps to maintain the required microhabitats of *I. rubilio*. However, the effects on, and response of the species to, herbicides are still unclear, and it is uncertain whether the species will retreat to the neighboring fields when herbicides are sprayed. Here, we recommend for the conservation of Taiwanese *I. rubilio* that: 1) the current government allowances for fallow water terraces be maintained in order to avoid sudden changes to habitats; and 2) low-frequency and low-dose herbicide application be continued; it is important to understand the interactions between *I. rubilio*, fallow or farmed water rice terraces, and herbicide use, which may benefit habitat creation in neighboring areas; that 3) *I. rubilio* be listed as a protected species in Taiwanto prevent the conversion of potential habitats; and 4) collaboration with local farmers and landlords through NGOs be encouraged, as has been done to conserve *C. melanurum* at Gongliao (Environmental Ethics Foundation of Taiwan, 2013). NGOs offer a beneficial mechanism to pass on knowledge on species and conservation to farmers, which has proven to benefit species conservation (Silva-Andrade et al., 2016). NGOs can also help with local environmental education and promoting conservation consciousness for future generations. The government can provide additional allowances to farmers when *I. rubilio* appears in their fields and/or when farmers engage in wildlife-friendly farming in their fields and in this manner support conservation efforts.

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