Received: 3 November 2019 | Revised: 1 October 2020 | Accepted: 14 October 2020

DOI: 10.1111/jeb.13725

SPECIAL ISSUE

JOURNAL OF Evolutionary Biology eseb WILEY

Genomic landscape of reproductive isolation in *Lucania* killifish: The role of sex loci and salinity

Emma L. Berdan[1] | **Rebecca C. Fuller**[2] | **Genevieve M. Kozak**[3]

[1]Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden

[2]Department of Animal Biology, University of Illinois Urbana-Champaign, Champaign, IL, USA

[3]Department of Biology, University of Massachusetts-Dartmouth, Dartmouth, MA, USA

Correspondence
Emma L. Berdan, Department of Marine Sciences, University of Gothenburg, Gothenburg, Sweden.
Email: emma.berdan@gmail.com

Funding information
National Science Foundation (NSF) Early Faculty Development (CAREER), Grant/Award Number: DEB-0953716; National Science Foundation Dissertation Improvement, Grant/Award Number: DEB-1110658; University of Illinois Research Board, Grant/Award Number: 11153

Abstract

Adaptation to different environments can directly and indirectly generate reproductive isolation between species. Bluefin killifish (*Lucania goodei*) and rainwater killifish (*L. parva*) are sister species that have diverged across a salinity gradient and are reproductively isolated by habitat, behavioural, extrinsic and intrinsic post-zygotic isolation. We asked if salinity adaptation contributes indirectly to other forms of reproductive isolation via linked selection and hypothesized that low recombination regions, such as sex chromosomes or chromosomal rearrangements, might facilitate this process. We conducted QTL mapping in backcrosses between *L. parva* and *L. goodei* to explore the genetic architecture of salinity tolerance, behavioural isolation and intrinsic isolation. We mapped traits relative to a chromosome that has undergone a centric fusion in *L. parva* (relative to *L. goodei*). We found that the sex locus appears to be male determining (XX-XY), was located on the fused chromosome and was implicated in intrinsic isolation. QTL associated with salinity tolerance were spread across the genome and did not overly co-localize with regions associated with behavioural or intrinsic isolation. This preliminary analysis of the genetic architecture of reproductive isolation between *Lucania* species does not support the hypothesis that divergent natural selection for salinity tolerance led to behavioural and intrinsic isolation as a by-product. Combined with previous studies in this system, our work suggests that adaptation as a function of salinity contributes to habitat isolation and that reinforcement may have contributed to the evolution of behavioural isolation instead, possibly facilitated by linkage between behavioural isolation and intrinsic isolation loci on the fused chromosome.

KEYWORDS

behavioural isolation, centric fusion, chromosomal rearrangements, salinity tolerance, speciation

1 | INTRODUCTION

Local adaptation often plays a critical role in speciation when the force of natural selection is channelled into the generation of reproductive isolation (i.e. ecological speciation, Nosil, 2012; Schluter, 2009). This occurs in two ways: (1) natural selection may directly generate reproductive isolating barriers such as habitat isolation, immigrant inviability, or extrinsic post-zygotic isolation; or (2) the force of natural selection may contribute to reproductive isolation indirectly (i.e. as a by-product) (Cruz, Carballo, Conde-Padin, & Rolan-Alvarez, 2004; Kay, 2006; Lowry et al., 2008; Moser et al., 2016; Nosil, 2012; Nosil et al., 2009; Nosil et al., 2005; Ramsey et al., 2003; Rundle & Nosil, 2005; Schemske, 2010; Schluter, 2001). The strength of divergent natural selection can be indirectly funnelled into the creation of other forms of reproductive isolation via physical linkage between adaptive loci and loci that contribute to other barriers (but are not under natural selection themselves) or through pleiotropy where a given locus is under natural selection via its effect on one trait, with indirect effects on traits influencing reproductive isolation. Environmental factors requiring adaptation in different traits at multiple life stages may play a larger role in generating both direct and indirect reproductive isolation as they affect many loci in the genome and provide stronger barriers to migration, increasing divergence between the species (Feder & Nosil, 2010; Nosil et al., 2009). Studies of genomic architecture are particularly useful in determining contributions (direct or linked) of natural selection to reproductive isolation by testing for signatures of natural selection on barrier loci (Presgraves et al., 2003) or genomic signatures of selection on hybrids along ecological clines (Grahame et al., 2006; Schaefer et al., 2016; Sung et al., 2018). Genome-wide association studies often find co-localization of loci contributing to ecological traits and loci contributing to reproductive isolation (Bay et al., 2017; Renaut et al., 2011). However, linkage disequilibrium between loci coding for ecological traits and those contributing to reproductive isolation is consistent with ecological speciation but can also be caused by spatial subdivision among species (Beirne, Welch, Loire, Bonhomme, & David, 2011). Genetic mapping studies can help differentiate between these two possibilities. Much of this prior work has focused on terrestrial or aquatic freshwater systems. The indirect effects of natural selection on reproductive isolation in marine and coastal species remain less well studied.

An important environmental factor driving local adaptation in coastal aquatic environments is salinity. The transition from fresh water to salt water is marked by a rapid shift in communities in nearly all groups of aquatic organisms, indicating the strength of this boundary (Godfrey & Wooten, 1979, 1981; Gunter, 1945, 1950, 1961). Multiple studies have suggested salinity has played a significant role in speciation in teleost fish (Betancur et al., 2015; Hrbek & Meyer, 2003; Huyse, Van Houdt, & Volckaer, 2004; Lee & Bell, 1999; Whitehead, 2010). In particular, salinity often contributes to habitat isolation among closely related species (Torres-Dowdall et al., 2013). Environmental salinity requires complex physiological adaptation because, in high salinity environments, organisms are subject to ion influxes and loss of water from tissues. Conversely, in low salinity environments, fluxes of water into tissues and loss of ions to the environment occurs (Evans, 2008; Evans et al., 2005). This complex adaptation causes physiological divergence within species in many tissues and life stages (Seehausen & Wagner, 2014; Taylor, 1999; Whitehead et al., 2011). Previous work suggests the genomic basis of this important trait may be dispersed across the genome on multiple chromosomes or linkage groups (Berg et al., 2015; Brennan et al., 2018). Whereas it is clear that salinity tolerance directly contributes to habitat isolation, extrinsic isolation, and immigrant inviability, the contribution of salinity to other reproductive isolating barriers via linked selection remains unknown.

Linked selection can only generate reproductive isolation if traits that contribute to isolating barriers are coupled to traits under divergent natural selection (Butlin & Smadja, 2018). In general, the coupling of different isolating barriers is necessary for the completion of speciation (i.e. the full cessation of gene flow). However, coupling is particularly difficult when speciation occurs with gene flow as recombination directly opposes divergence by homogenizing allelic combinations, thus breaking down linkage disequilibrium between reproductive barriers (Butlin, 2005; Felsenstein, 1981). Chromosomal rearrangements have been hypothesized to facilitate speciation with gene flow because recombination is reduced between homologous chromosomes with different arrangements. Both theoretical and empirical work has shown that the genes underlying reproductive isolation are less likely to become decoupled and homogenized via gene flow when they co-occur in areas of low recombination (Charlesworth & Barton, 2018; Faria & Navarro, 2010; Hoffmann & Rieseberg, 2008; Kirkpatrick & Barton, 2006; Lowry & Willis, 2010; Noor et al., 2001; Wellenreuther & Bernatchez, 2018; Wellenreuther et al., 2019). Thus, genomic features such as rearrangements may allow the environment to play a larger role in speciation by facilitating tight physical linkage between loci under divergent natural selection and loci conferring other reproductive isolating barriers. Rearrangements may also contribute to speciation by coupling multiple reproductive isolation barriers unrelated to the environment (Butlin & Smadja, 2018). If a chromosomal rearrangement has facilitated divergence, then the expectation would be that ecologically important traits and reproductive isolating barriers should map to the rearranged region.

In this study, we examine the contribution of divergent natural selection for salinity tolerance to the evolution of behavioural and intrinsic post-zygotic isolation between *Lucania goodei* and *L. parva* and ask whether a chromosomal rearrangement has facilitated the speciation process. *Lucania goodei* and *L. parva* are closely related species (Duggins et al., 1983; Whitehead, 2010) that differ in salinity tolerance. *Lucania goodei* is found primarily in freshwater sites (restricted mainly to Florida and southern Georgia), whereas *L. parva* can be found in fresh, brackish, and marine habitats as far west as central Mexico and as far north as Massachusetts (Lee, 1980). Within Florida, the two species ranges overlap by ~ 12%–19%. The species co-occur in either brackish pools along the coast or freshwater portions of rivers that are tidally influenced (Fuller & Noa, 2008).

Differential adaptation to salinity between the two species is present at multiple life stages (Dunson & Travis, 1991; Fuller, 2008; Fuller et al., 2007). In the early life-history stage, *L. parva* has high survival across all salinities (0.2–30 ppt). In contrast, *L. goodei* has high survival in fresh and slightly brackish salinities (0–10 ppt) but has low survival at higher salinities (20–30 ppt) (Fuller et al., 2007). These differences in salinity tolerance are reflected in genetic differences in coding sequence and expression differences between the two species (Berdan & Fuller, 2012b; Kozak et al., 2014).

Hybrids between *L. parva* and *L. goodei* can be found in nature at low levels (Hubbs, Walker, & John, 1943) in the portions of the range where the two species co-occur. Several reproductive isolating barriers other than habitat isolation exist. There is reduced viability of hybrid offspring at high salinities and reduced overall fitness of F2 offspring and backcrosses to *L. goodei* (Fuller, 2008; Fuller et al., 2007). Previous work on *Lucania* indicates that a large genetic incompatibility is segregating between the two species that results in some hybrid males having drastically reduced fitness (Fuller, 2008). Offspring from hybrid F1 males (from crosses between *L. parva* females and *L. goodei* males) have reduced viability and nearly half die during the first few days of development. Besides these post-zygotic isolating barriers, assortative mating due to male and female preferences causes behavioural isolation between the two species (Berdan & Fuller, 2012a; Fuller et al., 2007; Kozak et al., 2015; St. John & Fuller, 2019). A pattern consistent with reinforcement is present where this behavioural isolation is heightened in areas where the two species co-occur (Gregorio et al., 2012; Kozak et al., 2015; St. John & Fuller, 2019).

In addition to phenotypic divergence, previous work has established that a chromosomal fusion has occurred in the lineage leading to *L. parva*. Karyotypes and genetic mapping indicate that two acentric chromosomes have been fused into a single metacentric chromosome in *L. parva* (a centric fusion), leading to 1N = 23 in *L. parva* compared to 1N = 24 in *L. goodei* (Berdan et al., 2014; Uyeno & Miller, 1971). The sex-determining region is currently unmapped in *Lucania*, but karyotypes of both species do not differ between males and females, suggesting that the sex chromosomes are homomorphic (Berdan et al., 2014; Uyeno & Miller, 1971). Homomorphic sex chromosomes are common among many fish and reptile species (Bachtrog, 2013; Bachtrog et al., 2014; Mank, Promislow, & Avis, 2005). Chromosomes containing the sex-determining region often have important effects in speciation because they can play a disproportionate role in intrinsic isolation and also tend to diverge faster than autosomes (Bachtrog et al., 2011; Coyne & Orr, 2004; Meisel & Connallon, 2013; Presgraves, 2018; Saether et al., 2007). Therefore, we sought to determine the nature of sex determination in this system and to map loci physically linked to the sex-determining region.

We used crosses between *L. goodei* and *L. parva* to map loci linked to the sex-determining region, early life stage survival at different salinities (hereafter referred to as 'salinity tolerance'), behavioural isolation (male attractiveness for each species), and intrinsic post-zygotic isolation (reduced male fertilization success and offspring viability). We wanted to determine the distribution of these traits in the genome and whether behavioural and post-zygotic isolation loci are physically linked to salinity loci, the chromosomal fusion, or the sex-determining region. To do this, we created a series of backcrossed pedigrees, phenotyped the backcrossed offspring for sex, salinity tolerance, male attractiveness, male fertilization success and offspring viability, and genotyped the offspring to conduct QTL mapping. We predicted that if salinity adaptation contributes to reproductive isolation indirectly via physical linkage, then loci that contribute to survival at high salinities should map to similar genomic regions as loci for other reproductive isolating traits. We predicted that if genomic features have facilitated coupling of adaptive loci and other reproductive isolating loci, then these traits should co-localize to the chromosomal fusion and/or the sex chromosome. If the fusion by itself contributes to the coupling of multiple reproductive isolating barriers (independent of salinity), then traits for other reproductive barriers, but not salinity tolerance, should map there.

2 | METHODS

2.1 | Field collections, animal husbandry, and crosses

Our goal was to QTL map sex determination, salinity tolerance and multiple components of reproductive isolation. To do this, we created three separate crosses among *Lucania* populations and genotyped the offspring (Crosses 1–3; see Table 1). Cross 1 was the main mapping backcross to *L. goodei* in which we genotyped all traits (Table 1). We chose to examine reproductive isolation and salinity tolerance from backcrosses into *L. goodei* because previous work had shown that significant genetic incompatibilities and reductions in survival in saltwater were present in these backcrosses. In contrast, backcrosses to *L. parva* show limited evidence for reduced survival and genetic incompatibilities (Fuller, 2008; Fuller et al., 2007). Hence, backcrosses into *L. goodei* were expected to produce offspring in

TABLE 1 Crosses used. For sample sizes for each assay see Figure S2, S3, and S4. For location see Figure 1

Cross	Population/Species 1	Population/Species 2	Backcross direction	Phenotypes
1	Delks *L. goodei*	Delks *L. parva*	*L. goodei*	Male Fertilization Success, Male Mating Success, Offspring Viability, Sex, Salinity Tolerance
2	Blue Springs *L. goodei*	Indian R. *L. parva*	Both	Sex
3	Pecos R. *L. parva*	Indian R. *L. parva*	Both	Sex

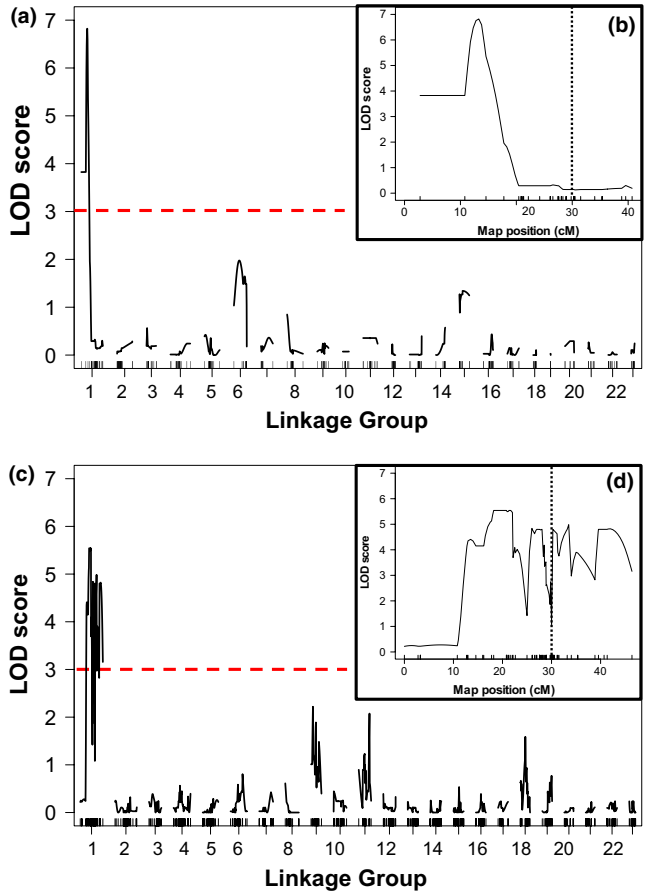

FIGURE 1 Location of the sex determination locus. LOD scores across the*L. parva*linkage map for cross 2 interspecies backcrosses to *L. parva* and *L. goodei* (a, b) and cross 3*L. parva* interpopulation cross (c, d). Red dashed line indicates LOD score = 3 (approximately equivalent to$p < .05$). Inset shows LOD score across the fused chromosome LG1 (b, d). Black dashed line separates the putative ancestral 1A and 1B portions of the chromosome (1B starts at 30cM)

which salinity tolerance and reproductive isolating barriers were segregating. For mapping of sex-linked loci, we used two additional mapping crosses (Crosses 2 and 3; see Table S1).

Individuals were collected using dip nets and seines between 2009 and 2011 (map in Figure S1) and housed as described in supplemental methods. We crossed fish by pairing a male and a female in a 38-litre (10 gallon) aquarium. For each cross, F1 families were generated in both directions from parental populations and pairs were checked for eggs every 2–3 days (see supplemental methods). Fry were raised to adulthood and then backcrossed to one or both parental populations (Table 1). All F1 and backcrosses were performed in fresh water. In all experiments, our freshwater source was dechlorinated city water treated with Start Right (Jungle Laboratories, Cibolo, Texas).

For the main mapping cross 1, parental adult *L. goodei* and *L. parva* were collected from a population where they co-occur: The Boat Ramp at Delk's Bluff near Ocala (Marion County, Florida). Families of all four possible types of backcrosses to *L. goodei* were made (Figure S2). A randomly selected portion of the offspring was raised to one month of age in either fresh or salt water, euthanized and later genotyped. Genotypes were compared between fresh and salt water in order to map the location of salinity tolerance (see below). The remaining freshwater offspring were raised to adulthood to assay male fertilization success and offspring viability (i.e. survival to hatching for offspring of the backcrossed males), behavioural isolation and sex determination (Figure S2).

For mapping crosses 2 and 3, F1s were created in both directions, raised to adulthood, and used to create all possible backcrosses in both directions. These offspring were genotyped to map the location of the sex-determining region. For cross 2, the parental populations were *L. goodei* from Blue Springs along the Santa Fe River, Florida, and *L. parva* from Indian River along Merritt Island, Florida. For cross 3, the parental populations were *L. parva* from Indian River, Florida and *L. parva* from the Pecos River, Texas (Figure S1).

2.2 | Predictions for loci linked to the sex-determining locus

Our goal was to map loci linked to the sex-determining region. We assumed that loci that are closely linked to the sex-determining region would not segregate in Mendelian ratios. Instead, the SNPs should exhibit classic signatures of a given sex determination system (Staelen et al., 2008; Star et al., 2016). As sexually dimorphic loci were not present on our linkage map, we scanned for linked loci that showed the expected species/population of origin patterns (see Figure S3–S4). In the case of an XX-XY system (male determining locus), F1r (*L. parva* ♀ × *L. goodei* ♂) males should pass on an *L. goodei* allele to male offspring and an *L. parva* allele to female offspring at loci linked to the sex-determining region. When backcrossed to *L. goodei*, we expected the resulting female offspring to be heterozygous and the male offspring to be homozygous for *L. goodei* alleles for loci linked to the sex-determining region (Figure S3). Similarly, F1 (*L. goodei* ♀ × *L. parva* ♂) males should pass on an *L. parva* allele to male offspring and an *L. goodei* allele to female offspring. When backcrossed to *L. goodei*, we expected the resulting male offspring to be heterozygous and the female offspring to be homozygous for *L. goodei* alleles. Predictions for a ZZ-ZW system are in the supplemental material (methods; Figure S3).

We assigned the offspring from diagnostic crosses with a sex genotype consistent with the predictions from a given sex determination system and mapped this as a trait. Adult males and females are visually identifiable by sexually dimorphic coloured anal fins (males have these, females do not; Fuller et al., 2007). Hence, we can assign them genotypes predicted from a hypothesized sex determination system, the identity of their parents and their sex (Figure S3). Our results were consistent with an XX-XY sex determination system (see results below), which is in keeping with the sex determination system of other members of Fundulidae (Chen & Ruddle, 1970). However, our sample size was low for offspring from diagnostic crosses for the XX-XY system (i.e. backcrosses involving F1r males).

To map the sex-determining region more finely, we used backcrossed offspring from two additional crosses (Cross 2: between *L. goodei* and *L. parva;* Cross 3: between *L. parva* populations; Table 1; Figures S3–S4; Supplemental Methods). To map the sex-determining region, we used the pure species maps from Berdan et al. (2014) (*L. goodei* map = 915 SNP markers, *L. parva* map = 766 SNP markers).

2.3 | Salinity tolerance assays

For the salinity tolerance assay, we used the main mapping backcross to *L. goodei* (Cross 1). We obtained clutches of backcrossed eggs from multiple families and divided the eggs between fresh water (0.2 ppt salinity, 32 families, 1,152 eggs total) and salt water (15 ppt salinity, 23 families, 521 eggs total). Eggs were placed in groups in 177-mL tubs treated with methylene blue (anti-fungal agent) in fresh water (~0.2 ppt) or salt water (15 ppt; see supplemental methods). We chose 15 ppt because it creates a significant osmoregulatory challenge without inducing excessive mortality. Fifteen ppt is greater than the isosmotic point (~10 ppt). Previous studies show that survival to fry to the eating stage is ~ 13% for *L. goodei* offspring raised in salinities 20–30 ppt compared to ~ 87% at 0.2 ppt (Fuller, 2008). We recorded the number of eggs that hatched and the number of fry that survived to one month of age. At one month of age, a subset of offspring was euthanized and preserved for genotyping (freshwater: 61 offspring from 18 families; saltwater: 84 offspring from 17 families). Additional offspring from the freshwater treatment were raised to adulthood to obtain adults for measurements of behavioural isolation and male fertilization success.

2.4 | Male behavioural isolation

We measured behavioural isolation between the backcrossed males (cross 1) and both *L. goodei* and *L. parva* females. We used a no-choice mating assay, which has been used successfully in previous studies of behavioural isolation in *Lucania* (Berdan & Fuller, 2012b; Fuller et al., 2007; Kozak et al., 2012; St. John & Fuller, 2019). Adult backcross males were placed in a 38-litre (10 gallon) aquarium with a stimulus female: either a female *L. goodei* or a female *L. parva*. We checked pairs every 2nd day for eggs over 20 days, recording the day on which eggs were first found and the total number of eggs produced. We obtained stimulus females from two populations – Delk's Bluff (6 trials with *L. goodei* females and 6 trials with *L. parva* females) and the Wakulla River (23 trials with *L. goodei* females and 27 *L. parva* females). Thirty-three males were tested in total; 29 males were tested with both *L. goodei* and *L. parva* females and another 4 were tested only with *L. parva* females. We randomized the order in which males were paired with *L. goodei* and *L. parva* females.

From these data, we recorded whether or not males mated as a binary variable (latency < 20 days = 1; latency ≥ 20 days = 0) and daily egg production rate, which served as indices of male attractiveness to and willingness to mate with stimulus females. We did not check fertilization status or survival of the eggs for this measure. St. John and Fuller (2019) found egg laying to be more robust than time measures of preference in *Lucania*. These fish are external fertilizers, so there is little opportunity for male seminal fluids to alter female mating rates. Furthermore, females lay ~ 1–2 eggs per spawning event (Breder & Rosen, 1966), so the number of eggs is approximately equal to the number of spawns. The disadvantage of no-choice mating assays is that it is difficult to determine precisely what attributes of males and females are being measured. In the *Lucania* system, both males and females have mating preferences (Kozak et al., 2015; St. John & Fuller, 2019). Hence, the assays may be detecting loci that affect both hybrid male attractiveness and hybrid male preference of females.

2.5 | Reduced male reproductive success

We assayed both the fertilization success (proportion of eggs fertilized) and the offspring viability (proportion of viable eggs that hatched) from the backcross males mated to females in the mating assays (N = 23 males). We checked all collected eggs under a light microscope to assess fertilization. We considered eggs that were already dead upon collection to be unfertilized. We saved the fertilized eggs and measured their survival until hatching as our metric of 'offspring viability'. After mating and fertilization trials, males were subsequently euthanized with MS-222 and stored in ethanol at −20°C.

2.6 | Genotyping

DNA isolation protocols are described in the supplemental methods. For cross 1, DNA was extracted from 173 offspring from the salinity tolerance assay (61 freshwater, 84 saltwater), 33 males from the male behavioural isolation and intrinsic isolation assays, and 27 females. For cross 2 and 3, DNA from 50 adults and 36 adults were isolated, respectively (Figure S4c). Samples were diluted to a concentration of 75 ng/μl prior to genotyping.

All genotyping was done using a single custom designed Illumina Infinium Beadchip as described in Berdan et al. (2014). The Bead Chip contained probes for genotyping of 4,545 SNPs: 1,679 putatively species-specific loci (used in crosses 1 and 2), 1,369 segregating among *L. parva* populations (used in cross 3) and 1,497 segregating among *L. goodei* populations (not used in this study).

All DNA samples were spread across twelve 96-well plates and genotyped on the Illumina Infinium Bead Chip, scanned using the iScan System (Illumina) at the Keck Center for Comparative and Functional Genomics at the University of Illinois in 2011–2013. There was no indication that backcross type (Figure S2) influenced the general segregation of markers using a quasi-binomial glm model with the proportion of homozygous genotype calls as a dependent variable and a likelihood ratio test for difference between models

with and without cross type as an explanatory variable (χ^2_3 deviance = 0.100, $p = 0.57$).

2.7 | Backcross linkage map

L. goodei and *L. parva* linkage maps have been previously constructed and genome size in *L. goodei* was previously estimated to be 1.3 Gb (Berdan et al., 2014). We created a hybrid map because (a) map length differs between *L. goodei* and *L. parva* (605 cM and 392 cM respectively) and (b) we did not know whether recombination in hybrids differs from the parental species. We constructed a backcross linkage map from the backcrossed offspring from cross 1 ($N = 189$ individuals). We constructed the map in Joinmap 4.0 (Van Ooijen, 2006) following methods described in Berdan et al. (2014). We considered all hybrids to come from a single family, and we only constructed the map using species-specific SNPs markers that were in Hardy-Weinberg equilibrium (222 markers total) and that showed the correct inheritance pattern (heterozygous in all F1 parents and homozygous in all *L. goodei* parents).

2.8 | QTL mapping—sex determination

All QTL mapping, other loci association tests, and visualization were performed in R v.3.5 (R Core Team, 2018), using the rQTL (Broman & Sen, 2009) and ggplot (Wickham, 2016) packages. We tested whether our sex-specific genotypes were consistent with either an XX-XY or a ZZ-ZW sex determination system. To do this, we used the predicted genotypes for sex-linked loci to map the sex-determining region on our linkage maps (Figures S3–S4). We used scanone with a binary model to calculate LOD scores and determined significance and the 95% Bayesian credible interval using 5,000 permutations. For cross 1, we used 60 genotyped individuals and the new backcross map. For cross 2, we used 50 genotyped backcross offspring and species-specific SNPs which had been previously mapped on the *L. parva* and *L. goodei* linkage maps (Berdan et al., 2014) (353 markers *L. goodei* map, 355 *L. parva* map). For Cross 3, we used 36 genotyped backcrossed individuals (Figure S4), filtered SNP data, identified alleles that were fixed between parental population (Kozak et al., 2014) and used the 821 markers out of these that had a position on the *L. parva* map.

2.9 | Salinity tolerance genotype testing

We sought to determine the location of loci associated with salinity tolerance. To do this, we compared the frequency of the different genotypes across the genome among offspring that survived in fresh and salt water. We considered only offspring that were raised to 1 month of age and then euthanized so as to directly compare the same window of mortality between fresh and saltwater (inclusion of adults raised in fresh water does not alter the results). Along a given linkage group, some markers were in complete linkage and were removed (28 of 172 markers removed). Previous work indicates that juveniles of both *L. goodei* and *L. parva* survive well in fresh water. We therefore used the frequency of the SNP genotypes among the 61 freshwater offspring as the expected frequency and asked whether the frequencies in saltwater differed from expected proportions using a chi-square test ($df = 1$). We corrected for multiple testing by using the Benjamini and Hochberg (1995) method as implemented in R with 'p.adjust'.

2.10 | Mapping gametic disequilibrium—interactions among loci

Genetic incompatibilities among loci are predicted to generate distortions in genotype frequencies in surviving backcrossed individuals. Incompatible allele combinations will be reduced or absent among the survivors. The goal here was to determine whether backcrossed offspring differed in their probability of survival due to interactions among genotypes located on different linkage groups. To do this, we tested for nonrandom patterns of genotypes, using a chi-square analysis. We only included backcrossed individuals that had been raised in fresh water ($N = 121$ individuals, including offspring and adults) to avoid the distorting effects of differential survival in salt water. We considered both offspring that were raised until one month of age (and then killed) as well as offspring that were raised to adulthood. Analyses that excluded adult backcrossed offspring ($n = 61$) had little effect on the qualitative results, but reduced power. Along a given linkage group, some of the markers were in complete linkage, so we used one representative marker from each linkage block. We also only considered patterns among loci located on different linkage groups. We performed a total of 10,675 tests. For each test, we calculated chi-square statistic ($df = 1$), the associated p-value and the frequencies of the four combinations of genotypes (homozygous at both locus 1 and 2, heterozygous at both locus 1 and 2, homozygous at locus 1/heterozygous at locus 2 and vice versa). Again, we used the false discovery rate correction in 'p.adjust'.

2.11 | QTL mapping—phenotypes of backcrossed males

We sought to determine the location of QTL related to multiple aspects of reproductive isolation: male fertilization success, offspring viability as a function of male identity and behavioural isolation of backcross males (egg production and whether or not mating occurred) with both *L. goodei* and *L. parva* females (sample size: Figure S2; phenotypic distribution: Figure S5–S7). Backcrossed males were paired separately with *L. goodei* and *L. parva* stimulus females. We performed QTL analyses separately for all traits. In total, we had six traits that we mapped in backcrossed males: whether or not a male mated with *L. goodei*, whether or not a male mated with *L. parva*, egg production with *L. goodei*, egg production with *L. parva*,

male fertilization success and offspring viability. For each of these traits, the QTL mapping was done in rQTL using the backcross linkage map and scanone with standard interval mapping ('em') (Broman & Sen, 2009). We calculated the significance of LOD scores using 5,000 permutations and the 95% Bayesian credible interval for any significant QTL identified. Inclusion of backcross types (BC i-iv) as a covariate or estimating LOD thresholds separately for the sex chromosome (Broman et al., 2006) did not qualitatively change any of our findings (Table S2).

3 | RESULTS

3.1 | Backcross map

The backcross linkage map contained a total of 172 placed markers on 23 linkage groups, which were numbered based on syntenic markers shared with the *L. goodei* and *L. parva* maps (see Berdan et al., 2014). In both the *L. parva* map and the backcross map, linkage group 1 represented a fusion of two linkage groups (1A and 1B) from *L. goodei*. The total length of our map was 448 cM, which is intermediate between the lengths of our previously published maps for *L. goodei* (605 cM) and *L. parva* (392 cM) (Berdan et al., 2014).

3.2 | Loci linked to the sex-determining region

There was support for an XX-XY sex determination system that mapped to the fused chromosome. There was little support for a ZZ-ZW system in cross 1, as the predicted ZZ-ZW genotypes failed to map to the hybrid map (LOD < 1.32, $p > .53$; $N = 44$ informative individuals). In contrast, the XX-XY predicted genotypes in cross 1 mapped to chromosome 1 at 0 cM near marker 05836 (LOD = 3.35, $p = 0.02$, 95% Bayesian Credible Interval 0–12 cM; $N = 16$ individuals; Figure S8a,b). Likewise, both crosses 2 and 3 provided strong support for an XX-XY sex determination system that mapped to one the end of the fused chromosome (Figure 1; Figure S8). In cross 2 (second *L. parva* – *L. goodei* interspecies cross), the predicted XX-XY genotypes mapped to chromosome 1A at 2 cM between markers 13121 and 14413 on the *L. goodei* map (LOD = 5.21, $p < .001$, $N = 50$; 95% Bayesian Credible Interval 0.5–3 cM; Figure S8c,d). Using these same data and the *L. parva* linkage map, the sex-determining region mapped to chromosome 1 at 10.5 cM near marker 13,005 (LOD = 6.82, $p < .001$, $N = 50$; 95% Bayesian Credible Interval 9–11 cM; Figure 1a,b; a location of 12cM on chromosome 1 in the *L. parva* corresponds to a location 1cM on the backcross map; Table S3). Using cross 3 (*L. parva* population backcrosses) and the *L. parva* map, the predicted XX-XY genotypes mapped to chromosome 1 at marker 11321 at 20.8 cM (LOD = 5.54, $p < .001$, $N = 36$; 95% Bayesian Credible Interval 13–44 cM; Figure 1c,d). Thus, the data supported an XX-XY sex determination system that consistently mapped to the chromosome 1A portion of the fused chromosome in all 3 crosses.

3.3 | Salinity tolerance

Survival in salt water was approximately half of that in fresh water (Figure S5a, Table S4, freshwater = 40.3 ± 0.037% SE, $n = 29$; saltwater = 24.4 ± 0.046%, $N = 19$; for families where 5 or more eggs were tested). This survival rate is similar to that found previously in Fuller et al. (2007) and Fuller (2008) for backcrosses to *L. goodei* (freshwater survival: 65%, saltwater survival: 32%).

In our experiment, salinity tolerance loci are diagnosable by an overabundance of heterozygotes (i.e. individuals with an *L. parva* allele) in salt water compared to fresh water. We compared the proportion of homozygous (*L. goodei*) and heterozygous genotypes at each marker between fresh and saltwater rearing conditions (Table 2). We examined QTL at the linkage group level (i.e. a maximum of one QTL per linkage group) and only considered linkage groups with more than one significant locus as being involved in adaptation to salinity as a way to avoid potential false positives that could be caused by genotyping errors. Linkage groups with multiple markers where heterozygotes were under-represented in fresh water and over-represented in salt water were as follows: 3, 6, 7, 12 and 17 (Table 2, Table S5, Figure 2). The effects were particularly strong for linkage group 7, where the heterozygotes were 1.9 times as abundant in salt water (~0.65) as they were in fresh water (~0.34). Loci at linkage group 16 showed the opposite pattern to our expectations, where heterozygous individuals were common among freshwater and rare among saltwater offspring. An additional 5 linkage groups (11, 18, 19, 21, 22) had a single significant marker implicated in salinity tolerance (Table 2, Table S5). Of these, linkage group 18 showed the expected pattern with an overrepresentation of heterozygotes in salt water. Linkage group 11,19, 21 and 22 showed the opposite pattern with an overrepresentation of heterozygotes in fresh water relative to salt water. Only linkage group 21 was implicated in intrinsic isolation, whereas linkage group 11 was implicated in behavioural isolation (see below).

3.4 | Gametic disequilibrium—interactions among loci

The chromosomal fusion was implicated in genetic incompatibilities. Survival of backcrossed offspring in fresh water was low (~40%), and the surviving backcrossed offspring were a nonrandom subset that had favourable combinations of alleles at different loci. Twenty-six of 10,675 tests for interactions among genotypes at loci on different linkage groups remained significant after correcting for multiple tests. All of these interactions among loci involved an overrepresentation of offspring that had concordant genotypes (i.e. either homozygous for the *L. goodei* specific marker at both loci or heterozygous at both loci). Individuals with mixed genotypes (i.e. homozygous at one locus, but heterozygous at another) were either absent or under-represented. Although there were 26 significant interactions, these involved loci on only five pairs of linkage groups (Table 3). There were multiple significant interactions involving loci on linkage group 1 and both linkage

TABLE 2 Salinity associated loci: loci showing significant different proportions of heterozygotes in freshwater versus saltwater

Marker	Linkage Group	Position (cM)	Proportion of heterozygotes in freshwater	Proportion of heterozygotes in saltwater	Chi-square *p*-value	FDR *p*-value
09418	7	16.96	0.34	0.65	2.10E−09	3.01E−07
00141	7	17.49	0.34	0.64	8.42E−09	5.88E−07
14667	7	0.00	0.18	0.41	1.23E−08	0.0000
14398	17	24.83	0.44	0.68	0.0000	0.0005
18723	17	25.90	0.45	0.67	0.0000	0.0011
11877	21	0.00	0.54	0.33	0.0001	0.0027
00137	17	25.89	0.46	0.67	0.0001	0.0027
13073	6	0.00	0.48	0.68	0.0002	0.0034
13872	18	9.84	0.33	0.51	0.0003	0.0052
11937	3	0.00	0.34	0.52	0.0006	0.0079
15386	12	6.62	0.38	0.57	0.0006	0.0079
10789	3	0.00	0.35	0.52	0.0008	0.0092
05062	12	6.66	0.39	0.57	0.0008	0.0092
11514	3	0.08	0.34	0.51	0.0009	0.0094
14634	3	3.34	0.34	0.51	0.0012	0.0106
11023	12	6.11	0.41	0.58	0.0012	0.0106
10999	16	0.00	0.62	0.45	0.0013	0.0106
12180	19	7.45	0.57	0.40	0.0017	0.0132
11538	16	3.18	0.61	0.44	0.0018	0.0132
05635	3	0.00	0.36	0.52	0.0018	0.0132
09531	3	0.00	0.37	0.53	0.0020	0.0136
16266	11	34.49	0.59	0.43	0.0026	0.0162
11737	12	6.87	0.41	0.57	0.0026	0.0162
03290	3	0.00	0.36	0.52	0.0028	0.0168
15226	12	6.88	0.41	0.57	0.0038	0.0215
12650	6	16.40	0.40	0.55	0.0041	0.0227
07450	16	2.65	0.59	0.44	0.0053	0.0280
11889	22	10.68	0.57	0.43	0.0071	0.0364
06697	6	16.41	0.40	0.54	0.0100	0.0491

Note: Results from chi-square test with $df = 1$.

Abbreviations: cM, CentiMorgan; FDR, False discovery rate.

groups 13 and 16. One interaction between linkage group 1 and linkage group 13 involved a marker very close to the sex-determining region (marker 13005). There were also significant interactions between linkage groups 13 and 16, linkage groups 21 and 22, and linkage groups 23 and 2. Due to our somewhat low sample size ($N = 121$), a two-fold difference in the abundance of concordant genotypes versus mixed genotypes was required for a marker to be inferred as highly significant after FDR correction (Table S6).

3.5 | Behavioural isolation

As expected, behavioural isolation was higher when backcrossed males were paired with *L. parva* females than with *L. goodei* females. We found that 75% of males mated at least once with *L. goodei* females during the 20-day period, but only 48% of males mated with *L. parva* females. Similarly, backcrossed males had higher daily egg production with *L. goodei* (1.78 eggs/day) than they did with *L. parva* (0.61 eggs/day) (Figure S6).

The number of eggs produced in pairings with *L. goodei* females mapped to chromosome 1 at 32 cM (LOD = 2.89, $p = .01$, $N = 29$; Figure 3a). Males that were homozygous for the *L. goodei* allele had higher mating success than heterozygotes that carried an allele from *L. parva* (homozygotes: 3.41 eggs/day, heterozygotes: 0.77 eggs/day; Figure S9). There were no QTL identified for the probability of a male mating with *L. goodei* females, which is not surprising as most males successfully mated with *L. goodei*.

Mating success with *L. parva* females mapped to two linkage groups, but the genotypic pattern did not match our expectation that heterozygotes should have increased success with *L. parva* (backcrosses could only be heterozygous for *L. parva* alleles, not

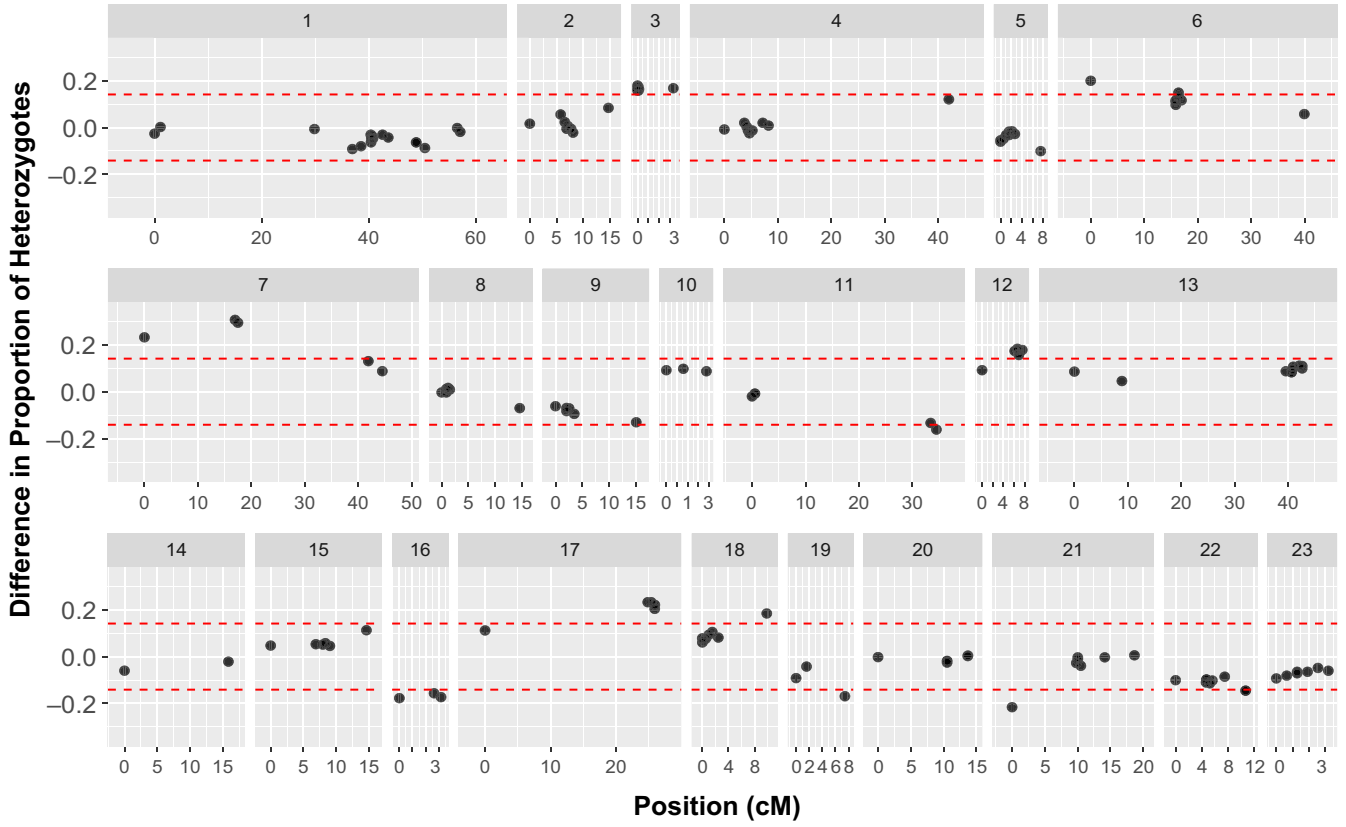

FIGURE 2 Salinity tolerance loci. Difference in proportion of heterozygous individuals in salt versus freshwater plotted for loci across all 23 linkage groups. Linkage group numbers listed above, position of loci in centiMorgans (cM) on the hybrid map shown. Different linkage groups separated by white partitions. Red lines indicate FDR cut-offs. LG 3, 6, 7,12, 17 showed multiple outliers in the predicted positive direction. See Table 2for loci names

homozygous). Whether or not a male mated with *L. parva* females mapped to linkage group 1, marker 13870 at 57 cM (LOD = 2.87, $p = .021$, $N = 33$; Figure 3b) and heterozygotes were less likely to mate with female *L. parva* than were homozygotes (90% of homozygotes mated with *L. parva* compared to 27% of heterozygotes; Figure S9). Likewise, the number of eggs laid when males were mated to *L. parva* females mapped to chromosome 11 at 16.5 cM (LOD = 6.2, $p = .01$, $N = 33$) (Figure 3c), but the pattern was one where heterozygotes had lower egg production with *L. parva* females than individuals that were homozygous for the *L. goodei* allele (Figure S9).

3.6 | Fertilization success and offspring viability as a function of male genotype

Fertilization success and offspring viability did not differ between males when mated *L. goodei* or *L. parva* females (fertilization success: *L. goodei* females = 0.52 ± 0.12 SE, *L. parva* females 0.57 ± 0.12 SE; offspring viability: *L. goodei* females = 0.70 ± 0.09 SE, *L. parva* females = 0.77 ± 0.11). Fertilization success (proportion of fertilized eggs) of backcrossed males was generally bimodal with males having high or low success (Figure S7). Fertilization success of backcrossed males mapped to a single QTL located on linkage group 7 at 25 cM (LOD = 4.15, $p = .03$, $N = 23$; Figure 4a). Male fertilization success was 2.6 times higher in heterozygotes (0.64) than *L. goodei* homozygotes (0.24) (Figure S9). Offspring viability was defined as the proportion of fertilized eggs surviving to hatching. Offspring viability as a function of paternal genotype mapped to linkage group 1 at 9 cM (LOD = 3.47, $p = .04$, $N = 20$; Figure 4b) in the same region where sex determination maps and offspring viability was 2.4 times higher in homozygotes (0.78) than in heterozygotes (0.32) (Figure S5, Figure S7).

4 | DISCUSSION

In this study, we investigated the role of salinity adaptation in the evolution of behavioural and intrinsic isolation in the killifish *Lucania goodei* and *L. parva* by genetically mapping salinity tolerance, sex determination, behavioural isolation and intrinsic isolation. We found that salinity tolerance has a polygenic basis, mapping to multiple linkage groups, but with little co-localization with other reproductive isolating barrier loci. This pattern provides little evidence that selection via physical linkage or pleiotropy has contributed much to the evolution of behavioural or intrinsic isolation among *Lucania* species. This study is consistent with adaptation to salinity contributing to reproductive isolation through habitat isolation, extrinsic isolation and/or immigrant inviability.

TABLE 3 Genotypes between markers on different linkage groups with significant frequency distortion

Linkage groups		*p*-value	# genotypes				Marker ID	
LG Marker 1	LG Marker 2	FDR	AABB	AABb	AaBB	AaBb	Marker 1	Marker 2
1 - sex	13	1.53E−23	57	0	0	64	**13005**	02161
2	23	1.53E−23	55	0	0	66	03425	11531
21	22	2.10E−23	57	0	0	62	02541	06333
2	23	2.10E−23	54	0	0	65	17258	11531
2	23	3.67E−23	55	1	0	65	15948	11531
2	23	3.67E−23	54	0	1	66	14340	11531
21	22	8.33E−23	57	1	0	61	23270	06333
21	22	4.27E−22	57	0	2	60	03323	06333
21	22	4.27E−22	57	0	2	60	03556	06333
21	22	6.34E−22	57	0	2	59	03555	06333
22	21	2.46E−21	56	2	1	60	06333	06712
1*	13	1.26E−18	44	0	0	51	*10924*	02161
1 - sex	13	5.93E−11	46	8	8	52	**11211**	02161
1 - sex	13	8.93E−07	40	7	14	45	11521	02161
13	16	0.0066	38	16	19	48	02161	12642
1 - sex	16	0.0066	38	19	16	48	**13005**	12642
13	16	0.012	37	16	20	48	02161	04992
13	16	0.012	37	16	20	48	02161	17027
1 - sex	16	0.012	37	20	16	48	**13005**	04992
1 - sex	16	0.012	37	16	20	48	**13005**	17027
16	13	0.025	36	21	16	48	01506	02161
1 - sex	16	0.025	36	21	16	48	**13005**	01506
13	16	0.036	36	17	19	45	02161	13269
1 - sex	16	0.036	36	17	19	45	13005	13269
13	16	0.046	35	16	22	48	02161	11538
1 - sex	16	0.046	35	22	16	48	13005	11538

Note: Genotypes refers to the number of individuals that are homozygous for the *L. goodei* marker at both loci (AABB), are heterozygous at both loci (AaBb), or are homozygous at one locus but heterozygous at another (AABb and AaBB). 1-sex indicates a marker on linkage group1 located within the sex determining region (marker ID in bold); 1* indicates a marker on linkage group 1 that is adjacent to the sex determining region (marker ID in italics). *p*-value from a chi-square test with $df = 1$.

Abbreviation: FDR, False discovery rate.

We also found evidence that a fusion between an autosome and the chromosome with the sex-determining region has occurred in *L. parva*. Several of the genetic incompatibilities we mapped were located on the fused sex chromosome, with *L. parva* alleles causing gametic disequilibrium (segregation distortion) and offspring inviability (reduced hatching success of offspring). These results provide preliminary evidence that the chromosomal fusion may harbour loci related to reproductive isolation. Below, we discuss these results in more detail.

4.1 | Adaptation to salinity and speciation in lucania

Salinity tolerance mapped to numerous locations in the *Lucania* genome. Admittedly, some of these loci may be false positives. Still, salinity tolerance in *Lucania* is almost certainly polygenic as decades of research have revealed that salinity tolerance in teleosts is a complex trait that involves multiple tissues (e.g. gills, kidneys) and physiological pathways (Evans, 2008; Evans et al., 2005; Larsen et al., 2011; Laverty & Skadhauge, 2012). Indeed, our own previous work examining both gene expression and coding changes in *Lucania* indicates that salinity tolerance is a multi-locus trait (Berdan & Fuller, 2012b; Kozak et al., 2014). Other studies of the genomic basis of salinity tolerance in teleosts have revealed similarly distributed genetic architectures. *Lucania* is a member of the Fundulidae family (Whitehead, 2010). In *Fundulus heteroclitus*, a genome-wide association study found salinity tolerance loci on at least 5 different chromosomes (Brennan et al., 2018). In salmonids, between 3 (in *Oncorhynchus mykiss*) and 10 (*Salmo salar* and *Salvelinus alpinus*) linkage groups are involved in salinity tolerance (Norman et al., 2012). Loci associated with salinity

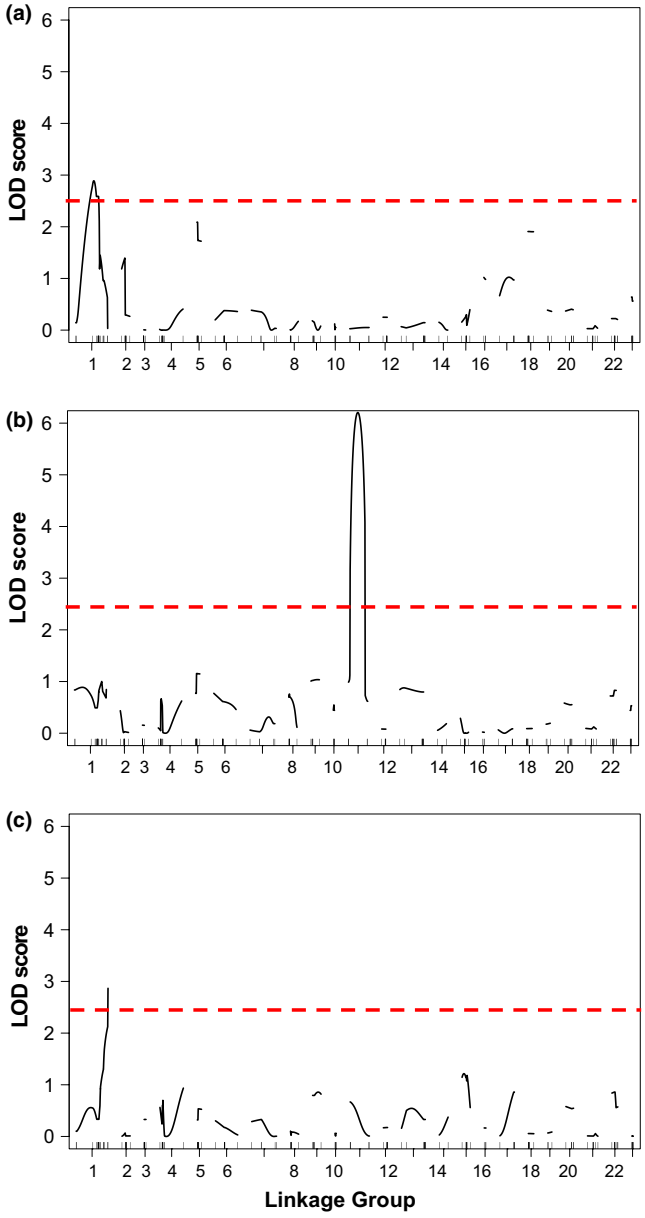

FIGURE 3 LOD scores from QTL mapping of behavioural isolation. (a) number of eggs produced when male mated to*L. goodei*,(b) number of eggs produced when male mated to*L. parva*, C) male probability of mating with*L. parva*.Red dashed line indicates LOD score = 2.5 (approximately equivalent to$p < .05$)

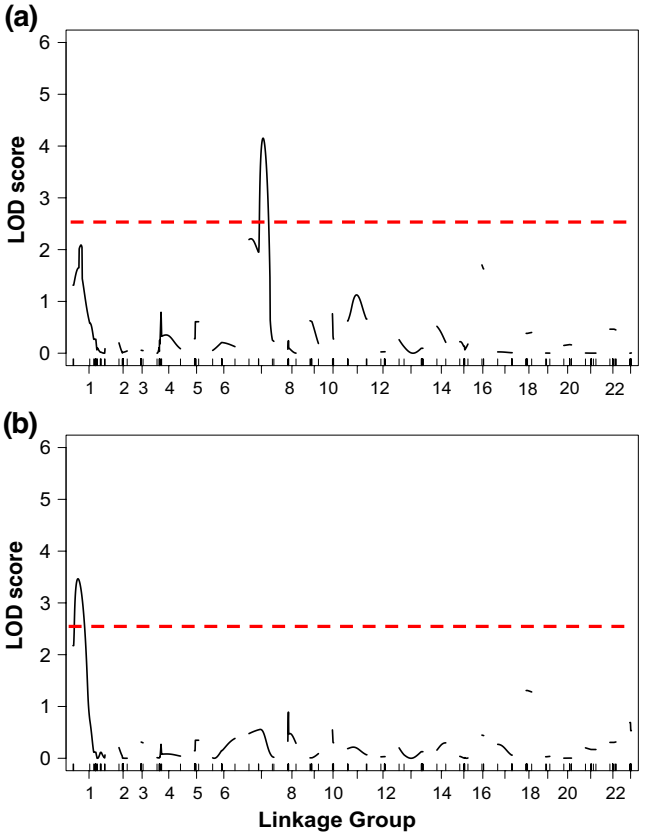

FIGURE 4 LOD scores from QTL mapping of male fertilization and offspring viability. (a) Male fertilization success, (b) male offspring viability. Red dashed line indicates the LOD score = 2.5, approximately equivalent to$p < .05$

map to 11 different linkage groups in Atlantic cod (*Gadus morhua)* (Berg et al., 2015). It is not yet clear if this type of genetic architecture will facilitate or hinder divergence during speciation with gene flow. If many targets of divergent selection are spread across the genome, this increases the potential for the coupling of adaptive loci with other isolating barriers; however, the effect of divergent selection at each locus will be diluted. Therefore, whether polygenic adaptation leads to the evolution of other reproductive isolating barriers via linked selection should depend on the number of loci, their physical positions and the strength of selection.

Our current study provides little support for the hypothesis that the force of divergent natural selection has played a major role in the evolution of behavioural and intrinsic isolation. Of the five linkage groups where heterozygotes with *L. parva* alleles survived better at high salinity, only linkage group 7 affected both salinity tolerance and male fertilization success. However, the pattern in fertilization success was not consistent with this linkage group increasing reproductive isolation. Rather than *L. parva* alleles conferring low fertility, backcrossed individuals that were homozygous for *L. goodei* alleles on linkage group 7 were more likely to be infertile. Our finding that areas of the genome associated with salinity tolerance differ from those contributing to other forms of reproductive isolation is in keeping with prior evidence that sympatry between *L. goodei* and *L. parva* led to increased behavioural isolation among *L. parva* populations, regardless of salinity habitat (Kozak et al., 2015). Novel adaptations can become coupled with genetic incompatibilities due to spatial subdivision of populations as indicated by theoretical models (Bierne et al., 2011). Given that karyotypes differ among species and intrinsic isolation maps to the fused chromosome, our current data cannot rule out an incompatibility-first model, with coupling of salinity tolerance and incompatibilities in *Lucania* occurring later.

Two caveats deserve mention before the hypothesis of reproductive isolation as an indirect outcome of salinity adaptation is rejected. First, this study had a relatively low sample size for a QTL study. Hence, only loci with very large effects could be detected and small

effects can be important in reproductive isolation (Ellison et al., 2011). Furthermore, sparse marker coverage may have led to missed QTL, broader intervals, and the potential of considering multiple small effect QTL as a single large effect QTL. Second, this study mapped salinity tolerance only at the earliest life-history stage of egg/larval survival. We chose this life stage because previous work has shown that both species can tolerate fresh water well in the egg/larval fish stage and that *L. goodei* have reduced survival in salt water in this stage relative to *L. parva*. However, juvenile and adult *L. parva* have reduced survival relative to *L. goodei* when they are forced to overwinter in fresh water (Fuller et al., 2007). We did not map salinity tolerance at later life stages, although these may conceivably be important to the evolution of other reproductive isolating barriers. Given these caveats, our current understanding of the *Lucania* system is that differential adaptation to salinity appears to contribute to habitat isolation (Fuller & Noa, 2008), but does not result in correlated responses in other traits that promote behavioural or intrinsic isolation.

4.2 | Sex determination, chromosomal rearrangements and speciation

The male sex-determining region (XX-XY) mapped to the fused chromosome in both hybrid (*L. goodei* × *L. parva*) and pure *L. parva* crosses. This suggests that the centric fusion in *L. parva* occurred between the *Lucania* chromosome with the sex-determining region and an autosome. Combined with the pattern of reduced fertility in F1r males (with *L. parva* mothers), but not F1r females (Fuller, 2008), this finding is consistent with Haldane's Rule of the heterogametic sex being more likely to exhibit sterility or inviability (Turelli & Moyle, 2007). Although all karyotypic evidence points to the *Lucania* sex chromosomes being homomorphic (Berdan et al., 2014; Uyeno & Miller, 1971), it is possible that F1r males are potentially hemizygous for some loci in the male determining region itself (Peichel et al., 2020), particularly if there has been some change in the genetic content of the chromosome accompanying the fusion. In fishes, sex chromosomes are often involved in fusions because of sexually antagonistic selection or male-mutation bias (Bachtrog, 2013; Bachtrog et al., 2011; Kitano & Peichel, 2012; Pennell et al., 2015). However, unlike many other known fusions in fish (Kitano & Peichel, 2012; Kitano et al., 2009), our fusion does not appear to represent a neo-Y system with unfused × chromosomes, because both males and females possess fused chromosomes (Berdan et al., 2014). Future work can investigate the differences that have arisen in between the autosome in *L. goodei* and the homologous portion in *L. parva* that is now sex-linked, similar to previous work with *Drosophila* species (Counterman, Ortíz-Barrientos, & Noor, 2004).

The chromosomal fusion may contribute to reproductive isolation between *L. goodei* and *L. parva* as several genetic incompatibilities map there (Figure 5; Table 4). Chromosomal fusions often differentiate populations and have been shown both theoretically and empirically to facilitate adaptation (Dobigny et al., 2017; Franchini et al., 2010; Guerrero & Kirkpatrick, 2014; Wellband et al., 2019). QTLs for behavioural isolation mapped to the fused chromosome, including number of eggs laid with *L. goodei* and the probability of mating with *L. parva* females. We found that the fused chromosome contained a locus associated with offspring viability. Additionally, loci on the fused chromosome appeared to interact with loci on linkage groups 13 and 16 to cause an incompatibility (segregation distortion). Overall, of 6 reproductive isolating barriers mapped, 4 mapped to the fused chromosome. However, this may be a result of a mapping bias caused by regions of low recombination when QTL loci are spread across the genome (Noor et al., 2001). Further work is needed to clarify the role of the fusion to reproductive isolation in *Lucania*. We also identified an incompatibility segregating between two other pairs of linkage groups (2 with 23 and 21 with 22). The interaction between linkage group 21 and 22 is interesting because it involves markers that mapped to a putative translocation. These markers map to linkage group 21 in one species and linkage group 22 in the other (Berdan et al., 2014). Our data suggest there are at least several segregating incompatibilities among multiple linkage groups that contribute to the reduced fitness previously observed in backcrosses to *L. goodei* (Fuller, 2008; Fuller et al., 2007).

Finally, we note that intrinsic isolation is asymmetric in *Lucania*, in line with Darwin's corollary to Haldane's rule (Turelli & Moyle, 2007). Additionally, in the F1 generation, F1r (*L. parva* ♀ × *L. goodei* ♂) males suffer a 50% reduction in early egg survival relative to parental males and F1 (*L. goodei* ♀ × *L. parva* ♂) males (Fuller, 2008). We did not map this incompatibility in this study, although the incompatibility was expressed in our F1 generation, with some F1r males having no fertility and being excluded as sires (Table S4). It is possible that the incompatibility was selected against in backcross iv (F1r ♂ × *L. goodei* ♀), as males from this cross tended to have higher male fertilization success and offspring viability than male offspring from the other three backcrosses. Unfortunately, we do not have the power to determine if these reductions in offspring viability represent the same incompatibilities across generations here. The major takeaway is that we have mapped loci important to reproductive isolation preventing gene flow from *L. parva* into *L. goodei*, but the reproductive barriers preventing gene flow from *L. goodei* into *L. parva* remain unknown.

4.3 | Implications for reinforcement

Potential linkage of behavioural and intrinsic isolation on the fused chromosome is intriguing given prior work in *Lucania*, suggesting that reduced hybrid fitness may have caused increased species-specific preferences in areas where the two species co-occur (Gregorio et al., 2012; Kozak et al., 2015). This process is often referred to as reinforcement, where costly mating with heterospecifics generates selection for altered mating traits and preference (Servedio & Noor, 2003). Reinforcement can only occur when post-zygotic isolation is present. *Lucania goodei* and *L. parva* are fairly genetically diverged with an estimated genome-wide F_{ST} of 0.38 and intrinsic post-zygotic incompatibilities are present (Fuller, 2008; Kozak et al., 2014), suggesting that these two species have been isolated

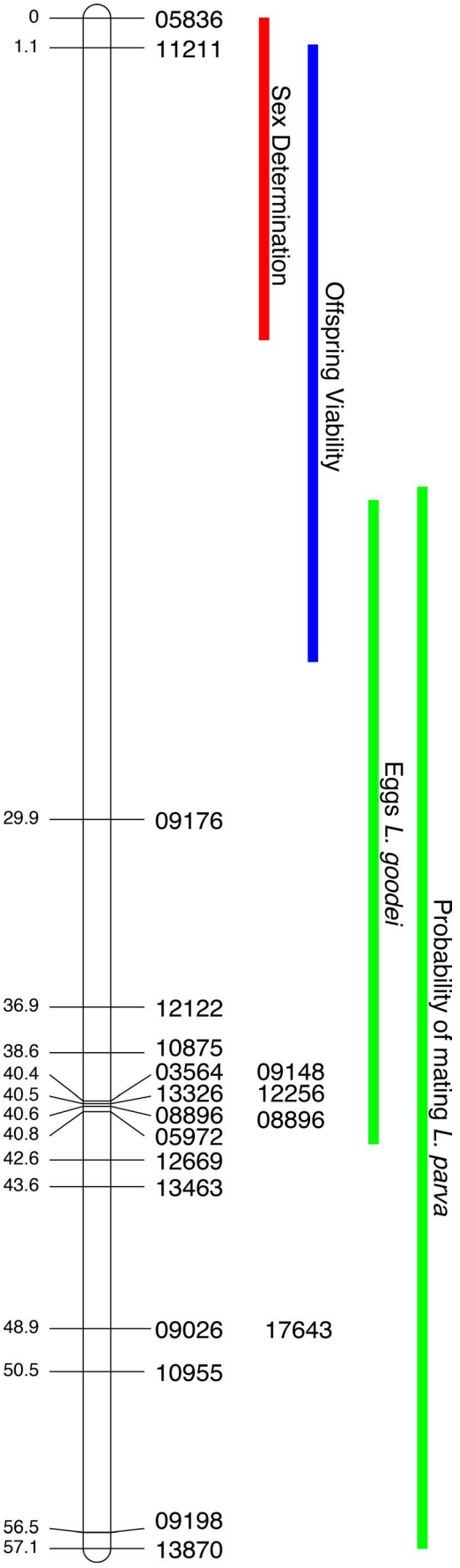

FIGURE 5 Sex-determining and isolating loci mapping to Linkage group 1. Bayesian credible intervals for sex determination and isolating traits (solid rectangles) mapped relative to position (in cM) along linkage group 1 (the fused chromosome) from the hybrid linkage map. Blue indicates hybrid incompatibility loci; green indicates behavioural isolation; sex determination region shown in red. The ancestral autosomal portion (chromosome 1B) is located at ~ 40–57 cM on this hybrid map. This figure was made with LinkageMapView (Ouellette et al., 2017).

for a significant amount of time. We hypothesize that the initial stages of divergence likely occurred via local adaptation and habitat isolation, after which many of the reproductive isolating barriers that we mapped in this study may have evolved.

Our findings combined with our previous work (Gregorio et al., 2012; Kozak et al., 2015) add to the growing support for the importance of chromosomal rearrangements involving the sex-determining region as facilitators of reinforcement. In order for reinforcement to occur, incompatibilities and behavioural isolation must be coupled with one another (Butlin & Smadja, 2018; Ortíz-Barrientos, Grealy, & Nosil, 2009). Physical linkage, with subsequent reduced recombination between loci, is one of the strongest ways to generate coupling. Fusions can generate linkage disequilibrium in two ways: by bringing previously unlinked loci together and by reducing recombination (Dumas & Britton-Davidian, 2002; Franchini et al., 2010). An example of this occurs in Japan Sea sticklebacks (*Gasterosteus nipponicus*), where the Y-chromosome has fused to an autosome containing a behavioural isolation locus (Kitano et al., 2009). We found preliminary evidence that major effect QTL for behavioural isolation and hybrid incompatibilities may be located on the fused sex chromosome in *Lucania*. However, additional studies with larger sample sizes and an enhanced ability to detect QTL of smaller effect are needed to determine the true effect of our detected QTL, as Beavis effects may have upwardly biased these (Xu, 2003), and to rule out false positives. Such studies are needed to definitively determine whether incompatibility loci are linked to behavioural isolation loci in *Lucania*.

Both of our QTL for mating success with *L. parva* were in the opposite direction than anticipated. Individuals homozygous for *L. goodei* alleles had higher success than heterozygous individuals. There are several possible explanations for this. First, these QTL could be false positives caused by a small sample size and the difficulties of properly measuring behavioural responses. Second, an *L. parva* allele may potentially only increase mating success with *L. parva* females when mating in saltwater environments. However, Berdan and Fuller (2012b) investigated the effect of salinity on behavioural isolation between *L. goodei* and *L. parva* and found little effect of salinity. Third, behavioural sterility could be occurring. Behavioural sterility occurs when admixed individuals fail to mate due to a defect (neurological, pheromonal, etc.) that renders them incapable of correctly courting the parental species (Coyne & Orr, 2004; Naisbit et al., 2001; Stratton & Uetz, 1986). Notably, our crossing design allowed backcross individuals to be homozygous for *L. goodei* alleles but only heterozygous for *L. parva* alleles. One possibility is

TABLE 4 Summary of locations of isolating barriers. Grey shading indicates that a QTL for the specified trait localized on the specified linkage group

	Intrinsic Isolation		Behavioural Isolation				Adaptation	Sex determination
Linkage Group	Gametic Disequilibrium (Incompatibilities)	Male Fertilization Success	Male Offspring Viability	Eggs Laid with *L. parva* ♀	Probability of Mating with *L. parva* ♀	Eggs Laid with *L. goodei* ♀	Differential Survival in Fresh versus Saltwater	Sex-Determining Locus
1					*			
2								
3								
4								
5								
6								
7		*						
8								
9								
10								
11				*				
12								
13								
14								
15								
16							*	
17								
18								
19								
20								
21								
22								
23								

Note: A * indicates that the expected pattern was reversed.

that being heterozygous for *L. parva* alleles in an *L. goodei* genetic background is detrimental for effectively courting any female; a defect that would only manifest in certain backcrosses. Preferences of males and females show evidence for character displacement in *L. parva* and *L. goodei* (Gregorio et al., 2012; Kozak et al., 2015); however, the traits upon which these preferences are based have not yet been identified. Additional investigation of the male trait differences associated with *L. parva* genotypes and preferences of *L. parva* females against admixed males will be needed to clarify this result.

In summary, previous work in *Lucania* indicates that differences in salinity tolerance have profound effects on habitat distribution (Fuller & Noa, 2008), ecological interactions (Dunson & Travis, 1991), survival across different life stages (Fuller, 2008; Fuller et al., 2007), physiology (Berdan & Fuller, 2012b) and genomic differentiation (Kozak et al., 2014) in *Lucania*. Salinity tolerance in *L. parva* from the egg stage to small juvenile is polygenic and distributed across the genome. However, there is currently no evidence that adaptation to salinity contributes to the evolution of reproductive isolating barriers that prevent *L. goodei* and *L. parva* from collapsing into a hybrid swarm in areas where they co-occur. Instead, a chromosomal rearrangement has created a fused chromosome in *L. parva* that contains both the sex-determining region and loci that contribute to some forms of reproductive isolation. Theory predicts reinforcement is more likely to occur when incompatibility loci and mate preference loci are located on sex chromosomes than when they are on autosomes, because of reduced recombination and the greater efficiency of natural selection on sex chromosomes (as recessive alleles are exposed in the heterogametic sex) (Hall & Kirkpatrick, 2006; Lemmon & Kirkpatrick, 2006; Servedio & Saetre, 2003). Our results suggest that ecological adaptation as a function of salinity may be important in the early stages of differentiation in allopatry, but that chromosomal rearrangements involving the sex chromosome may contribute to reinforcement when species come back into secondary contact.

ACKNOWLEDGEMENTS

We thank the editor and three anonymous reviewers for helpful comments that improved the manuscript. This work was funded by a National Science Foundation (NSF) Early Faculty Development (CAREER) grant (DEB-0953716) to R.C.F, a National Science Foundation Dissertation Improvement Grant to R.C.F and E.L.B. (DEB-1110658) and a University of Illinois Research Board Award 11153. This work was approved by the University of Illinois Institutional Animal Care and Use Committee (protocols 08183 and 09306).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/jeb.13725.

DATA AVAILABILITY STATEMENT

R Studio files with code for the QTL mapping, salinity tolerance analysis, and the gametic disequilibrium analysis as well as their associated data files can be found on dryad: doi.org/10.5061/dryad.6wwpzgmvp.

ORCID

Emma L. Berdan https://orcid.org/0000-0002-6435-4604
Rebecca C. Fuller https://orcid.org/0000-0002-5744-8012
Genevieve M. Kozak https://orcid.org/0000-0001-6413-1403

REFERENCES

Bachtrog, D. (2013). Y-chromosome evolution: Emerging insights into processes of Y-chromosome degeneration. *Nature Reviews Genetics*, *14*, 113–124. https://doi.org/10.1038/nrg3366

Bachtrog, D., Kirkpatrick, M., Mank, J. E., McDaniel, S. F., Pires, J. C., Rice, W., & Valenzuela, N. (2011). Are all sex chromosomes created equal? *Trends in Genetics*, *27*, 350–357. https://doi.org/10.1016/j.tig.2011.05.005

Bachtrog, D., Mank, J. E., Peichel, C. L., Kirkpatrick, M., Otto, S. P., Ashman, T.-L., Hahn, M. W., Kitano, J., Mayrose, I., Ming, R., Perrin, N., Ross, L., Valenzuela, N., & Vamosi, J. C. (2014). Sex determination: Why so many ways of doing it? *PLoS Biology*, *12*, e1001899. https://doi.org/10.1371/journal.pbio.1001899

Bay, R. A., Arnegard, M. E., Conte, G. L., Best, J., Bedford, N. L., McCann, S. R., Dubin, M. E., Chan, Y. F., Jones, F. C., Kingsley, D. M., Schluter, D., & Peichel, C. L. (2017). Genetic coupling of female mate choice with polygenic ecological divergence facilitates stickleback speciation. *Current Biology*, *27*, 3344–3349. https://doi.org/10.1016/j.cub.2017.09.037

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Berdan, E. L., & Fuller, R. C. (2012a). Interspecific divergence of ionoregulatory physiology in killifish: Insight into adaptation and speciation. *Journal of Zoology*, *287*, 283–291. https://doi.org/10.1111/j.1469-7998.2012.00914.x

Berdan, E. L., & Fuller, R. C. (2012b). A test for environmental effects on behavioral isolation in two species of killifish. *Evolution*, *66*, 3224–3237. https://doi.org/10.1111/j.1558-5646.2011.01646.x

Berdan, E. L., Kozak, G. M., Ming, R., Rayburn, A. L., Kiehart, R., & Fuller, R. C. (2014). Insight into genomic changes accompanying divergence: genetic linkage maps and synteny of *Lucania goodei* and *L. Parva* Reveal a robertsonian fusion. *G3*, *4*, 1363–1372. https://doi.org/10.1534/g3.114.012096

Berg, P. R., Jentoft, S., Star, B., Ring, K. H., Knutsen, H., Lien, S., Jakobsen, K. S., & André, C. (2015). Adaptation to low salinity promotes genomic divergence in Atlantic cod (*Gadus morhua* L.). *Genome Biology and Evolution*, *7*, 1644–1663. https://doi.org/10.1093/gbe/evv093

Betancur, R., Ortí, G., & Pyron, R. A. (2015). Fossil-based comparative analyses reveal ancient marine ancestry erased by extinction in ray-finned fishes. *Ecology Letters*, *18*, 441–450. https://doi.org/10.1111/ele.12423

Bierne, N., et al. (2011). The coupling hypothesis: why genome scans may fail to map local adaptation genes. *Molecular Ecology*, *20*(10), 2044–2072.

Breder, C. M., & Rosen, D. E. (1966). *Modes of reproduction in fishes*. : American Museum of Natural History by the Natural History Press.

Brennan, R. S., Healy, T. M., Bryant, H. J., La, M. V., Schulte, P. M., & Whitehead, A. (2018). Integrative population and physiological genomics reveals mechanisms of adaptation in killifish. *Molecular Biology and Evolution*, *35*, 2639–2653. https://doi.org/10.1093/molbev/msy154

Broman, K. W., & Sen, Ś. (2009). *A guide to QTL mapping with R/qtl*. Springer-Verlag.

Broman, K. W., Sen, Ś., Owens, S. E., Manichaikul, A., Southard-Smith, E. M., & Churchill, G. A. (2006). The X chromosome in quantitative trait locus mapping. *Genetics*, *174*, 2151–2158. https://doi.org/10.1534/genetics.106.061176

Butlin, R. K. (2005). Recombination and speciation. *Molecular Ecology*, *14*, 2621–2635. https://doi.org/10.1111/j.1365-294x.2005.02617.x

Butlin, R. K., & Smadja, C. M. (2018). Coupling, reinforcement, and speciation. *The American Naturalist*, *191*, 155–172. https://doi.org/10.1086/695136

Charlesworth, B., & Barton, N. H. (2018). The spread of an inversion with migration and selection. *Genetics*, *208*, 377–382. https://doi.org/10.1534/genetics.117.300426

Chen, T. R., & Ruddle, F. H. (1970). A chromosome study of four species and a hybrid of the killifish genus *Fundulus* (Cyprinodontidae). *Chromosoma*, *29*, 255–267. https://doi.org/10.1007/bf00325942

Counterman, B. A., Ortíz-Barrientos, D., & Noor, M. A. (2004). Using comparative genomic data to test for fast-X evolution. *Evolution*, *58*, 656–660. https://doi.org/10.1111/j.0014-3820.2004.tb01688.x

Coyne, J., & Orr, H. A. (2004). *Speciation*. Sinauer Associates.

Cruz, R., Carballo, M., Conde-Padín, P., & Rolán-Alvarez, E. (2004). Testing alternative models for sexual isolation in natural populations of *Littorina saxatilis*: Indirect support for by-product ecological speciation? *Journal of Evolutionary Biology*, *17*, 288–293. https://doi.org/10.1111/j.1420-9101.2003.00689.x

Dobigny, G., Britton-Davidian, J., & Robinson, T. J. (2017). Chromosomal polymorphism in mammals: An evolutionary perspective. *Biological Reviews*, *92*, 1–21. https://doi.org/10.1111/brv.12213

Duggins, C. F., Karlin, A. A., & Relyea, K. G. (1983). Electrophoretic variation in the killifish genus *Lucania*. *Copeia*, *1983*, 564–570. https://doi.org/10.2307/1444415

Dumas, D., & Britton-Davidian, J. (2002). Chromosomal rearrangements and evolution of recombination: Comparison of chiasma distribution patterns in standard and Robertsonian populations of the house mouse. *Genetics*, *162*, 1355–1366. https://doi.org/10.1038/hdy.2014.69

Dunson, W. A., & Travis, J. (1991). The role of abiotic factors in community organization. *The American Naturalist*, *138*, 1067–1091. https://doi.org/10.1086/285270

Ellison, C. K., Wiley, C., & Shaw, K. L. (2011). The genetics of speciation: Genes of small effect underlie sexual isolation in the Hawaiian cricket *Laupala*. *Journal of Evolutionary Biology*, *24*, 1110–1119. https://doi.org/10.1111/j.1420-9101.2011.02244.x

Evans, D. H. (2008). Teleost fish osmoregulation: What have we learned since August Krogh, Homer Smith, and Ancel Keys. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *295*, R704–713. https://doi.org/10.1152/ajpregu.90337.2008

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews*, *85*, 97–177. https://doi.org/10.1152/physrev.00050.2003

Faria, R., & Navarro, A. (2010). Chromosomal speciation revisited: rearranging theory with pieces of evidence. *Trends in Ecology & Evolution*, *25*(11), 660–669.

Fuller, R. C., & Noa, L. A. (2008). Distribution and stability of sympatric populations of *Lucania goodei* and *L. parva* across Florida. *Copeia*, *2008*(3), 699–707.

Feder, J. L., & Nosil, P. (2010). The efficacy of divergence hitchhiking in generating genomic islands during ecological speciation. *Evolution*, *64*, 1729–1747. https://doi.org/10.1111/j.1558-5646.2009.00943.x

Felsenstein, J. (1981). Skepticism towards Santa Rosalia, or why are there so few kinds of animals? *Evolution*, *124–138*, https://doi.org/10.1111/j.1558-5646.1981.tb04864.x

Franchini, P., Colangelo, P., Solano, E., Capanna, E., Verheyen, E., & Castiglia, R. (2010). Reduced gene flow at pericentromeric loci in a hybrid zone involving chromosomal races of the house mouse *Mus musculus domesticus*. *Evolution*, *64*, 2020–2032. https://doi.org/10.1111/j.1558-5646.2010.00964.x

Fuller, R. C. (2008). Genetic incompatibilities in killifish and the role of environment. *Evolution*, *62*, 3056–3068. https://doi.org/10.1111/j.1558-5646.2008.00518.x

Fuller, R. C., McGhee, K. E., & Schrader, M. (2007). Speciation in killifish and the role of salt tolerance. *Journal of Evolutionary Biology*, *20*, 1962–1975. https://doi.org/10.1111/j.1420-9101.2007.01368.x

Grahame, J. W., Wilding, C. S., & Butlin, R. K. (2006). Adaptation to a steep environmental gradient and an associated barrier to gene exchange in *Littorina saxatilis*. *Evolution*, *60*, 268–278. https://doi.org/10.1111/j.0014-3820.2006.tb01105.x

Godfrey, R. K., & Wooten, J. W. (1979). *Aquatic and Wetland Plants of the Southeastern United States: Monocotyledons*, Athens, Georgia: The University of Georgia Press.

Godfrey, R. K., & Wooten, J. W. (1981). *Aquatic and Wetland Plants of the Southeastern United States: Dicotyledons*, Vol. *2*, Athens, Georgia: The University of Georgia Press.

Gunter, G. (1945). Studies on marine fishes of Texas. *Publications of Institute of Marine Science (University of Texas)*, *1*(l): 1–199.

Gunter, G. (1950). Seasonal population changes and distributions as related to salinity of certain invertebrates on the Texas coast, including the commercial shrimp. *Publications of Institute of Marine Science (University of Texas)*, *1*(2), 7–51.

Gunter, G. (1961). Some relations of estuarine organisms to salinity. *Limnology and Oceanography*, *6*(2), 182–190.

Gregorio, O., Berdan, E. L., Kozak, G. M., & Fuller, R. C. (2012). Reinforcement of male mate preferences in sympatric killifish species *Lucania goodei* and *Lucania parva*. *Behavioral Ecology and Sociobiology*, *66*, 1429–1436. https://doi.org/10.1007/s00265-012-1398-0

Guerrero, R. F., & Kirkpatrick, M. (2014). Local adaptation and the evolution of chromosome fusions. *Evolution*, *68*, 2747–2756. https://doi.org/10.1111/evo.12481

Hall, D. W., & Kirkpatrick, M. (2006). Reinforcement and sex linkage. *Evolution*, *60*(5), 908–921. https://doi.org/10.1111/j.0014-3820.2006.tb01170.x

Hoffmann, A. A., & Rieseberg, L. H. (2008). Revisiting the impact of inversions in evolution: From population genetic markers to drivers of adaptive shifts and speciation? *Annual Review of Ecology, Evolution, and Systematics*, *39*, 21–42. https://doi.org/10.1146/annurev.ecolsys.39.110707.173532

Hrbek, T., & Meyer, A. (2003). Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). *Journal of Evolutionary Biology*, *16*, 17–36. https://doi.org/10.1046/j.1420-9101.2003.00475.x

Hubbs, C. L., Hubbs, L. C., & Johnson, R. E. (1943). *Hybridization in nature between species of cyprinodont fishes*, *22*. University of Michigan Press.

Huyse, T., Van Houdt, J., & Volckaert, F. A. (2004). Paleoclimatic history and vicariant speciation in the "sand goby" group (Gobiidae, Teleostei). *Molecular Phylogenetics and Evolution*, *32*, 324–336. https://doi.org/10.1016/j.ympev.2003.11.007

Kay, K. M. (2006). Reproductive isolation between two closely related hummingbird pollinated neotropical gingers. *Evolution*, *60*, 538–552. https://doi.org/10.1111/j.0014-3820.2006.tb01135.x

Kirkpatrick, M., & Barton, N. (2006). Chromosome inversions, local adaptation and speciation. *Genetics*, *173*, 419–434. https://doi.org/10.1534/genetics.105.047985

Kitano, J., & Peichel, C. L. (2012). Turnover of sex chromosomes and speciation in fishes. *Environmental Biology of Fishes*, *94*, 549–558. https://doi.org/10.1007/s10641-011-9853-8

Kitano, J., Ross, J. A., Mori, S., Kume, M., Jones, F. C., Chan, Y. F., Absher, D. M., Grimwood, J., Schmutz, J., Myers, R. M., Kingsley, D. M., & Peichel, C. L. (2009). A role for a neo-sex chromosome in stickleback

speciation. *Nature*, *461*, 1079–1083. https://doi.org/10.1038/nature08441

Kozak, G. M., Brennan, R. S., Berdan, E. L., Fuller, R. C., & Whitehead, A. (2014). Functional and population genomic divergence within and between two species of killifish adapted to different osmotic niches. *Evolution*, *68*, 63–80. https://doi.org/10.1111/evo.12265

Kozak, G. M., Roland, G., Rankhorn, C., Falater, A., Berdan, E. L., & Fuller, R. C. (2015). Behavioral isolation due to cascade reinforcement in *Lucania* killifish. *The American Naturalist*, *185*, 491–506. https://doi.org/10.1086/680023

Kozak, G. M., Rudolph, A. B., Colon, B. L., & Fuller, R. C. (2012). Postzygotic isolation evolves before prezygotic isolation between fresh and saltwater populations of the rainwater killifish, *Lucania parva*. *International Journal of Evolutionary Biology*, *2012*. https://doi.org/10.1155/2012/523967

Larsen, P. F., Schulte, P. M., & Nielsen, E. E. (2011). Gene expression analysis for the identification of selection and local adaptation in fishes. *Journal of Fish Biology*, *78*, 1–22. https://doi.org/10.1111/j.1095-8649.2010.02834.x

Laverty, G., & Skadhauge, E. (2012). Adaptation of teleosts to very high salinity. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *163*, 1–6. https://doi.org/10.1016/j.cbpa.2012.05.203

Lee, C. E., & Bell, M. A. (1999). Causes and consequences of recent freshwater invasions by saltwater animals. *Trends in Ecology and Evolution*, *14*, 284–288. https://doi.org/10.1016/s0169-5347(99)01596-7

Lemmon, A. R., & Kirkpatrick, M. (2006). Reinforcement and the genetics of hybrid incompatibilities. *Genetics*, *173*, 1145–1155. https://doi.org/10.1534/genetics.105.048199

Lowry, D. B., Rockwood, R. C., & Willis, J. H. (2008). Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. *Evolution*, *62*, 2196–2214. https://doi.org/10.1111/j.1558-5646.2008.00457.x

Lowry, D. B., & Willis, J. H. (2010). A widespread chromosomal inversion polymorphism contributes to a major life-history transition, local adaptation, and reproductive isolation. *PLoS Biology*, *8*, e1000500. https://doi.org/10.1371/journal.pbio.1000500

Mank, J. E., Promislow, D. E., & Avise, J. C. (2005). Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. *Evolution*, *59*, 1570–1578. https://doi.org/10.1111/j.0014-3820.2005.tb01806.x

Meisel, R. P., & Connallon, T. (2013). The faster-X effect: Integrating theory and data. *Trends in Genetics*, *29*, 537–544. https://doi.org/10.1016/j.tig.2013.05.009

Moser, D., Frey, A., & Berner, D. (2016). Fitness differences between parapatric lake and stream stickleback revealed by a field transplant. *Journal of Evolutionary Biology*, *29*, 711–719. https://doi.org/10.1111/jeb.12817

Naisbit, R. E., Jiggins, C. D., & Mallet, J. (2001). Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *268*, 1849–1854. https://doi.org/10.1098/rspb.2001.1753

Noor, M. A., Cunningham, A. L., & Larkin, J. C. (2001). Consequences of recombination rate variation on quantitative trait locus mapping studies: Simulations based on the *Drosophila melanogaster* genome. *Genetics*, *159*, 581–588. https://doi.org/10.1101/787382

Noor, M. A., Grams, K. L., Bertucci, L. A., & Reiland, J. (2001). Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences*, *98*, 12084–12088. https://doi.org/10.1073/pnas.221274498

Norman, J. D., Robinson, M., Glebe, B., Ferguson, M. M., & Danzmann, R. G. (2012). Genomic arrangement of salinity tolerance QTLs in salmonids: A comparative analysis of Atlantic salmon (*Salmo salar*) with Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*). *BMC Genomics*, *13*, 420. https://doi.org/10.1186/1471-2164-13-420

Nosil, P. (2012). *Ecological speciation*. Oxford University Press.

Nosil, P., Harmon, L. J., & Seehausen, O. (2009). Ecological explanations for (incomplete) speciation. *Trends in Ecology and Evolution*, *24*, 145–156. https://doi.org/10.1016/j.tree.2008.10.011

Nosil, P., Vines, T. H., & Funk, D. J. (2005). Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution*, *59*, 705–719. https://doi.org/10.1111/j.0014-3820.2005.tb01747.x

Ortiz-Barrientos, D., Grealy, A., & Nosil, P. (2009). The genetics and ecology of reinforcement: Implications for the evolution of prezygotic isolation in sympatry and beyond. *Annals of the New York Academy of Sciences*, *1168*, 156–182. https://doi.org/10.1111/j.1749-6632.2009.04919.x

Ouellette, L. A., Reid, R. W., Blanchard, Jr., S. G., & Brouwer, C. R. (2017). LinkageMapView - rendering high resolution linkage and QTL maps. *Bioinformatics*, *34*(2), 306–307.

Peichel, C. L., McCann, S. R., Ross, J. A., Naftaly, A. F. S., Urton, J. R., Cech, J. N., Grimwood, J., Schmutz, J., Myers, R. M., Kingsley, D. M., & White, M. A. (2020). Assembly of the threespine stickleback Y chromosome reveals convergent signatures of sex chromosome evolution. *Genome Biology*, *21*, 1–31. https://doi.org/10.1186/s13059-020-02097-x

Pennell, M. W., Kirkpatrick, M., Otto, S. P., Vamosi, J. C., Peichel, C. L., Valenzuela, N., & Kitano, J. (2015). Y fuse? Sex chromosome fusions in fishes and reptiles. *PLoS Genetics*, *11*, e1005237. https://doi.org/10.1371/journal.pgen.1005237

Presgraves, D. C. (2018). Evaluating genomic signatures of "the large X-effect" during complex speciation. *Molecular Ecology*, *27*, 3822–3830. https://doi.org/10.1111/mec.14777

Presgraves, D. C., Balagopalan, L., Abmayr, S. M., & Orr, H. A. (2003). Adaptive evolution drives divergence of a hybrid inviability gene between two species of *Drosophila*. *Nature*, *423*, 715–719. https://doi.org/10.1038/nature01679

R Core Team (2018). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Ramsey, J., Bradshaw, H. D. Jr, & Schemske, D. W. (2003). Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution*, *57*, 1520–1534. https://doi.org/10.1111/j.0014-3820.2003.tb00360.x

Renaut, S., Nolte, A. W., Rogers, S. M., Derome, N., & Bernatchez, L. (2011). SNP signatures of selection on standing genetic variation and their association with adaptive phenotypes along gradients of ecological speciation in lake whitefish species pairs (*Coregonus* spp.). *Molecular Ecology*, *20*, 545–559. https://doi.org/10.1111/j.1365-294x.2010.04952.x

Rundle, H. D., & Nosil, P. (2005). Ecological speciation. *Ecology Letters*, *8*, 336–352. https://doi.org/10.1111/j.1461-0248.2004.00715.x

Saether, S. A., Saetre, G.-P., Borge, T., Wiley, C., Svedin, N., Andersson, G., Veen, T., Haavie, J., Servedio, M. R., Bures, S., Kral, M., Hjernquist, M. B., Gustafsson, L., Traff, J., & Qvarnstrom, A. (2007). Sex chromosome-linked species recognition and evolution of reproductive isolation in flycatchers. *Science*, *318*, 95–97. https://doi.org/10.1126/science.1141506

Schaefer, J., Duvernell, D., & Campbell, D. C. (2016). Hybridization and introgression in two ecologically dissimilar *Fundulus* hybrid zones. *Evolution*, *70*, 1051–1063. https://doi.org/10.1111/evo.12920

Schemske, D. W. (2010). Adaptation and the origin of species. *The American Naturalist*, *176*, S4–S25. https://doi.org/10.1086/657060

Schluter, D. (2001). Ecology and the origin of species. *Trends in Ecology and Evolution*, *16*, 372–380. https://doi.org/10.1016/s0169-5347(01)02198-x

Schluter, D. (2009). Evidence for ecological speciation and its alternative. *Science*, *323*, 737–741. https://doi.org/10.1126/science.1160006

Seehausen, O., & Wagner, C. E. (2014). Speciation in freshwater fishes. *Annual Review of Ecology, Evolution, and Systematics*, *45*, 621–651. https://doi.org/10.1146/annurev-ecolsys-120213-091818

Servedio, M. R., & Noor, M. A. (2003). The role of reinforcement in speciation: Theory and data. *Annual Review of Ecology, Evolution, and Systematics*, *34*, 339–364. https://doi.org/10.1146/annurev.ecolsys.34.011802.132412

Servedio, M. R., & Sætre, G. P. (2003). Speciation as a positive feedback loop between postzygotic and prezygotic barriers to gene flow. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*, 1473–1479. https://doi.org/10.1098/rspb.2003.2391

St. John, M. E., & Fuller, R. C. (2019). The effects of experimental design on mating preferences and reproductive isolation in killifish. *Behavioral Ecology*, *30*, 92–100. https://doi.org/10.1093/beheco/ary150

Staelens, J., Rombaut, D., Vercauteren, I., Argue, B., Benzie, J., & Vuylsteke, M. (2008). High-density linkage maps and sex-linked markers for the black tiger shrimp (*Penaeus monodon*). *Genetics*, *179*, 917–925. https://doi.org/10.1534/genetics.107.080150

Star, B., Tørresen, O. K., Nederbragt, A. J., Jakobsen, K. S., Pampoulie, C., & Jentoft, S. (2016). Genomic characterization of the Atlantic cod sex-locus. *Scientific Reports*, *6*, 31235. https://doi.org/10.1038/srep31235

Stratton, G. E., & Uetz, G. W. (1986). The inheritance of courtship behavior and its role as a reproductive isolating mechanism in two species of *Schizocosa* wolf spiders (Araneae; Lycosidae). *Evolution*, *40*, 129–141. https://doi.org/10.1111/j.1558-5646.1986.tb05724.x

Sung, C. J., Bell, K. L., Nice, C. C., & Martin, N. H. (2018). Integrating Bayesian genomic cline analyses and association mapping of morphological and ecological traits to dissect reproductive isolation and introgression in a Louisiana Iris hybrid zone. *Molecular Ecology*, *27*, 959–978. https://doi.org/10.1111/mec.14481

Taylor, E. B. (1999). Species pairs of north temperate freshwater fishes: Evolution, taxonomy, and conservation. *Reviews in Fish Biology and Fisheries*, *9*, 299–324.

Torres-Dowdall, J., Dargent, F., Handelsman, C. A., Ramnarine, I. W., & Ghalambor, C. K. (2013). Ecological correlates of the distribution limits of two poeciliid species along a salinity gradient. *Biological Journal of the Linnean Society*, *108*, 790–805. https://doi.org/10.1111/bij.12031

Turelli, M., & Moyle, L. C. (2007). Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. *Genetics*, *176*, 1059–1088. https://doi.org/10.1534/genetics.106.065979

Uyeno, T., & Miller, R. R. (1971). Multiple sex chromosomes in a Mexican cyprinodontid fish. *Nature*, *231*, 452–453. https://doi.org/10.1038/231452a0

Van Ooijen, J. W. (2006). *JoinMap 4. Software for the calculation of genetic linkage maps in experimental populations*. Kyazma BV.

Wellband, K., Mérot, C., Linnansaari, T., Elliott, J. A. K., Curry, R. A., & Bernatchez, L. (2019). Chromosomal fusion and life history-associated genomic variation contribute to within-river local adaptation of Atlantic salmon. *Molecular Ecology*, *28*, 1439–1459. https://doi.org/10.1111/mec.14965

Wellenreuther, M., & Bernatchez, L. (2018). Eco-evolutionary genomics of chromosomal inversions. *Trends in Ecology and Evolution*, *33*, 427–440. https://doi.org/10.1016/j.tree.2018.04.002

Wellenreuther, M., Mérot, C., Berdan, E., & Bernatchez, L. (2019). Going beyond SNPs: The role of structural genomic variants in adaptive evolution and species diversification. *Molecular Ecology*, *28*, 1203–1209. https://doi.org/10.1111/mec.15066

Whitehead, A. (2010). The evolutionary radiation of diverse osmotolerant physiologies in killifish (*Fundulus* sp.). *Evolution*, *64*, 2070–2085. https://doi.org/10.1111/j.1558-5646.2010.00957.x

Whitehead, A., Roach, J. L., Zhang, S., & Galvez, F. (2011). Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *Proceedings of the National Academy of Sciences*, *108*, 6193–6198. https://doi.org/10.1073/pnas.1017542108

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer.

Xu, S. (2003). Theoretical basis of the Beavis effect. *Genetics*, *165*, 2259–2268. https://doi.org/10.1007/bf00288854

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Berdan EL, Fuller RC, Kozak GM. Genomic landscape of reproductive isolation in *Lucania* killifish: The role of sex loci and salinity. *J Evol Biol*. 2021;34:157–174. https://doi.org/10.1111/jeb.13725