

## Cryptic species of the gobioid paedomorphic genus *Schindleria* from Palau, Western Pacific Ocean

Takeshi Kon · Tetsuo Yoshino · Mutsumi Nishida

Received: 7 May 2010/Revised: 16 July 2010/Accepted: 20 July 2010/Published online: 24 August 2010  
© The Ichthyological Society of Japan 2010

**Abstract** The extremely paedomorphic fish *Schindleria* (Gobioidei, Schindleriidae) is widely distributed in the Indo-Pacific Ocean, inhabiting coral reef lagoons. At least 21 cryptic species (*Schindleria* spp. 1–21) have been discovered around the Ryukyu and Ogasawara Islands. As a first step in extending the survey areas to the entire Indo-Pacific Ocean, the present study collected and examined *Schindleria* in Palau, located approximately 2,200 km from the Ryukyu Islands, for any cryptic diversity. Molecular phylogenetic analysis using partial mitochondrial 16S rRNA sequences of 71 specimens revealed four new cryptic species of *Schindleria* spp. 22–25 (Palau 1–4) in Palau.

**Keywords** *Schindleria* · Palau · Mitochondrial DNA · Cryptic species

### Introduction

Paedomorphic taxa are highly likely to include cryptic species because of morphological uniformity resulting from their reduced body form. In fact, extraordinary cryptic diversity has been revealed by molecular phylogenetic analysis in the gobioid paedomorphic genus *Schindleria*

(Kon et al. 2007). The extremely paedomorphic fish *Schindleria* (Gobioidei, Schindleriidae), believed to include some of the smallest (8 mm long), lightest (2 mg), and youngest reproducing (23 days, 10 generations per year) vertebrates (Bruun 1940; Kon and Yoshino 2002; Watson and Walker 2004), is widely distributed in the Indo-Pacific Ocean, inhabiting coral reef lagoons (Bruun 1940; Jones and Kumaran 1964; Ozawa and Matsui 1979; Harris and Cyrus 1996; Young and Chiu 2000; Landaeta et al. 2002; Watson and Walker 2004; Abu El-Regal and Kon 2008). They are all characterized by a reduced larva-like form; for example, a slender, translucent and scaleless body.

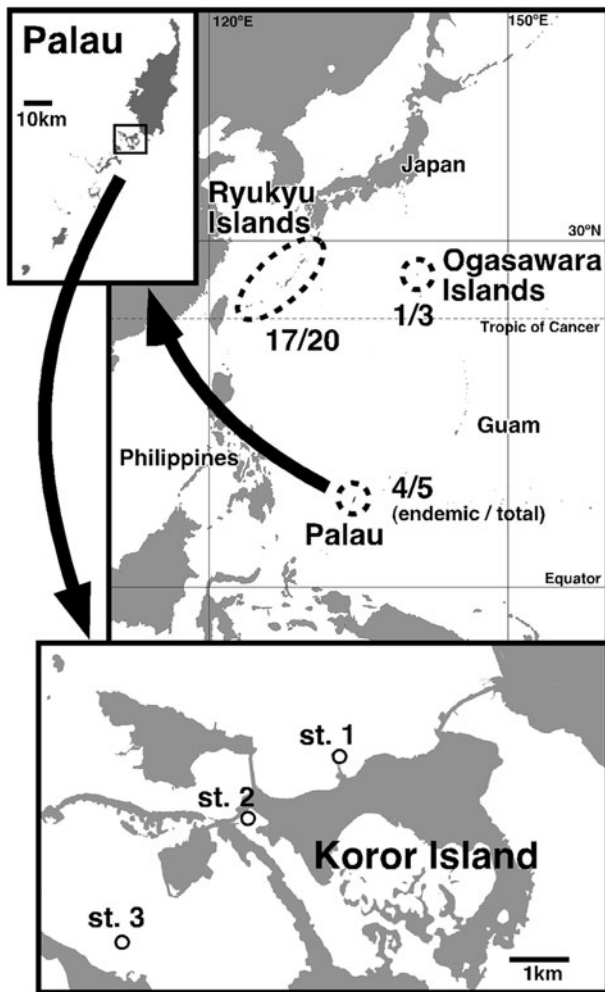
Only three nominal species, *Schindleria praematura*, *Schindleria pietschmanni* and *Schindleria brevipinguis*, have been recognized, but at least 21 cryptic species (*Schindleria* spp. 1–21) were discovered by Kon et al. (2007) in the Ryukyu and Ogasawara Islands, Japan, based on molecular phylogenetic analysis using partial mitochondrial 16S rRNA sequences. It is, however, uncertain whether many cryptic species are distributed in Japan are widespread or restricted to Japanese waters. As a first step towards extending our scope to the entire Indo-Pacific Ocean, the present study aimed to examine genetic characteristics of *Schindleria* collected in Palau, Micronesia, which is located approximately 2,200 km from the Ryukyu Islands.

### Materials and methods

**Sampling and observation.** Examples of *Schindleria* spp. were collected at night by hand-net and lamp at three localities (st. 1–3) around Koror Island in Palau, western Pacific Ocean (7°N, 134°E) in February 2009 (Fig. 1). All specimens were fixed and preserved in 99.5%

T. Kon (✉) · M. Nishida  
Department of Marine Bioscience,  
Atmosphere and Ocean Research Institute,  
The University of Tokyo, 5-1-5 Kashiwanoha,  
Kashiwa, Chiba 277-8564, Japan  
e-mail: kon@aori.u-tokyo.ac.jp

T. Yoshino  
Department of Marine Sciences, Faculty of Science,  
University of the Ryukyus, 1 Senbaru, Nishihara,  
Okinawa 903-0213, Japan



**Fig. 1** Locations of collection sites (st. 1–3) of *Schindleria* spp. in Palau. The three open circles indicate the sampling sites. Numbers beside the three regions (Ryukyu, Ogasawara, and Palau) indicate the number of detected species (endemic/total) according to molecular phylogenetic analysis

ethanol immediately after collection. Dorsal and anal fin-ray numbers of all specimens were counted. The external morphology of urogenital papillae (UP), which showed wide variation, was observed and photographed under a light microscope.

**DNA preparation, amplification, sequencing, and phylogenetic analysis.** All molecular protocols for DNA extraction and the amplification and sequencing of the partial mitochondrial 16S ribosomal RNA gene used in this study were identical to those of Kon et al. (2007). Newly determined sequences were deposited in DDBJ/EMBL/GenBank (accession nos. AB557889–557925, 557927–557930). The DNA sequences [41 newly determined sequences added to the data set of Kon et al. (2007); a total of 211 sequences] were aligned using CLUSTAL X, version 1.81 (Thompson et al. 1997) and then inspected and corrected by eye.

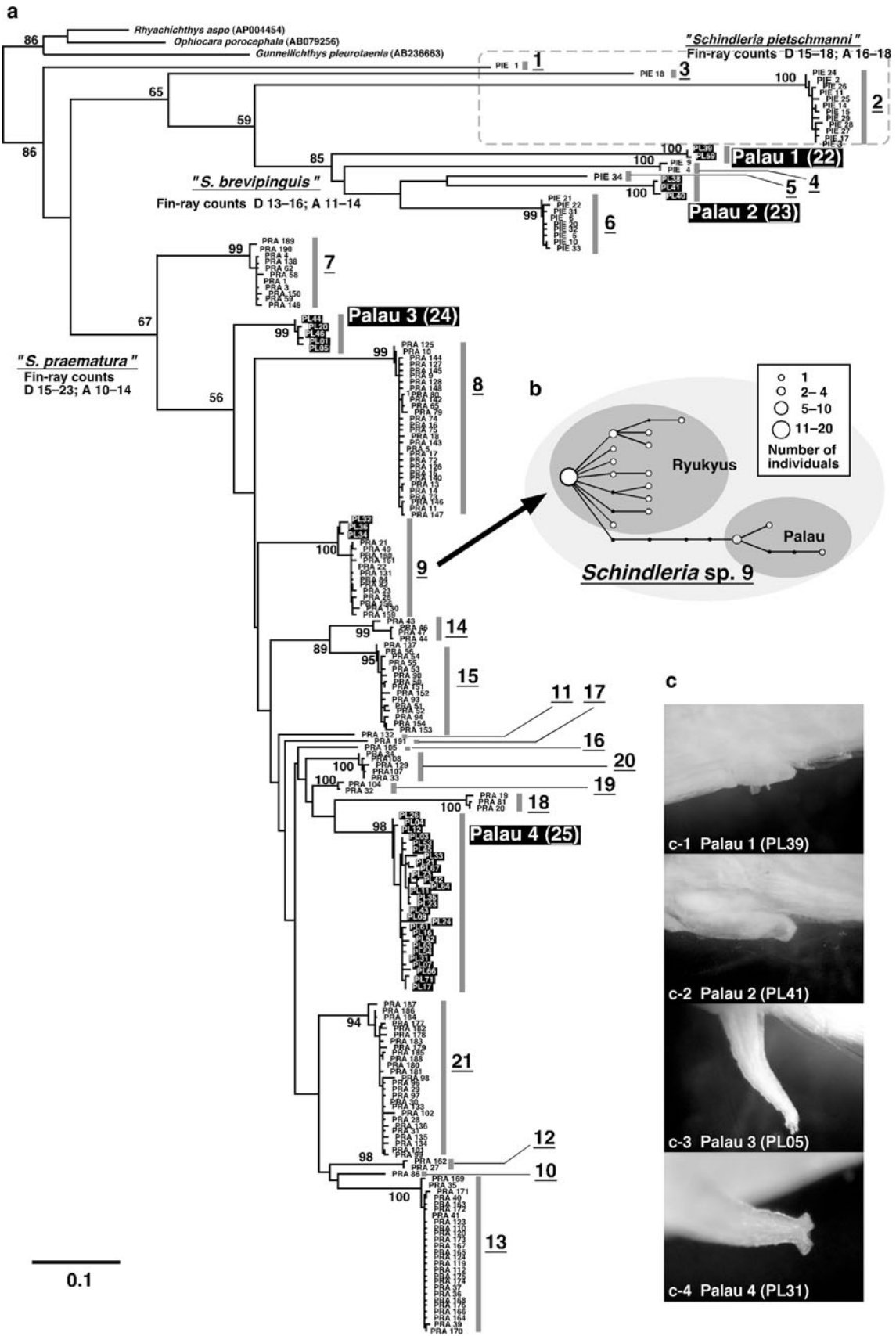
Maximum likelihood (ML) analysis for phylogenetic reconstruction was applied using RAxML (Stamatakis 2006). The GTR +  $\Gamma$  model (Yang 1994) (the model recommended by the author of the program) was used, and rapid bootstrap (BS) analysis was conducted with 300 replications (–f a option). This model performs BS analysis using GTRCAT, which is the GTR approximation with optimization of individual per-site substitution rates, and classifies those individual rates into a certain number of rate categories. After implementing the BS analysis, the program uses every fifth BS tree as a starting point to search for the ML tree using the GTR +  $\Gamma$  model of sequence evolution, and saves the top ten best-scoring ML trees (fast ML searches). Finally, RAxML calculates better likelihood scores (slow ML searches) for these ten trees and places BS probabilities on the best-scoring ML tree. *Rhyacichthys aspro* (AP004454, Rhyacichthyidae) (Miya et al. 2003), *Ophiocara porocephala* (AB079256, Eleotridae) (Mukai et al. 2002), and *Gunnellichthys pleurotaenia* (AB236663, Microdesmidae) (Kon et al. 2007) were used as outgroups to root all ML trees (Akihito et al. 2000; Thacker 2003, 2009).

**Population structure analyses for *Schindleria* sp. 9.** A statistical parsimony network (SPN) of *Schindleria* sp. 9, which was the only species comprising Ryukyuan and Palauan individuals, was constructed using TCS 1.18 (Clement et al. 2000), which employs the method of Templeton et al. (1992).

## Results and discussion

**New Palauan cryptic species.** DNA segments of 557 base pairs (bp) of the 16S rRNA gene were successfully sequenced and aligned for 71 Palauan specimens. A total of 250 nucleotide positions (44.9% in 557 bp) varied in all *Schindleria*; these variations defined 41 new haplotypes (Fig. 2a: PL). No haplotypes were shared by Palau and Japan.

The ML tree based on the 16S rRNA partial sequences showed five clades for Palauan *Schindleria* (Fig. 2a). Four of the five clades (clades Palau 1–4) are endemic to Palau. These four clades were composed of Palauan haplotypes only, and were supported by high bootstrap values ranging from 98 to 100% (Fig. 2a). Each of the four clades differed from the other clades by divergence values of uncorrected *p* distance of 0.09–0.26, which were similar to the gobioid congeneric inter-species values (0.01–0.19) (Kon et al. 2007). Moreover, the inter-clade values of the four clades were much greater than the intra-clade values (0.002–0.010). Therefore, these extant clades seem to have undergone relatively long and independent lineage-sorting processes. According to Kon et al.'s (2007) standards of DNA-based taxonomy for the genus *Schindleria*, we



◀ **Fig. 2** Phylogenetic position and morphology of urogenital papillae of Palauan *Schindleria*. **a** Maximum likelihood tree of *Schindleria* species based on the mitochondrial DNA 16S ribosomal RNA gene (557 bp). Numbers beside major internal branches indicate bootstrap probabilities based on 300 replicates. Haplotypes in white on a dark background indicate Palauan. Numerals (1–21) indicate species numbers. **b** Statistical parsimony network of *Schindleria* sp. 9. Each circle represents one haplotype. Size of each circle is proportional to the haplotype frequency. Small black dots represent putative (missing) haplotypes that were not observed. **c** Photographs of urogenital papillae in male specimens of new Palauan cryptic species; *c-1* Palau 1 (specimen code PL39), 11.4 mm in standard length (SL), morphological type of urogenital papillae is K (thick and short, lacking small projection); *c-2* Palau 2 (PL41), 11.3 mm SL, type D (rounded in anterior half, and hook-like in posterior half); *c-3* Palau 3 (PL05), 17.6 mm SL, type L (tip is shallowly bifurcate, each with a small lobe and lacking a small projection); *c-4* Palau 4 (PL31), 16.2 mm SL, type I (tip is bifurcate, each with a small lobe and lacking a small projection)

conclude that the differentiation of the 16S rRNA gene indicated that at least four cryptic species of *Schindleria* exist in Palau (*Schindleria* spp. 22–25).

The other clade of the above five for Palauan *Schindleria* corresponded to Kon et al.'s (2007) *Schindleria* sp. 9, which was distributed in the Ryukyus (Fig. 2a). In addition to finding the four cryptic species, the present analysis of mitochondrial 16S rRNA sequences also detected population differentiation in *Schindleria* sp. 9. A haplotype network based on the sequences showed a “dumbbell-like phylogeny” consisting of two subclades that differed by a minimum of five mutations, indicating that the Palauan population genetically diverged from the Ryukyuan population (Fig. 2b). Mukai et al. (2009) suggested that the Kuroshio Current, flowing from Palau and Guam's waters to the northeast following the Philippines and Ryukyu Islands, is a powerful transporter for fish larvae in this region, based on the genetic homogeneity of the gobiid *Bathygobius cocosensis* among populations in Guam, the Ryukyus, and Japan, suggesting that gene flow over these regions must have occurred recently and is ongoing. Additionally, a haplotype (ATP6/8 genes) shared by the pomacentrid *Dascyllus aruanus* populations of

Guam and the Ryukyus also suggested gene flow between the regions (McCafferty et al. 2002). However, the haplotype network of *Schindleria* sp. 9 suggested that gene flow between Palau (close to Guam) and the Ryukyus must have been limited recently. This difference in genetic structure may be due to differences in their dispersal ability (e.g., planktonic larval duration, homing of larval fish), although currently there is no ecological evidence to support this.

**Morphology of the Palauan *Schindleria*.** Fin ray counts and types of external morphology of UP in the five species collected from Palau (*Schindleria* spp. 9, 22–25) are summarized in Table 1. Two of the four Palauan species, *Schindleria* sp. 22 (Palau 1) and 24 (Palau 3), have unique UP [new types K (Fig. 2c-1) and L (Fig. 2c-3), respectively] among *Schindleria* species; therefore, these two species can be distinguished morphologically from the others. The other two Palauan species, *Schindleria* spp. 23 (Palau 2) and 25 (Palau 4), were indistinguishable morphologically from some *Schindleria* species (Table 1; Fig. 2c-3, c-4) (see Kon et al. 2007 for detailed data). *Schindleria* sp. 9 (Clade 9) comprising Palauan (PL32, 34, and 36) and Ryukyuan haplotypes was somewhat variable morphologically; the external morphology of the UP of the Palauan population (type I) differed from that of the Ryukyuan population (types A and H; Kon et al. 2007) (Table 1).

**Level of endemism of *Schindleria*.** Our molecular analysis indicated that a single species (*Schindleria* sp. 9) among a total of 25 species was distributed in both Japanese (Ryukyu and Ogasawara) and Palauan regions. Four of the five species collected are endemic to Palau. In Japanese waters, 17 of 20 and 1 of 3 species are endemic to Ryukyu and Ogasawara, respectively (Fig. 1) (see Kon et al. 2007 for detailed data). These results suggest that the level of endemism is high in *Schindleria*. Therefore, considering the global distribution of *Schindleria* (Eastern Africa to Easter Island), a high potential for undiscovered endemic species is conceivable for *Schindleria*. Worldwide high-density sampling will be necessary to clarify the hidden diversity of *Schindleria*.

**Table 1** Fin-ray counts and morphological types of the urogenital papillae of *Schindleria* species collected in Palau

Species (clades)	Fin-ray counts		Type of urogenital papillae <sup>a</sup>	Number of specimens <sup>b</sup>			
	Dorsal	Anal		St. 1	St. 2	St. 3	Total
22 (Palau 1)	14–16	11–13	K (new)	0	0	2	2
23 (Palau 2)	14–16	13–14	D	0	0	3	3
24 (Palau 3)	18–22	11	L (new)	0	0	7	7
25 (Palau 4)	16–22	10–13	I	1	2	52	55
9	18–20	11–13	I	0	0	4	4

<sup>a</sup> See Kon et al. (2007) and Fig. 2c

<sup>b</sup> See Fig. 1 for details of sampling sites

**Acknowledgments** We thank J. Sakaue (Marine Southern Laboratories) and T. Mori (Kitasato University) for their cooperation during the collection of specimens in Palau. This study was supported in part by Grants-in-Aid for Scientific Research to T. K. (20570084) and M. N. (19207007) from the Japan Society for the Promotion of Science.

## References

- Abu El-Regal M, Kon T (2008) First record of the paedomorphic fish *Schindleria* (Gobioidei, Schindleriidae) from the Red Sea. *J Fish Biol* 72:1539–1543
- Akihito, Iwata A, Kobayashi T, Ikeo K, Imanishi T, Ono H, Umehara Y, Hamamatsu C, Sugiyama K, Ikeda Y, Sakamoto K, Fumihito A, Ohno S, Gojobori T (2000) Evolutionary aspects of gobioid fishes based upon a phylogenetic analysis of mitochondrial cytochrome *b* genes. *Gene* 259:5–15
- Bruun AF (1940) A study of a collection of the fish *Schindleria* from South Pacific waters. *Dana Report* 21:1–12
- Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Mol Ecol* 9:1657–1659
- Harris SA, Cyrus DP (1996) Occurrence of Schindler's fishes, genus *Schindleria* (Teleostei: Gobioidei), at a small reef in the mouth of the Kosi Estuary, Kwazulu-Natal: a first record for southern Africa. *Bull Mar Sci* 59:228–234
- Jones S, Kumaran M (1964) On the fishes of the genus *Schindleria* Giltey from the Indian Ocean. *J Mar Biol Assoc India* 6:257–264
- Kon T, Yoshino T (2002) Extremely early maturity found in Okinawan gobioid fishes. *Ichthyol Res* 49:224–228
- Kon T, Yoshino T, Mukai T, Nishida M (2007) DNA sequences identify numerous cryptic species of the vertebrate: a lesson from the gobioid fish *Schindleria*. *Mol Phylogenet Evol* 44:53–62
- Landaeta MF, Veas R, Castro LR (2002) First record of the paedomorphic goby *Schindleria praematura*, Easter Island, South Pacific. *J Fish Biol* 61:289–292
- McCafferty S, Bermingham E, Quenouille B, Planes S, Hoelzer G, Asoh K (2002) Historical biogeography and molecular systematics of the Indo-Pacific genus *Dascyllus* (Teleostei: Pomacentridae). *Mol Ecol* 11:1377–1392
- Miya M, Takeshima H, Endo H, Ishiguro NB, Inoue JG, Mukai T, Satoh TP, Yamaguchi M, Kawaguchi A, Mabuchi K, Shirai SM, Nishida M (2003) Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Mol Phylogenet Evol* 26:121–138
- Mukai T, Yonezawa T, Nishida M (2002) First record of an eleotrid fish *Ophiocara porocephala* from two islands, Tanegashima and Amami-oshima, Japan. *IOP Diving News* 13:2–4
- Mukai T, Nakamura S, Nishida M (2009) Genetic population structure of a reef goby, *Bathygobius cocosensis*, in the northwestern Pacific. *Ichthyol Res* 56:380–387
- Ozawa T, Matsui S (1979) First record of the schindlerid fish, *Schindleria praematura*, from southern Japan and the South China Sea. *Jpn J Ichthyol* 25:283–285
- Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690
- Templeton AR, Crandall KA, Sing CF (1992) A cladistic-analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA-sequence data. 3. Cladogram estimation. *Genetics* 132:619–633
- Thacker CE (2003) Molecular phylogeny of the gobioid fishes (Teleostei: Perciformes: Gobioidei). *Mol Phylogenet Evol* 26:354–368
- Thacker CE (2009) Phylogeny of Gobioidei and placement within Acanthomorpha, with a new classification and investigation of diversification and character evolution. *Copeia* 2009:93–104
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL\_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882
- Watson W, Walker HJ (2004) The world's smallest vertebrate, *Schindleria brevipinguis*, a new paedomorphic species in the family Schindleriidae (Perciformes: Gobioidei). *Rec Austral Mus* 56:139–142
- Yang Z (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J Mol Evol* 39:306–314
- Young S-S, Chiu T-S (2000) New records of a paedomorphic fish, *Schindleria praematura* (Pisces: Schindleriidae), from waters of Taiwan. *Acta Zool Taiwan* 11:127–133