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TECHNICAL NOTE

Sex identification PCR–RFLP assay tested in eight species of *Sebastes* rockfish

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Abstract

The phenotypic identification of sex in *Sebastes* rockfish is difficult and often impractical from a management perspective, and the genetic basis of sex determination in the genus is currently uncertain. We tested a previously developed sex identification polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) assay in eight species of *Sebastes* rockfish. Results indicated that the association of this restriction site with sex is species-dependent.

Keywords Fisheries management · RAD-seq · Sex determination · Sex chromosome

Many species of *Sebastes* rockfish occur in the Northeast Pacific Ocean (Love et al. 2002), supporting large commercial and recreational fisheries that are jointly managed by federal and state governments (PFMC 2016). Rockfish are highly speciose with over 110 identified species, which exhibit significant diversity in ecology, morphology and coloration (Love et al. 2002; Hyde and Vetter 2007). Many species are of conservation concern; for example in the Puget Sound, the US National Marine Fisheries Service lists the yelloweye rockfish as threatened and the bocaccio rockfish as endangered, with both species managed under the US Endangered Species Act (Andrews et al. 2018). Sex is an important factor for the effective management of many fishes (Hanson et al. 2008). In multiple rockfish species, large, long-lived females exhibit the highest fecundity and are essential for the successful maintenance of populations (Berkeley 2006; Berkeley et al. 2004a, b; Hixon et al. 2014). Thus, a key reference point for fisheries, the spawning stock biomass, is a metric of the number of females in the population. Without real world information to base this number on, there is uncertainty, and ultimately the sustainability of the fisheries management is hindered. Sex identification is often impractical for rockfish using current methods. In many species the shape of urogenital papillae can distinguish males and females (Love et al. 2002; Worton and Rosenkranz 2003; Andrews et al. 2018), but this trait is only present in reproductively mature adults, and differences can be small and difficult to detect, leading to misidentification (Love et al. 2002; Worton and Rosenkranz 2003). The examination of gonads is a more reliable method of sex identification, but lethal dissection is undesirable during a fisheries survey and mutilation of product is unattractive for fish intended for market. Furthermore, these results are confounded by the fact that gonads are typically underdeveloped in juveniles, despite sex ratio estimates for upcoming cohorts being essential for fisheries management. In short, the fisheries management community needs a relatively cheap and non-invasive technic for sexing rockfish.

The genetic basis of sex determination is uncertain in *Sebastes* and only a few studies have addressed the topic. Despite most fish having homomorphic sex chromosomes (Charlesworth and Mank 2010), two cytogenetic studies by Anderson (1979) and Ida et al. (1982), respectively identified exclusively male and female heteromorphic sex chromosomes in two separate sets of eight *Sebastes* species (none of which are sampled in this study). These cytogenetic results

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suggest that there may be both male and female heterogametic sex determination systems in *Sebastes*. More recently, a double digest restriction site-associated DNA sequencing (ddRAD-seq) study (Fowler and Buonaccorsi 2016) investigated the sex-determination system of two closely related, sister species of rockfish: gopher rockfish *Sebastes carnatus* and black-and-yellow rockfish *S. chrysomelas*. This study identified male-specific loci in both species and concluded that these species most likely have a male heterogametic sex determination system with a recently evolved Y sex chromosome (Fowler and Buonaccorsi 2016). Another RAD-seq study on deacon rockfish *S. diaconus* identified 92 loci that readily distinguished males and females (Vaux et al. 2019). These loci were shared between the sexes and it is possible that the observed variation reflected either pseudoautosomal regions, or differential selection between males and females, which could be elevated if sex chromosomes have not yet evolved in the species (Vaux et al. 2019).

A genetic assay could simplify sex identification for *Sebastes* rockfishes. Using their ddRAD-seq data, Fowler and Buonaccorsi (2016) identified a male-specific *MluCI* restriction site (AATT) within gopher rockfish for one locus (Sch.182255-210250). Oligonucleotide primers (Sch.182255-210250.F:5′-GGGTAGCACATTTCTGCAACC-3′ and Sch.182255-210250.R: 5′-ATCAAGTACACTCTTCTCTGTTGTGCT-3′) were developed to flank this restriction site. A polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) assay using these primers and the *MluCI* enzyme was tested, and males and females of both gopher and black-and-yellow rockfish were successfully distinguished.

We tested this assay in 8 species of *Sebastes* rockfish (Table 1), including gopher, black-and-yellow, black *S. melanops*, blue *S. mystinus*, canary *S. pinniger*, deacon *S. diaconus*, widow *S. entomelas*, and yellowtail rockfish *S. flavidus*. Five of the sampled species: blue, deacon, black, widow and yellowtail rockfish, are closely related, in addition to the gopher and black-and-yellow rockfish being sister within a separate clade (Hyde and Vetter 2007). A total of 32 individuals were analyzed, with two males and two females for each species, except for black rockfish that had one male, one female and two individuals of unknown sex (Table 1). Samples of gopher and black-and-yellow rockfish collected off southern California and used in Fowler and Buonaccorsi (2016) were provided by the authors. All remaining samples were collected off central Oregon by the Oregon Department of Fish and Wildlife. Sample details for all individuals are available online (Dryad: https://doi.org/10.5061/dryad.1jwstqjqx). Sex was determined by the examination of gonads via lethal dissection.

The PCR–RFLP method developed by Fowler and Buonaccorsi (2016) was followed, with products run for 40 min on a 2% agarose electrophoresis gel at 50 V with a 1500 bp ladder using SYBR Safe (ThermoFisher) DNA stain. Amplified PCR products were isolated and purified using gel excision (QIAquick Gel Excision Kit, Qiagen). The Big Dye

Table 1 Sampling and results for the eight tested *Sebastes* rockfish species

Common name	*Sebastes* species	N	*MluCI* digest success	Male restriction site	Female restriction site
Gopher rockfish	*S. carnatus* (Jordan & Gilbert, 1880)	4 (2 M, 2 F)	Males-only	AATT	
Black-and-yellow rockfish	*S. chrysomelas* (Jordan & Gilbert, 1881)	4 (2 M, 2 F)	Both sexes	AATT/AGTT	AATT/AGTT
Black rockfish	*S. melanops* Girard, 1856	4 (2 M, 2 F)	Both sexes	AATT	AATT
Blue rockfish	*S. mystinus* (Jordan & Gilbert, 1881)	4 (1 M, 1 F, 2 U)	Neither sex	AGTT	AGTT
Canary rockfish	*S. pinniger* (Gill, 1864)	4 (2 M, 2 F)	Neither sex	AGTT	AGTT
Deacon rockfish	*S. diaconus* Frable et al. 2015)	4 (2 M, 2 F)	Both sexes	AATT/AGTT	AATT/AGTT
Widow rockfish	*S. entomlas* (Jordan & Gilbert, 1880)	4 (2 M, 2 F)	Neither sex	AGTT	AGTT
Yellowtail rockfish	*S. flavidus* (Ayres, 1862)	4 (2 M, 2 F)	Both sexes	AATT	AATT

Columns list the number of individuals (N) per species, sex (male = M, female = F, unidentified = U), success of the *MluCI* digest, and predicted bases for the *MluCI* restriction site

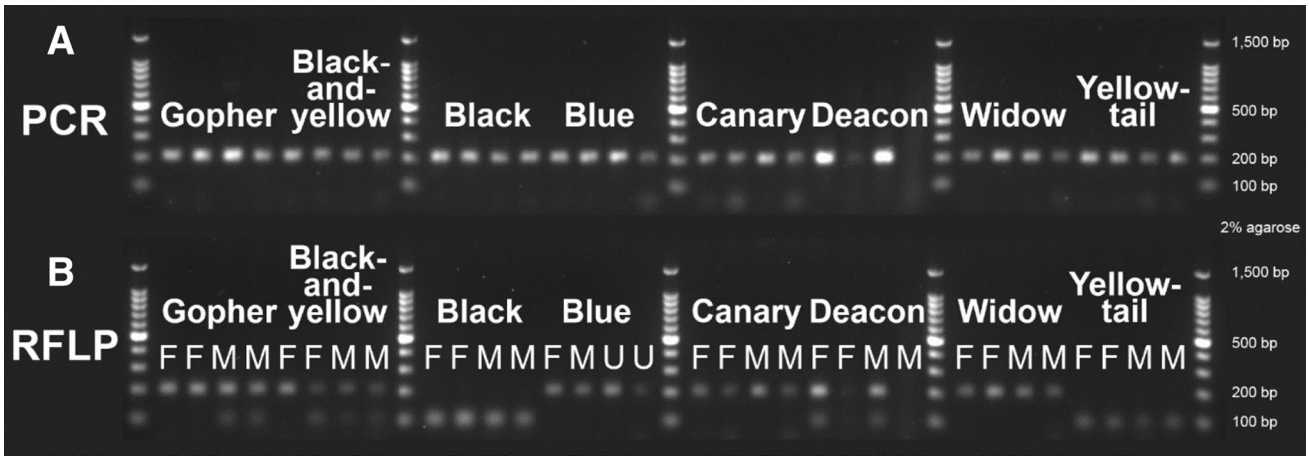

Fig. 1 Results for the PCR–RFLP assay shown using gel electrophoresis photos. **a** Results for the PCR amplification of the Sch.182255-210250 locus in eight species of rockfish. The presence of 220 bp bands indicates the successful amplification of the target locus for all samples except one male deacon rockfish. **b** Results for the *MluCI* digest that followed the PCR amplification. Samples exhibited an undigested 220 bp PCR product band (e.g. Blue rockfish), or a fully digested ~ 110 bp band (e.g. black rockfish), or two bands with the undigested and digested products (e.g. gopher rockfish). The digest is only sex-diagnostic in the gopher rockfish, where females have one undigested band and males have two bands, most likely because the *MluCI* restriction site only occurs on the putative Y-chromosome copy of the target locus

Terminator v3.1 cycle sequencing kit (ThermoFisher) was used to sequence 5 µl of purified PCR product in 20 uL reactions with 250 nM primer with a thermal cycler protocol of 25 cycles of 96 °C for 10-s, 50 °C for 5-s, and 60 °C for 4-min. Products were Sanger sequenced on an Applied Biosystems 3730XL analyzer.

Target PCR products were amplified in all species and samples, except for one male deacon rockfish (Fig. 1a). Sequence data for gopher rockfish were available from the previous study (Fowler and Buonaccorsi 2016), and at least one male and one female of the seven remaining species were successfully sequenced (see Dryad supplement). The assay successfully identified the sex of gopher rockfish (Fig. 1b), matching results reported in Fowler and Buonaccorsi (2016). However, in black-and-yellow rockfish, one female was digested in addition to the two males (Fig. 1b). Sequence data for black-and-yellow rockfish indicated that the *MluCI* restriction site was absent in one female but heterozygous in one male (Table 1). The *MluCI* restriction site appeared species-dependent rather than sex-dependent for the 6 remaining rockfish species. Sequencing results indicated the restriction site was present in both sexes for black, deacon and yellowtail rockfish, and absent in both sexes for blue, canary and widow rockfish (Table 1; Fig. 1b). All individuals of deacon rockfish were heterozygous for the restriction site (Table 1).

The successful amplification of the target region (220 bp) in all samples using the same pair of primers indicates that the locus is conserved among *Sebastes* rockfishes. However, the *MluCI* restriction site within this locus does not appear to be male-specific outside of gopher rockfish. The PCR–RFLP assay is therefore unlikely to be a reliable sex identification tool and thus inform management efforts in other *Sebastes* species. Since the result of the PCR–RFLP assay depends only upon the occurrence of the *MluCI* restriction site within the target Sch.182255-210250 locus, it is not possible to use these results to speculate about the genetic basis of sex determination in the seven remaining rockfish species. The target locus may in fact occur consistently on nascent sex chromosomes in all species, but the occurrence of the *MluCI* restriction site within this locus appears to be sorted randomly among lineages. Improved reference genomes for rockfish are required to determine whether this locus consistently occurs within the same genomic position across species, and further research would need to test whether the same region is used for sex determination in all taxa. The presence of the restriction site did not appear to follow a phylogenetic pattern (Hyde and Vetter 2007); the sister species (Frable et al. 2015) of blue and deacon rockfish exhibited absence and presence, respectively, and there was no consistent pattern among the five closely related species (blue, deacon, black, widow and yellowtail) as well (Table 1).

Other male-specific loci, such as Locus 170028, identified by Fowler and Buonaccorsi (2016) in gopher and black-and-yellow rockfish may provide a consistent sex identification assay among *Sebastes* species, as it was identified in two species. Likewise, some of the 92 loci that were highly differentiated between male and female deacon rockfish may be useful for sex identification in that species (Vaux et al. 2019). However, it is important to remember that RAD-seq methods

are unlikely to identify reliable sex-linked loci across multiple species. The restriction sites used for RAD-seq typically have an uneven distribution through a genome dependent on linkage disequilibrium, and the retained loci generally represent a small fraction of a genome (Lowry et al. 2017). The sequenced loci will therefore differ depending on the genome size and chromosome arrangements among species, and large parts of the genome may be entirely absent (Lowry et al. 2017). The method developed by Fowler and Buonaccorsi (2016) for identifying sex-specific loci is statistically robust, and it is intriguing that we were able to successfully amplify the same 220 bp product in all eight rockfish species. However, it would be highly fortunate for the DNA sequence of the target locus to be sufficiently conserved across all taxa, so that the *MluCI* restriction site is exclusive to the males of all species. Further genetic research, likely using whole-genomic sequencing methods to develop more complete reference genomes, should yield improved information about the genetic basis of sex determination across the *Sebastes* rockfish genus.

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