



Trioecy in the Marine Mussel *Semimytilus algosus* (Mollusca, Bivalvia): Stable Sex Ratios Across 22 Degrees of a Latitudinal Gradient

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Trioecy, the co-occurrence of males, females and hermaphrodites in natural populations, is a relatively common sexual system in plants, but is rare and poorly understood in animals. Previously, the marine mussel, *Semimytilus algosus*, has been described as a simultaneous hermaphrodite and there are reports of unisex animals in the wild. Here, we confirm trioecy in this bivalve, the first report of trioecy in the phylum Mollusca. We examined the frequencies of females, males and hermaphrodites in seven natural populations along a latitudinal gradient of ~2500 km and carried out a phylogenetic analysis with mussel species of the family Mytilidae with known sexual systems. Hermaphrodites (~95.3%), females (~3.6%), and males (~1.1%) were found in all seven populations across the species' range in Chile. The consistent sex ratios across all populations suggest that the sexual system is stable in space. Phylogenetic analysis (mtDNA) of members of the family Mytilidae indicates that this species alone has developed trioecy from dioecious ancestors. As unisex animals were the least common in the wild, it is likely that these animals, especially males, might be in the process of being lost in this species, and that trioecy might be an intermediate step towards gynodioecy or hermaphroditism. The reproductive characteristics of *S. algosus* make it an excellent model species for the study of the evolution of sexual systems in animals and also possibly the processes underlying sex determination in molluscs.

Keywords: Chile, gonochorism, intertidal mussels, mtDNA, Mollusca, reproduction, sexual ratio, simultaneous hermaphroditism

INTRODUCTION

One of the fundamental challenges of evolutionary biology is to understand breeding systems and to determine how and why different breeding systems exist (Darwin, 1876; Maynard Smith, 1978). Whilst a range of different breeding systems exist amongst the Animalia (reviewed by Weeks, 2012), there are two common sexual systems, dioecy (or gonochorism), in which individuals are either male or female during their reproductive lives but not both, and hermaphroditism, in which animals produce both eggs and sperm during their breeding lives (Avisé and Mank, 2009;

Leonard, 2010). Most animals are, however, dioecious and it is estimated that hermaphroditism occurs in only 5–6% of species (Jarne and Auld, 2006).

Mixed sexual systems are found in populations that contain a mixture of hermaphrodites and single-sex individuals, such as androdioecy (co-occurrence of males and hermaphrodites), gynodioecy (co-occurrence of females and hermaphrodites) and trioecy (males, female and hermaphrodites). Trioecy in natural populations is a rare and poorly understood sexual system in animals (Weeks, 2012; Armoza-Zvuloni et al., 2014). Two key questions related to breeding systems have long been of interest to evolutionary biologists. First, under what conditions do different sexual systems exist (Ghiselin, 1969; Maynard Smith, 1978)? And second, how stable are such sexual systems or are they simply transition states to another sexual systems (Charnov, 1982; Charlesworth, 1984)? With regard to the first question, a number of different factors have been identified or proposed as being important in promoting the existence of different breeding systems, in particular in plants (Barrett, 2002; Jones et al., 2013; Petry et al., 2016; Ansaldi et al., 2018), sometimes within populations of the same species in close geographic proximity. For example, Fleming et al. (1998) describe an example in the Sonoran Desert columnar cactus (*Pachycereus pringlei*) where trioecy occurs near the roosts of its major pollinator, the bat *Leptonycteris curasoae*, whilst gynodioecy occurs >50 km away from roosts. The abundance of the bat pollinators is suggested to play a critical role in the maintenance of this mixed sexual system. Significantly, in particular with regard to the second point, Fleming et al. (1998) note that trioecy can be a stable sexual system in this cactus when bat pollinators are limited in their abundance.

In their review of mixed sexual systems (which did not include trioecy) Eppley and Jesson (2008) tested the hypothesis that an organism's ability to move to find a mate influences its mating system (e.g., Ghiselin, 1969; Heath, 1977; Charnov, 1979; Puurtinen and Kaitala, 2002). They showed that taxa with well developed mate search capabilities tend to have separate sexes, whereas sessile taxa and those species with poorly developed mate search capabilities tend to exhibit increased hermaphroditism. Thus, sessile organisms such as plants and many marine invertebrates may be expected to exhibit greater levels of hermaphroditism than motile organisms. Following from this, it is often considered that different reproductive strategies appear during the evolutionary transition between dioecy and hermaphroditism or vice versa (Charlesworth, 2006; Weeks, 2012; Weeks et al., 2014a,b). This sort of transition may have occurred multiple times and is better understood in plants than in animals (Weiblen et al., 2000; Weeks, 2012). Unlike in plants, transitions in animals are mostly from dioecious systems to hermaphrodites (Ghiselin, 1969; Eppley and Jesson, 2008; Weeks, 2012; Sasson and Ryan, 2017). This reproductive transition has received less attention both theoretically and empirically (Ghiselin, 1974; Charnov, 1982; Weeks, 2012), probably due to the lack of plausible models that allow for hypothesis testing. Therefore, the study of populations of a species where hermaphrodite and unisex animals co-occur is crucial to aid

our understanding not only of the reproductive dynamics of the species, but also the evolution of the sexual system.

In bivalve molluscs, the presence of separate sexes (dioecy) is the most frequent reproductive strategy (Collin, 2013; Gosling, 2015). Only 9% of species exhibit hermaphroditism (Heller, 1993), and these are mainly associated with certain groups (e.g., oysters of the genera *Ostrea* and *Crassostrea*). However, hermaphroditic individuals have been found in species that are considered to be strictly dioecious (Ghiselin, 1969; Heller, 1993). For example, there are reports of hermaphroditic animals in some populations of mytilids (true mussels), but they usually correspond to isolated cases that represent <<1% of the population (Vinuesa, 1977; Saavedra et al., 1997; Alfaro et al., 2001; Barber et al., 2005; Montenegro et al., 2010; Al-Barwani et al., 2013; Abdel Razek et al., 2017; Torroglosa and Giménez, 2019). Whilst these occurrences of hermaphrodites amongst the Bivalvia may be natural but very rare, in other instances the phenomenon of hermaphroditism has been attributed to ecotoxic events (Nice et al., 2003; Ciocan et al., 2012; Dublinowska et al., 2016) or drastic population fluctuations (Adjei-Boateng and Wilson, 2016), which probably alter the mechanism of sexual differentiation (Yusa, 2009). However, only a few studies have performed an in-depth analysis of the causes behind the appearance of bisexual animals in dioecious species (Morton, 1991; Romo-Piñera et al., 2009; Dublinowska et al., 2016).

Semimytilus algosus (Gould 1850) is a marine bivalve mollusc belonging to the family Mytilidae, which inhabits the lower intertidal zone of the Pacific coastline of South America (Broitman et al., 2001; Navarrete et al., 2008). It has a wide distribution, from Manta, Ecuador (0°56'S) to Maicolpue, Chile (40°35'S) (personal observation, Pablo Oyarzún). González et al. (1980) first described the hermaphroditism of *S. algosus* from Concepción, near the BíoBío river, Chile (36°48'S), and reported that gonad tissue is located in two compartments: on one side of the mantle there is an intense yellow tissue corresponding to the male tissue, and on the opposite side there is the female tissue that has a dark brown colour. Subsequently, Garrido (1996) analysed the *S. algosus* population of Mehuín (southern Chile, 39°26'S) and noted that, in addition to hermaphrodites, some individuals exhibited gonadal tissue of a single-sex (i.e., unisex animals). This information, collected from two geographically distinct sites and at different times, raises the possibility that *S. algosus* has a mixed reproductive system – trioecy – and may therefore represent an opportunity to understand how an unusual reproductive system has evolved in a predominantly dioecious group, the family Mytilidae (true marine mussels). The objectives of the present work were: (1) to test our hypothesis that the mussel, *S. algosus*, has evolved a sexual system based on trioecy, (2) to examine the spatial stability of the sexual system across multiple independent (non-interbreeding) populations along a natural latitudinal gradient of ~ 2500 km, and (3) determine the phylogenetic position of this mussel within the broader evolutionary context of the family Mytilidae of the phylum Mollusca, that is known to exhibit dioecy.

TABLE 1 | Site survey information in Chile, number of *Semimytilus algosus* specimens collected (*N*), date of collection, geographical coordinates of sites and percentage of males, females and hermaphrodites.

Sites	Coordinates		Date	<i>N</i>	Female (%)	Male (%)	Hermaphroditism (%)
Arica	18°27'50.49"	70°18'23.79"	04.03.2018	607	4.8	0.6	94.6
Zapallar	32°33'06.12"	71°27'40.50"	25.02.2017	599	2.2	0.5	97.3
Maitencillo	32°38'52.89"	71°26'19.08"	23.02.2017	612	2.1	0.4	97.5
Colcura	37°07'06.77"	73°09'16.69"	10.12.2017	502	3.4	1.2	95.4
Mehuín	39°25'32.18"	73°13'00.89"	16.09.2017	589	4.4	2.3	93.3
Calfuco	39°46'49.64"	73°23'33.99"	12.10.2017	597	2.2	1.3	96.5
Maicolpue	40°35'35.08"	73°44'09.87"	29.11.2017	602	6.1	1.2	92.7
Mean					3.6	1.1	95.3

MATERIALS AND METHODS

Sampling Sites

Semimytilus algosus specimens were collected haphazardly from seven intertidal rocky coastal sites in Chile, spanning approximately 22 degrees of latitude and nearly 2500 km (Table 1). In total, 4108 mussels were sampled between February 2017 and March 2018. Population-specific samples were collected in different months, during early spring to late autumn. Field observations indicate that gonads are developing or in spawning condition for most of the year (González et al., 1980) i.e., there is no tightly synchronised period of spawning activity as reported elsewhere for other mytilids. Specimens of *Brachidontes rodriguezii* (from Las Grutas, Argentina), *Choromytilus chorus* (from Colún, Chile), *Mytilus chilensis* (from Quillaiepe, Chile) and *Perumytilus purpuratus* (from Calfuco, Chile) were also collected for molecular analysis only (details in subsequent section). This was to permit comparison of the sexual system type of *S. algosus* (a member of the family Mytilidae) to the reproductive strategies that these mytilid species all possess (dioecy), given the relatively close evolutionary affinities of all of the species. All mussels were kept alive in seawater at low temperature during their transfer to the Systematics Laboratory of the Instituto de Ciencias Marinas y Limnológicas (ICML) at the Universidad Austral de Chile, Valdivia.

Gonad Dissection, Histological Procedure and Analyses

All 4108 *S. algosus* mussels were dissected to analyse the gonad tissue (Table 1). Gonad was carefully removed from the shell; gonadal smears (Torrado and Mikhailov, 1998) and microscopic observation of germ cells were performed in the 4108 mussels analysed. Gonad colouration (females = brown, males = yellow – González et al., 1980) was also recorded in order to test this phenotypic characteristic.

For histological procedures, 20 hermaphroditic mussels per site were examined. All unisex animals (male: 2–14; female: 13–37 per sites) were also included in the histological procedure (see Table 1). A piece of the middle region of the gonad of each specimen was extracted and fixed in 10% formalin for 10 days. Alcoholic dehydration, inclusion, sectioning (7 μm) and staining (hematoxylin-eosin) were performed according to Oyarzún et al. (2018). Gonad development was quantified

following standard terminology as applied to bivalve molluscs. Individual mussels were sexed (male, female, hermaphrodite) and sex ratios calculated for each of the seven populations.

A one-way ANOVA was performed to assess whether there were differences in maximum length (larger size = older age – Gray et al., 1997) with respect to the reproductive category (hermaphrodites, males and females) to assess whether sexual categories were related to or independent of size. Normality was tested using the Kolmogorov–Smirnov test corrected by Lilliefors; Levene's test was used to test for equality of variances. A statistical power test was also performed. The χ^2 test of independence (3 × 7 matrix) was used to assess whether the frequencies of hermaphrodites, females and males varied amongst sites. These analyses were carried out using the “corrplot” package implemented in R-project (R Development Core Team, 2008).

DNA Extraction, Amplification, Sequencing, and Alignment

Each *S. algosus* mussel was dissected and a small piece of mantle edge tissue was preserved in 95% ethanol and stored at 4°C. Approximately 25 mg of mantle tissue was isolated from each individual and total genomic DNA was extracted using a genomic E.Z.N.A.® Tissue DNA kit according to the manufacturer's instructions. Partial cytochrome *c* oxidase subunit I (COI) mitochondrial gene sequence was amplified using the primers SemMytF1/HCO2198 (De Greef et al., 2014) for *S. algosus*, CO1aF/CO1aR (Trovant et al., 2013) for *P. purpuratus*, and LCO1490/HCO2198 (Folmer et al., 1994) for the other three species. Standard PCR amplifications were carried out in 25 μL reaction mixtures containing 2 μL template DNA, 0.2 mM total dNTP, 2 mM MgCl₂, 0.4 mM of each primer, 1U of Taq (Invitrogen™), the manufacturer-supplied PCR buffer, and sterile distilled water. The PCR conditions involved an initial denaturation step at 93°C for 3 min, followed by 35 cycles at 93°C for 30 s, annealing at 52°C for 30 s (*S. algosus* 42°C × 30 s) and elongation at 72°C for 1 min, followed by further elongation at 72°C for 5 min.

All PCR amplification products were scored on 2% agarose gels stained with SYBR® Safe DNA and photographed under a blue-light transilluminator (Invitrogen™). For every gel, the sizes of amplified fragments were estimated from a 100 bp DNA ladder (Invitrogen™). Amplicons were purified and

sequenced by Macrogen (Seoul, South Korea). Both sequence directions were determined, using the individual primers from the original reaction. The DNA sequence (601 bp in length) was edited using Geneious® 11.0.4 (Biomatters Ltd., Auckland, New Zealand). All new sequences were deposited in GenBank under accession numbers MT103127–MT103143. Sequences of additional mytilids were obtained from GenBank (**Supplementary Table S1**). All nucleotide sequences were aligned using MAFFT v.7 (Katoh and Standley, 2013) under the iterative method of global pairwise alignment (G-INS-i) (Katoh et al., 2005), and default settings were chosen for all parameters.

Phylogenetic Analyses

Phylogenetic trees were constructed using Maximum Likelihood (ML) and Bayesian inference (BI). Evolutionary models and partitioning strategies were evaluated with PartitionFinder v2.1.1 (Lanfear et al., 2017), which chose the best partition under the BIC criterion (Schwarz, 1978). A ML tree was inferred using GARLI v2.0 (Bazin et al., 2014) with branch support being estimated by non-parametric bootstrap (200 replicates). Bayesian analyses were performed using MrBayes v3.2 (Ronquist et al., 2012). Each Markov chain was started from a random tree and run for 5.0×10^7 generations with every 1000th generation sampled from the chain. Stationarity was checked as suggested in Nylander et al. (2004). All sample points prior to reaching the plateau phase were discarded as “burn-in”, and the remaining trees were combined to find the *a posteriori* probability of phylogeny. Analyses were repeated four times to confirm that they all converged on the same results. Posterior probability values > 0.90 were taken as statistical support for a clade being present on the true tree (Huelsenbeck and Rannala, 2004).

RESULTS

Gonadal Analysis of *S. algosus*

In total, 4108 specimens of *S. algosus* with a shell length range of 11.44–38.70 mm were analysed. We had a probability 0.92 to detect (with threshold $p = 0.05$) an effect size of 4.97 with our sample size of $n > 75$ for each of three groups. The one-way ANOVA showed no significant differences amongst reproductive category type (hermaphrodites, males and females) as a function of mussel size ($F_{[2,1402]} = 2.776$; $P > 0.05$) (see size frequency – **Supplementary Figure S2**). *S. algosus* exhibited typical gonadal characteristics of mussels, with the two gonadal lobes (one on the left and one on right) containing almost all of the gonad. In hermaphroditic animals, one mantle lobe was male gonad (yellow) whilst the other lobe was female (dark brown), so the gonad was separated into a male and female portion (**Figure 1**), in all cases. The histological sections and the smears corroborated that tissue coloration is a sexual characteristic (**Figure 1**).

The gonads from unisex animals did not exhibit gametogenic differences compared to the hermaphroditic animals. In the female gonadal tissue, oocytes were connected to the follicular wall and were previtellogenic and/or vitellogenic (**Figure 1A**). Male tissue exhibited radially arranged spermatozoa in the lumen

(**Figure 1B**). In both males and females, little connective tissue was observed (**Figure 1**).

Sex Ratios

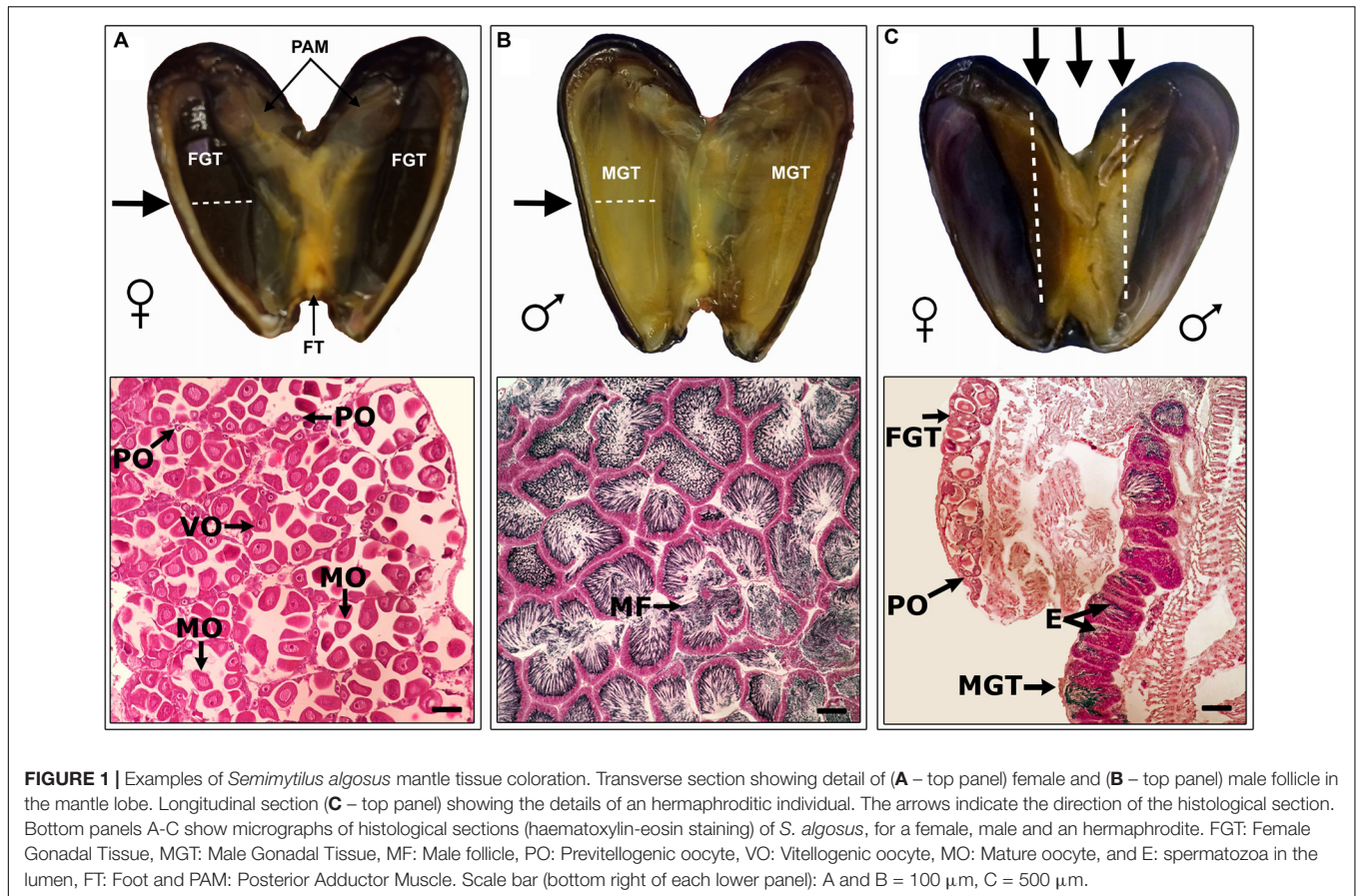
Unisex animals (males and females) and simultaneous hermaphrodites were found at all sites (**Table 1**). Significant differences were observed in the frequencies of hermaphrodites, females and males amongst populations ($\chi^2 = 41.967$; $df = 12$; P -value < 0.05). However, these differences were strongly influenced by the values obtained for males from Mehuín (2.3%) and females from Maicolpue (6.1%) (refer to **Supplementary Figure S1**). Hermaphrodites occurred at greatest abundance in all populations (92.7–97.5%), females corresponded to 2.1–6.1% of the mussels, and males occurred at the lowest frequency in all populations (0.4–2.3%) (**Table 1**).

Phylogenetic Relationships

Testing for the best fit substitution model resulted in the selection of models TrN + I + G (1st, best fit), TVM + G (2nd) and TrN + G (3rd) for COI. The BI and ML analyses revealed almost identical topologies with two major clades strongly supported (**Figure 2**). Clade 1 included the subfamilies Arcuatulinae, Crenellinae, Musculinae, and Mytilinae. Clade 2 included *S. algosus* and members of the subfamily Brachidontinae (*Mytilaster*, *Brachidontes*, *Ischadium*, *Geukensia*, and *Perumytilus*) (**Figure 2** and **Supplementary Table S1**), and within Clade 2, *Brachidontes rodriguezii* was sister to the rest of the genera. The clustering of *S. algosus* (trioecy) with *P. purpuratus* (dioecy) was strongly supported (*a posteriori* probability = 1.0; bootstrap support of 99%; **Figure 2**). Based on our phylogenetic reconstruction, *S. algosus* is placed for the first time as part of the monophyletic subfamily Brachidontinae.

DISCUSSION

Building on earlier reports of an unusual, but unconfirmed type of sexual system (González et al., 1980; Garrido, 1996), we report here, for the first time, the occurrence of trioecy in the South American intertidal marine mussel, *S. algosus*, which is also the first report of trioecy amongst members of the phylum Mollusca, the second-most speciose animal phylum (Kershaw, 1983). Animal trioecy is extremely rare, and, to the best of our knowledge, examples are limited to the sea anemones *Aiptasia diaphana* (Armoza-Zvuloni et al., 2014) and *Bartholomea annulata* (Jennison, 1981), the green hydra *Hydra viridissima* (Kaliszewicz, 2011) and the nematodes *Auanema rhodensis*, *A. freiburgensis* (Kanzaki et al., 2017) and *Rhabditis* sp. SB347 (Chaudhuri et al., 2011, 2015). Sex ratios differ considerably from one case to another. This can make it difficult to search for new cases of sexual trimorphism, because trioecy can be confused with the intersex state. This is a topic that needs further examination, although given the relatively rare occurrence of trioecy this may not be easy to achieve. Trioecy in animals is thought to be so rare because it represents an evolutionary unstable step from one mating system to another



(Leonard, 2018), but this does not completely explain its greater rarity in animals than in plants.

The Trioecy of *S. algosus*

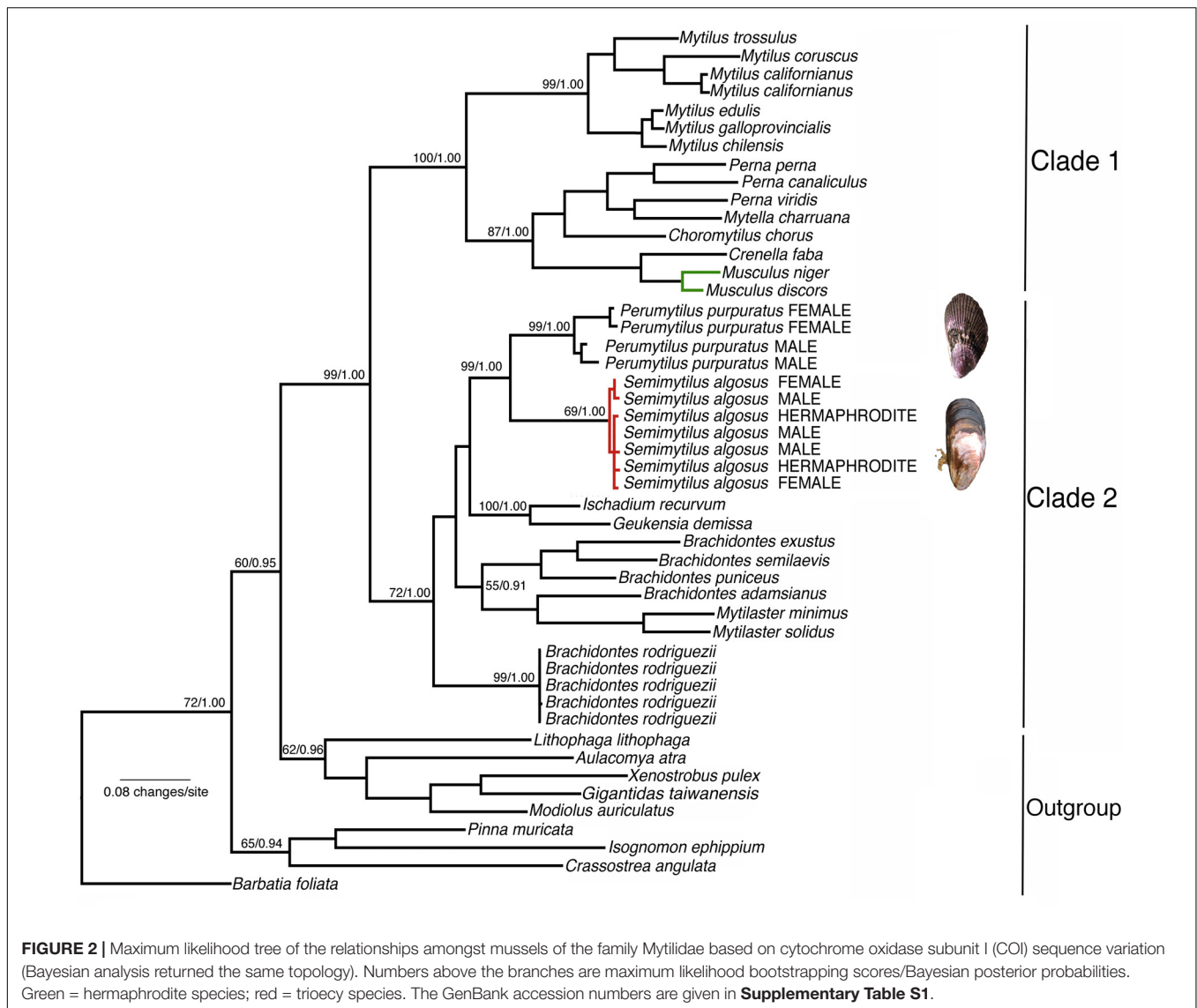
Analysis of gonad tissue from seven geographically distinct populations of the marine mussel, *S. algosus*, has identified the co-occurrence of hermaphrodites, females and males. In stark contrast to other reports of trioecy amongst animals where it is usually reported that hermaphrodites are scarce and/or exhibit abnormal development (Greve, 1996; Labruna et al., 2002; Maeno and Tanaka, 2007; Yoshizawa et al., 2009; Armoza-Zvuloni et al., 2014; Chaudhuri et al., 2015; Kanzaki et al., 2017), in *S. algosus* the hermaphroditic animals were the most abundant sex, whilst the unisex individuals combined represented \sim 5% of each population. The gonadal tissues of the three sexual phenotypes showed normal gametogenic development, i.e., the presence of follicles with cells at different stages of development. Both male and female gametes had characteristics similar to the germ cells described in other mytilid species (González et al., 1980; Toro et al., 2002; Oyarzún et al., 2010, 2011).

One of the most striking points from our results is the relative constancy of the ratio of the three sexes across the seven populations, that span more than 22 degrees of latitude, a distance of \sim 2500 km, from the subtropical north to the cool temperate south of Chile. Across all populations, regardless

of environment, the ratio of hermaphrodites:females:males was remarkably similar, at a mean of 95.3:3.6:1.1. This suggests that the trioecious state in *S. algosus* is stable over a large part of the range of the species. Our results are in contrast to those for others species, where it has been reported how factors such as pollinator density or environmental variation can influence the sexual system of a species, even over reasonably small spatial scales (Fleming et al., 1998; Barrett, 2002; Jones et al., 2013; Petry et al., 2016; Ansaldi et al., 2018). In addition, we rule out the possibility of *S. algosus* shows a different size in each reproductive category (ANOVA reveals that sexual category is independent of shell length, when length is a surrogate for age), as has been observed in other molluscs (e.g., *Ostrea chilensis* – DiSalvo et al., 1983).

Hermaphroditism in *S. algosus*

Hermaphroditism is a rarity amongst the species of the family Mytilidae (see **Supplementary Table S1**). Mytilid hermaphrodites are considered to be an exception produced by some abnormality in the determination of sex (Yusa, 2009; Dublinowska et al., 2016). However, the hermaphroditic individuals of *S. algosus* are expected to be the main sexual phenotype involved in a population's reproductive output given that they are the most abundant group by far.



Species with simultaneous hermaphroditism are subject to the probability of self-fertilisation (Liu et al., 2011). The hypothesis of "reproductive assurance" suggests that self-fertilisation occurs because animals that are capable of reproducing on their own ensure the persistence of the population in environments where mixed sex individuals are not available (Theologidis et al., 2014). It has, however, also been suggested that self-fertilisation may be associated with low reproductive potential and, therefore, with a loss of biological fitness (Charlesworth and Charlesworth, 1987). As a newly-reported invasive species in South Africa, *S. algosus* exhibits a high reproductive output and a high recruitment rate, factors that are likely to have contributed to its invasion success (De Greef et al., 2014; Zeeman et al., 2018). These reproductive traits are believed to have given *S. algosus* an advantage over the endemic mytilids, *Choromytilus meridionalis* and *Aulacomya atra*, both of which are dioecious, resulting in a stable coexistence of the three sexual phenotypes for *S. algosus* and suggesting that

S. algosus has an effective mechanism to avoid self-fertilisation (see later).

The gonads of *S. algosus* contained female and male gametes that were located independently. This gametic organisation is the most common form reported in hermaphroditic molluscs – it is known as a type I gonad (Sastry, 1979; Soria et al., 2002). The localisation of the gonads in separate compartments is probably a reproductive strategy to avoid self-fertilisation because the animal can control gametic emissions (spawning activity) independently (personal observation – P. Oyarzún). This suggestion is supported by the fact that Garrido (1996) observed an asynchrony in the spawning of the male and female gametes of the hermaphroditic mussels of *S. algosus*. Thus, *S. algosus* appears to employ a pre-zygotic mechanism (i.e., spawning asynchrony, possibly under direct control of gamete release) rather than a post-zygotic mechanism (e.g., some form of larval developmental incompatibility) to prevent self-fertilisation.

Trioecy as an Unstable Transitional State in Sexual Systems

It has been suggested that trioecy is an unstable state in a transition to another state such as dioecy, androdioecy or gynodioecy (Maurice and Fleming, 1995; Wolf and Takebayashi, 2004; Leonard, 2018). Supporting evidence for this suggestion has been obtained from experimental work on *Caenorhabditis elegans* that reverts to androdioecy after only a few generations (Stewart and Phillips, 2002; Cutter, 2005). However, the transition from dioecy to hermaphroditism, one of the reproductive states required to achieve trioecy in *S. algosus*, has had less attention than other changes (Weeks, 2012), despite having evolved several times within animals (Eppley and Jesson, 2008; Sasson and Ryan, 2017).

The transition from dioecious to hermaphrodite has been more common than *vice versa* in animals (Sasson and Ryan, 2017), although the transition mechanism is not well understood. There is evidence that androdioecious species (hermaphrodites + males) have evolved from dioecious species (Weeks et al., 2014b). Therefore, androdioecy is expected to be an intermediate reproductive system that emerges in the transition from dioecy to hermaphroditism. Such a transition may begin with the gain of an alternative sex in a unisex individual (male or female), then this mutant hermaphrodite is expected to invade the population (Ghiselin, 1974; Charnov, 1982; Weeks, 2012). However, this process is expected to be gradual (Leonard, 2018), suggesting that at the beginning of the transition, from one reproductive system to another, a sexual trimorphism – trioecy – is produced.

The reproduction of marine mussels is strongly rooted in dioecy (Gosling, 2015 and **Supplementary Table S1**), a reproductive strategy commonly found in most bivalve species (Heller, 1993; Collin, 2013). However, *S. algosus* shows a mixed reproductive system (hermaphrodites + females + males), where hermaphrodites are the most abundant and males the least abundant in each populations (mean value of ~1.1%). We interpret this as evidence of selection against the male sexual phenotype. This suggests that the factors which regulate female abundance in trioecious populations are independent of the factors that regulate male abundance. Although we do not know how the three sexes can coexist, we speculate that the trioecy of *S. algosus* is a transient reproductive system close to gynodioecy (transition hypothesis = dioecy trioecy gynodioecy hermaphroditism). Further investigations are needed on the interaction between males, females and hermaphrodites in populations to address the causes that promote the transitions from one sexual system to another.

Interactions Between Ecology, Life-History Characteristics, and Sexual System

It has been suggested that key life-history characteristics such as motility (or lack of it), population density, and environmental variation may all affect a species' breeding system. In many respects, *S. algosus* is a "typical" marine intertidal mussel. It is sessile as an adult, it occurs at high densities in wild populations

(Broitman et al., 2001; Navarrete et al., 2008; Zeeman et al., 2018), and population numbers are very large (100,000s to millions of individuals). It co-occurs with other mytilid species such as *Perumytilus purpuratus* and *Mytilus chilensis*, both of which are dioecious species. It is tolerant of environmental variation, given that it occurs over ~40 degrees of latitude. Despite analyses pointing to the importance of factors such as motility versus a sessile life form or breeding density within a population, which has relevance to gamete interactions (Eppley and Jesson, 2008; Avise and Mank, 2009), there is no obvious reason why *S. algosus* has evolved trioecy as a sexual system when all of its close relatives within the family Mytilidae have evolved and retained dioecy.

The Evolutionary Origin of Trioecy in *S. algosus*

The phylogenetic analysis performed in this study produced stable topologies that are congruent with other studies that use multiple genes (e.g., Trovant et al., 2015). Although the monophyly of the established subfamilies is not supported (specifically the Mytilinae subfamily), our results indicate for the first time that *S. algosus* is a member of the Brachidontinae subfamily. We are aware of the limitations of using only a single mitochondrial marker. However, mtDNA data has been very helpful in understanding the phylogenetic relationships in the Mytilidae (e.g., Hilbish et al., 2000; Terranova et al., 2007; Gérard et al., 2008). In fact, the largest genetic database for this group comes from mtDNA sequences (mainly COI and 16S). For this reason, we consider the use of COI was appropriate for our study, but we recognise that other markers and/or a greater number of markers may have yielded slightly different, but probably qualitatively similar, results.

Phylogenetic analysis of the mitochondrial DNA cytochrome *c* oxidase subunit I (COI) region indicates that *S. algosus* is closely related to dioecious species (e.g., *P. purpuratus* – within clade 2), and also reveals the independent evolution of sequential hermaphroditism (but not trioecy) in the bivalve genus *Musculus* (within clade 1). The question remains, however, why has trioecy developed in *S. algosus*, when other marine mussels with very similar ecologies and life-history characteristics are dioecious? We do not know the answer to this question (yet), but we speculate that it may be related to the mechanism of sex determination. For example, in *Rhabditis* nematodes, sex is genetically determined with males being XY and females XX; if XX larvae are exposed to environmental conditions that produce a dauer larva, they become simultaneous hermaphrodites as adults (Chaudhuri et al., 2015; Kanzaki et al., 2017). Therefore, environmental sex determination and genetic sex determination interact in this example of trioecy. Although there are several studies on the determination of sex in bivalves (reviewed by Breton et al., 2017), several aspects of the mechanism(s) involved remain unknown. The evolution of the sexes and/or origin of hermaphroditism are topics that researchers have struggled to resolve (i.e., Baldi et al., 2009; Geng et al., 2014), and these efforts have still not been sufficient to answer critical questions. *S. algosus* as a new model species may help shed new light on the evolution of sexual systems in general, and specifically in the Mollusca.

DATA AVAILABILITY STATEMENT

This manuscript contains previously unpublished data. The access numbers of the molecular data (sequences) are available - MT103127–MT103143 (GenBank) and the graphical representation of the data can be found in the **Supplementary Material**.

ETHICS STATEMENT

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of Universidad Austral de Chile committee. The protocol was approved by the biosafety institution of the Universidad Austral de Chile (N°008/17). The animals involved in this study were minor invertebrates (Mollusca: Bivalves: Mytilidae).

AUTHOR CONTRIBUTIONS

PO and JN conceived and designed the study, carried out the fieldwork and preparation of the manuscript.

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JT and JG conducted the analysis of the results and helped with preparing the final draft. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00348/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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