Vol. 627: 195–200, 2019
https://doi.org/10.3354/meps13082

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published September 26

NOTE

Sex chromosomes and sex-specific molecular markers in Indo-Pacific combtooth blennies (Blenniidae, *Istiblennius*)

Peter J. Hundt[1,2,*], Emma B. Liddle[3], Stuart V. Nielsen[4,5], Brendan J. Pinto[4,6], Tony Gamble[2,4,6]

[1]Department of Fisheries, Wildlife and Conservation Biology, University of Minnesota, St. Paul, MN 55108, USA
[2]Bell Museum of Natural History, University of Minnesota, Saint Paul, MN 55113, USA
[3]Department of Biology, Grinnell College, Grinnell, IA 50112, USA
[4]Department of Biological Sciences, Marquette University, Milwaukee, WI 53233, USA
[5]Florida Museum of Natural History, Division of Herpetology, Gainesville, FL 32611, USA
[6]Milwaukee Public Museum, Milwaukee, WI 53233, USA

ABSTRACT: Sex-specific genetic markers, markers found in one sex but not the other, can be used to recognize a species' sex chromosome system in cases where traditional karyotyping fails. Species with male-specific markers have an XX/XY system, while species with female-specific markers have a ZZ/ZW system. Here, we used data from restriction site-associated DNA sequencing, or RADseq, from 2 species of combtooth blenny, *Istiblennius lineatus* and *I. steindachneri*, to identify an excess of female-specific genetic markers, which points to ZZ/ZW sex chromosomes. We used PCR to validate the sex-specificity of one of these female-specific restriction site-associated DNA markers in an additional *Istiblennius* species, *I. edentulus*. We observed no sex-specific PCR amplification in 2 other *Istiblennius* species and 2 *Blenniella* species. This *Istiblennius* ZZ/ZW system, when combined with cytogenetic data from the literature illustrating an XX/XY system in *Parablennius*, establishes a transition between sex chromosome systems within Blenniidae.

KEY WORDS: Blenny · Fish · RADseq · Endocrine disrupting chemicals · Biomonitoring

1. INTRODUCTION

Vertebrate sex-determining systems can broadly be divided into environmental and genotypic systems with numerous transitions between them across the tree of life (Bachtrog et al. 2014). There are abundant transitions between sex-determining systems not only among major vertebrate clades but also within clades, and transitions even occur between species within genera (Bachtrog et al. 2014). Teleost fish are particularly diverse and exhibit repeated evolution of sex chromosomes, including XX/XY and ZZ/ZW systems, environmental sex determination, and sequential hermaphroditism (Devlin & Nagahama 2002). The clade Ovalentaria (>4800 species) exemplifies this diversity and comprises some of the most well-studied fish lineages regarding sex determination, including Cichlids (Cichlidae), medaka (Adrianichthyidae), guppies and swordtails (Poeciliidae), and neotropical silversides (Atherinopsidae) (Mank et al. 2006). As well studied as these fishes are, the sex-determining systems of most other ova-

*Corresponding author: hundt002@umn.edu

lentarians remain poorly known (Mank et al. 2006). Here, we identify sex chromosomes in several combtooth blenny species (herein referred to as blennies) and highlight the diversity of blenny sex-determining mechanisms.

Blennies (~404 species) are small (most <100 mm total length), cryptobenthic fishes and are among the most ubiquitous species in tropical marine habitats (Fricke et al. 2019). Like many fishes, blennies possess diverse morphological and behavioral traits associated with reproduction, including striking sexual dimorphism. These sexually dimorphic characters can be useful for identifying a blenny's phenotypic sex, but in some species the distinction becomes more complex due to alternate reproductive strategies. Several blenny species have at least 2 behaviorally distinct male phenotypes: nesters and sneakers. Nesters are the largest males that guard a nest and care for eggs, while sneakers are small males without adult male secondary sexual traits that dash into nests guarded by larger males to release sperm (Oliveira et al. 2001). Despite the prevalence of phenotypic and behavioral information available about blenny reproduction and sexual dimorphism, relatively little is known about sex determination and sex chromosomes. Only 1 species, the tentacled blenny *Parablennius tentacularis*, has a described sex chromosome system, XX/XY (Carbone et al. 1987). Although other blenny species have been karyotyped, no other sex chromosomes have been identified (Carbone et al. 1987, Caputo et al. 2001).

Restriction site-associated DNA sequencing, or RADseq, can be used to develop sex-specific genetic markers and identify a species' sex chromosome system, particularly when cytogenetically distinct sex chromosomes are absent (Gamble 2016). This involves generating RADseq data from multiple males and females and identifying sex-specific restriction site-associated DNA (RAD) markers, that is, RAD markers found in one sex but not the other (Gamble & Zarkower 2014). These sex-specific RAD markers are presumed to be on sex-limited parts of the genome, such as the Y or W chromosomes. Species with an excess of male-specific markers have an XX/XY system, whereas species with an excess of female-specific markers have a ZZ/ZW system (Gamble et al. 2015). Here, we used RADseq data to identify female-specific RAD markers, and thus a ZZ/ZW sex chromosome system, in 3 blenny species (*Istiblennius*). These *Istiblennius* ZZ/ZW systems, when considered in light of karyotypic data from the literature, demonstrate a transition in sex chromosome systems within Blenniidae.

2. MATERIALS AND METHODS

We extracted genomic DNA using the Qiagen® DNeasy Blood and Tissue extraction kit from 7 adult male and 6 adult female *Istiblennius lineatus* and 4 adult male and 4 adult female *I. steindachneri* (Table S1 in the Supplement at www.int-res.com/articles/suppl/m627p195_supp.pdf). RADseq libraries were constructed following previously published protocols (Etter et al. 2011, Gamble et al. 2015). Libraries were pooled and sequenced using paired-end 125 bp reads on an Illumina® HiSeq2500.

Raw Illumina reads were demultiplexed, trimmed, and filtered using the process_radtags function in Stacks (v1.48) (Catchen et al. 2011). We used RADtools 1.2.4 (Baxter et al. 2011) to generate candidate alleles for each individual and candidate loci across all individuals using previously described parameters (Gamble et al. 2015). A custom python script was used to identify putative sex-specific markers from the RADtools output (Gamble et al. 2015). We examined the RADseq data using this bioinformatic pipeline twice. First, we analyzed just 7 male and 6 female *I. lineatus*. Second, we analyzed all of the *I. lineatus* and *I. steindachneri* samples together. The small number of *I. steindachneri* samples prevented us from analyzing that species on its own. We assembled forward and reverse reads from the confirmed sex-specific RAD markers and designed PCR primers to validate the sex specificity of these RAD markers using Geneious R10 (Kearse et al. 2012). We used BLAST (Altschul et al. 1990) to identify the possible identity of the female-specific RAD markers.

3. RESULTS

Analyses of *Istiblennius lineatus* RADseq data recovered 79 692 RAD markers with 2 or fewer alleles including 3 confirmed male-specific RAD markers and 16 confirmed female-specific RAD markers. Confirmed sex-specific markers are a subset of the total number of sex-specific RAD markers that excludes RAD markers occurring in the raw reads files of the opposite sex, which are likely false positives. Analyses of RADseq data from 11 male and 10 female samples, combining *I. lineatus* and *I. steindachneri* samples, recovered 84 333 RAD markers with 2 or fewer alleles including zero male-specific markers and 4 confirmed female-specific RAD markers. These 4 RAD markers were a subset of the larger set of *I. lineatus* female-specific RAD markers.

From this pool of 4 confirmed female-specific RAD markers, we identified 2, Blen1 and Blen4, which consistently amplified in a sex-specific manner in *I. lineatus* samples (Fig. 1). Primer sequences (5′ to 3′) were Blen1-F1 AAC ACT TGT CAG TAG AGG CAG G and Blen1-R1 CCT TGT GTT GTT TTT CAA GCC G (PCR fragment size = 478 bp), and Blen4-F1 CCC GTT TTG TCT TTC GGT CAA A and Blen4-R1 ACC TTT AGC GAG TTG TTG CTC (PCR fragment size = 544 bp). Female-specific amplification of Blen4 in 4 male and 4 female *I. steindachneri* samples prompted us to assess its utility in males and females of 3 additional *Istiblennius* species, *I. dussumieri*, *I. edentulus*, and *I. zebra*, and 2 *Blenniella* species, *Blenniella leopardus* and *B. bilitonensis* (Table S1 in the Supplement). We observed no sex-specific amplification in any of these species (results not shown) except for *I. edentulus*, which produced 2 bands in female samples and a single band in male samples, consistent with a ZZ/ZW sex chromosome system. The top band that amplifies in both sexes corresponds to a Z allele, while the lower, female-specific band corresponds to the W allele (Fig. 1D). Blen1 primers did not amplify in a sex-specific manner in other species.

BLAST of the female-specific RAD markers against teleosts in the RefSeq genome database resulted in multiple hits for 3 markers, including Blen1 and Blen4, and no hits in the remaining marker. These multiple BLAST hits, including many matches within a single genome, are a signature of repetitive elements, which are highly enriched on the sex-limited sex chromosomes, the Y and W (Charlesworth et al. 1994). Indeed, further examination of these sequences using RepeatMasker (Smit et al. 2014) found either LINE elements or simple repeats in all 4 female-specific RAD markers.

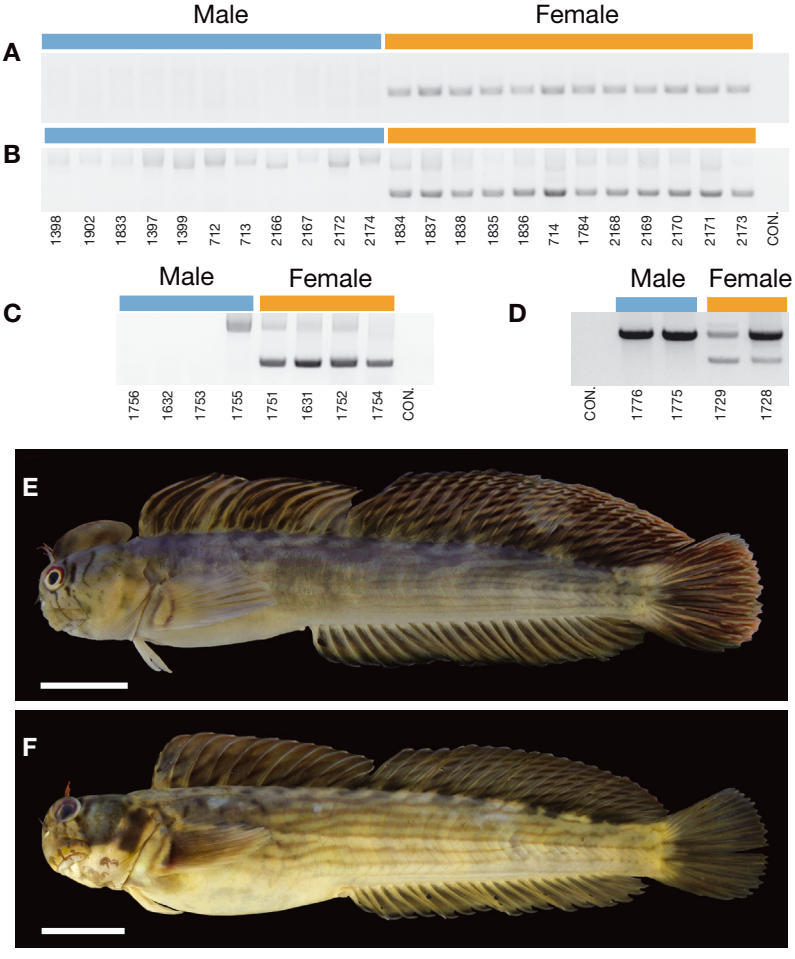

Fig. 1. Female-specific PCR amplification of (A) restriction site-associated DNA (RAD) marker Blen1 in 11 male and 12 female *Istiblennius lineatus* (sample IDs below [B]); (B) RAD marker Blen4 in 11 male and 12 female *I. lineatus*; (C) RAD marker Blen4 in 4 male and 4 female *I. steindachneri*; and (D) RAD marker Blen4 in 2 male and 2 female *I. edentulus*. (E) Adult male (KAUM 16407) and (F) female (KAUM 16329) *I. lineatus* collected in Terengganu, Malaysia, illustrating sexual dimorphism. Photos by Hiroyuki Motomura. KAUM: Kagoshima University Museum. Scale bars = 1 cm. Gel lanes labelled CON. are negative controls for each set of PCR reactions

4. DISCUSSION

An excess of female-specific RAD markers and PCR amplification of a subset of these markers only in females indicate a ZZ/ZW sex chromosome system. Female-specific amplification of the Blen4 marker in 3 species, the sister species *Istiblennius lineatus* and *I. steindachneri* along with *I. edentulus*, is evidence of a shared sex chromosome system (Fig. 1). Lack of sex-specific amplification does not automatically mean that *I. dussumieri* or *I. zebra* lacks the ZZ/ZW sex chromosome system found in its congeners but rather that there is simply a failure to PCR amplify that marker. The sex-limited sex chromosomes, the W and Y chromosomes, are evolutionarily dynamic, and thus PCR of Y- and W-specific markers may not work, even among closely related species (Gamble & Zarkower 2014). Given that *I. dussumieri* is nested within the

clade of ZZ/ZW *Istiblennius* species (Fig. 2) (Hundt et al. 2014), it is likely that *I. dussumieri* shares the same sex chromosome system as its congeners (although we cannot confirm this with our data). Alternately, *I. dussumieri* could represent a transition away from the shared ZZ/ZW system of the other *Istiblennius* species, which would not be unusual given the dynamic sex chromosome transitions seen in related Ovalentarians (Fig. 2A) (Devlin & Nagahama 2002, Mank et al. 2006). The phylogenetic position of *I. zebra* is currently unknown, but the argument above for *I. dussumieri* is relevant here too. Similarly, failure to amplify the sex-specific RAD marker in the 2 *Blenniella* species should be interpreted cautiously, and further research is needed to identify sex chromosomes in these species.

ZZ/ZW sex chromosomes in *Istiblennius*, along with the XX/XY sex chromosomes in *Parablennius tentacularis* (Carbone et al. 1987, Caputo et al. 2001), indicate at least 1 transition between sex chromosome systems in blennies (Fig. 2B). Using RADseq to identify sex chromosomes in additional blenny species would help identify the precise number of transitions and where the phylogeny transitions have occurred. Furthermore, a robust phylogenetic hypothesis of sex chromosome evolution for blennies would allow for in-depth investigation of how sex chromosomes may influence the evolution of the diverse sexually dimorphic traits and alternative reproductive strategies in the clade.

Due to their abundance and limited home ranges, blennies and other coastal benthic fishes have been recommended as ideal sentinel species for monitoring endocrine-disrupting pollutants and other contaminants in coastal marine environments (Lima et al. 2008, Barhoumi et al. 2012). Although many blenny species have been proposed to be included in monitoring programs, no PCR-based assay exists to identify genotypic sex in any blenny species. Non-invasive methods for determining phenotypic sex exist for some blennies, e.g. papilla morphology or ano-genital distance (Ferreira et al. 2010). However,

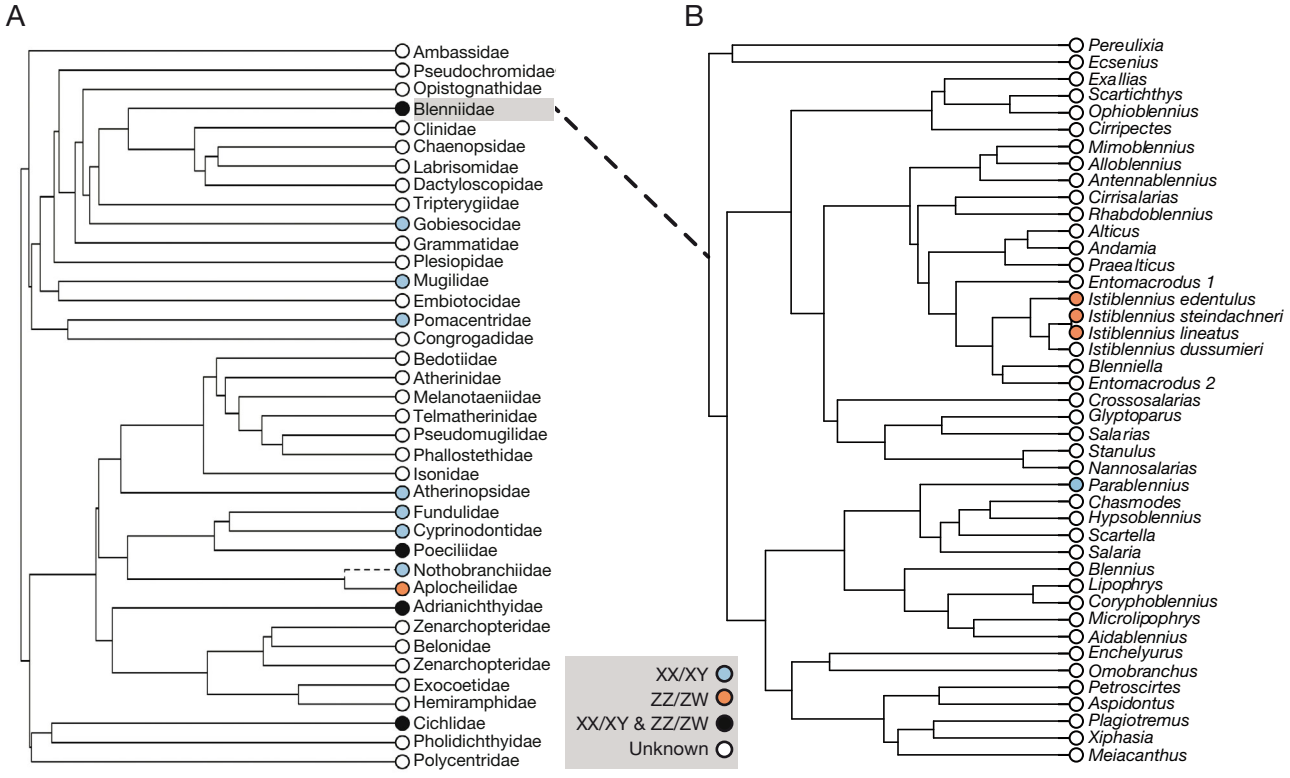

Fig. 2. (A) Phylogenetic position of combtooth blennies (Blenniidae [in gray box]) within clade Ovalentaria. Sex chromosome data from Devlin & Nagahama (2002), Takehana et al. (2007), Schultheis et al. (2009), Dor et al. (2016), and this study. Phylogeny follows Betancur-R et al. (2013), except for placement of Nothobranchiidae (dashed branch, which was not included in Betancur-R et al. 2013), based on phylogeny from Pohl et al. (2015). (B) Relationships among blenny genera with additional resolution within *Istiblennius*, following Hundt & Simons (2018). Circles at tips indicate sex chromosome system within each clade: XX/XY (blue), ZZ/ZW (orange), both XX/XY and ZZ/ZW (black), and unknown (white). Relative branch lengths between the 2 trees are independent

these traits become subjective outside of breeding season and, of course, may not be concordant with genotypic sex when endocrine-disrupting chemicals are present. Thus, sex-specific genetic markers that can identify genotypic sex are an important tool in any biomonitoring program. The Blen4 PCR primers described here can identify genotypic sex in at least 3 *Istiblennius* species and may be useful for investigating the impact of endocrine-disrupting chemicals in tropical marine environments. These *Istiblennius* species are among the most common and abundant fishes in nearshore rocky habitats in the Indo-Pacific and may serve as sentinels for detection of chemical pollutants throughout their large distributions. Developing PCR-based assays for genotypic sex in additional blenny species would both facilitate their use in biomonitoring and enhance our understanding of blenny sex chromosome evolution.

Data archive. NCBI BioProject: PRJNA553554; NCBI SRA accessions: SAMN12238311 to SAMN12238331.

Acknowledgements. We thank H. Motomura for photos; A. Simons (Bell Museum of Natural History, University of Minnesota) for access to specimens; and J. Egan, H. Motomura, M. Meguro, G. Ogihara, T. Yoshida, H. Nishiyama, Y. Nakamura, H. Endo, K. Takahashi, K. Hirota, N. Nakayama, A. Murase, Dr. G. Cooke, C. Morgans, C. H. Kuo, and U. S. Chew for field work help. Research was conducted under University of Minnesota animal care and use protocol 1005A82295. Funding was from Marquette University startup funds to T.G.; East Asia and Pacific Summer Institute (EAPSI)-Japan Society for Promotion of Science and National Science Foundation (NSF OISE 1210051), EAPSI-National Science Council of Taiwan (NSF OISE 1414788), and Lerner Gray Memorial Fund for Marine Research (American Museum of Natural History) to P.H.; and NSF DBI1659595 to A. Abbott and E. Blumenthal. The authors declare no conflict of interests.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bachtrog D, Mank JE, Peichel CL, Kirkpatrick M and others (2014) Sex determination: why so many ways of doing it? PLOS Biol 12:e1001899

Barhoumi S, Messaoudi I, Gagné F, Kerkeni A (2012) Spatial and seasonal variability of some biomarkers in *Salaria basilisca* (Pisces: Blennidae): implication for biomonitoring in Tunisian coasts. Ecol Indic 14:222–228

Baxter SW, Davey JW, Johnston JS, Shelton AM, Heckel DG, Jiggins CD, Blaxter ML (2011) Linkage mapping and comparative genomics using next-generation RAD sequencing of a non-model organism. PLOS ONE 6: e19315

Betancur-R R, Broughton RE, Wiley EO, Carpenter K and others (2013) The tree of life and a new classification of bony fishes. PLOS Curr 2015:5

Caputo V, Machella N, Nisi-Cerioni P, Olmo E (2001) Cytogenetics of nine species of Mediterranean blennies and additional evidence for an unusual multiple sex-chromosome system in *Parablennius tentacularis* (Perciformes, Blenniidae). Chromosome Res 9:3–12

Carbone P, Vitturi R, Catalano E, Macaluso M (1987) Chromosome sex determination and Y autosome fusion in *Blennius tentacularis* Brunnich, 1765 (Pisces, Blennidae). J Fish Biol 31:597–602

Catchen JM, Amores A, Hohenlohe P, Cresko W, Postlethwait JH (2011) Stacks: building and genotyping loci de novo from short-read sequences. G3 (Bethesda) 1: 171–182

Charlesworth B, Sniegowski P, Stephan W (1994) The evolutionary dynamics of repetitive DNA in eukaryotes. Nature 371:215–220

Devlin RH, Nagahama Y (2002) Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208: 191–364

Dor L, Shirak A, Rosenfeld H, Ashkenazi I and others (2016) Identification of the sex determining region in flathead grey mullet (*Mugil cephalus*). Anim Genet 47:698–707

Etter PD, Bassham S, Hohenlohe PA, Johnson EA, Cresko WA (2011) SNP discovery and genotyping for evolutionary genetics using RAD sequencing. In: Orgogozo V, Rockman MV (eds) Molecular methods for evolutionary genetics. Springer, New York, NY, p 157–178

Ferreira F, Santos MM, Reis Henriques MM, Vieira NM, Monteiro NA (2010) Sexing blennies using genital papilla morphology or ano genital distance. J Fish Biol 77: 1432–1438

Fricke R, Eschmeyer WN, Fong JD (2019) Eschmeyer's catalog of fishes: species by family/subfamily. https://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp (accessed 9 February 2019)

Gamble T (2016) Using RAD-seq to recognize sex-specific markers and sex chromosome systems. Mol Ecol 25: 2114–2116

Gamble T, Zarkower D (2014) Identification of sex-specific molecular markers using restriction site associated DNA sequencing. Mol Ecol Resour 14:902–913

Gamble T, Coryell J, Ezaz T, Lynch J, Scantlebury DP, Zarkower D (2015) Restriction site-associated DNA sequencing (RAD-seq) reveals an extraordinary number of transitions among gecko sex-determining mechanisms. Mol Biol Evol 32:1296–1309

Hundt PJ, Simons AM (2018) Extreme dentition does not prevent diet and tooth diversification within combtooth blennies (Ovalentaria: Blenniidae). Evolution 72: 930–943

Hundt PJ, Iglésias SP, Hoey AS, Simons AM (2014) A multilocus molecular phylogeny of combtooth blennies (Percomorpha: Blennioidei: Blenniidae): multiple invasions of intertidal habitats. Mol Phylogenet Evol 70:47–56

Kearse M, Moir R, Wilson A, Stones-Havas S and others (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Lima D, Santos MM, Ferreira AM, Micaelo C, Reis-Henriques MA (2008) The use of the shanny *Lipophrys pholis* for pollution monitoring: a new sentinel species for the northwestern European marine ecosystems. Environ Int 34:94–101

Mank JE, Promislow DEL, Avise JC (2006) Evolution of alternative sex-determining mechanisms in teleost fishes. Biol J Linn Soc 87:83–93

Oliveira RF, Canario AVM, Grober MS (2001) Male sexual polymorphism, alternative reproductive tactics, and androgens in combtooth blennies (Pisces: Blenniidae). Horm Behav 40:266–275

Pohl M, Milvertz FC, Meyer A, Vences M (2015) Multigene phylogeny of cyprinodontiform fishes suggests continental radiations and a rogue taxon position of *Pantanodon*. Vertebr Zool 65:37–44

Schultheis C, Böhne A, Schartl M, Volff JN, Galiana-Arnoux D (2009) Sex determination diversity and sex chromosome evolution in poeciliid fish. Sex Dev 3:68–77

Smit AFA, Hubley R, Green P (2014) RepeatMasker. Open-4.0. 2013–2015. www.repeatmasker.org

Takehana Y, Naruse K, Hamaguchi S, Sakaizumi M (2007) Evolution of ZZ/ZW and XX/XY sex-determination systems in the closely related medaka species, *Oryzias hubbsi* and *O. dancena*. Chromosoma 116:463–470

Editorial responsibility: Philippe Borsa, Montpellier, France

Submitted: March 11, 2019; Accepted: July 21, 2019
Proofs received from author(s): September 6, 2019