

SEX RATIO VARIATION IN THE *LESSONIA NIGRESCENS* COMPLEX (LAMINARIALES, PHAEOPHYCEAE): EFFECT OF LATITUDE, TEMPERATURE, AND MARGINALITY¹

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Little is known about variation of sex ratio, the proportion of males to females, in natural populations of seaweed, though it is a major determinant of the mating system. The observation of sexual chromosomes in kelps suggested that sex is partly genetically determined. However, it is probably not purely genetic since the sex ratio can be modified by environmental factors such as salinity or temperature. In this paper, sex ratio variation was studied in the kelp *Lessonia nigrescens* Bory complex, recently identified as two cryptic species occurring along the Chilean coast: one located north and the other south of the biogeographic boundary at latitude 29°–30° S. The life cycle of *L. nigrescens* is characterized by an alternation of microscopic haploid gametophytic individuals and large macroscopic fronds of diploid sporophytes. The sex ratio was recorded in progenies from 241 sporophytic individuals collected from 13 populations distributed along the Chilean coast in order (i) to examine the effect of an environmental gradient coupled with latitude, and (ii) to compare marginal populations to central populations of the two species. In addition, we tested the hypothesis that the sex ratios of the two cryptic species would be affected differently by temperature. First, our results demonstrate that sex

ratio seems to be mainly genetically determined and temperature can significantly modify it. Populations of the northern species showed a lower frequency of males at 14°C than at 10°C, whereas populations of the southern species showed the opposite pattern. Second, both species displayed an increased variation in sex ratio at the range limits. This greater variation at the margins could be due either to differential mortality between sexes or to geographic parthenogenesis (asexual reproduction).

Key index words: cryptic species; gametophyte; latitude; *Lessonia nigrescens*; marginal populations; Phaeophyceae; sex determination; sex ratio; temperature

Abbreviations: ESD, environmental sex determination; GSD, genetic sex determination; SST, sea surface temperatures

The expression of a given sexual phenotype is defined by genetic or environmental factors, or a combination of both (Nakamura 2009). The most studied type of genetic sex determination (GSD) involves sex chromosomes. Sex determination in diploid mammals and some diploid plants depends on the combination of sexual chromosomes, where an individual with XX becomes female (i.e., homogametic sex), while an individual with XY becomes

¹Received 4 December 2009. Accepted 20 July 2010.

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male (i.e., heterogametic sex). In some other groups, including birds, butterflies, and some fishes, females are heterogametic (ZW), and males are homogametic (ZZ). Alternatively, one or more mating-type loci determine mating compatibility in organisms such as the green algae *Chlamydomonas* and *Volvox* (Ferris et al. 2010), and some fungi where two incompatibility types are known (Charlesworth et al. 2005). In haploid or haploid–diploid life cycles, sex determination could directly rely on sexual chromosomes (e.g., gametophytes bearing X are females, and those bearing Y are males), whereas diploid individuals with both sexual chromosomes (XY) produce meiotic spores. This was clearly demonstrated in the dioecious liverwort *Marchantia polymorpha*, where male (bearing Y chromosome) and female (bearing X chromosome) are morphologically identical until the male and female organs differentiate (Tanurdzic and Banks 2004). Thus, segregation of sexual chromosomes during meiosis of the diploid phase ensures equal proportions of female and male gametophytes. Whatever the mechanism of GSD is, sex ratios are often close to 0.5 in natural populations (i.e., 1 male:1 female) (Uller et al. 2007).

However, the sexual phenotypes of species with GSD can be ultimately defined by environmental conditions (environmental sex determination [ESD]) (Kraak and Pen 2002). It is well known that incubation temperature of eggs in some reptiles (Sarre 2006) and water temperatures during larval development in amphibians modify sex ratios (for review, see Nakamura 2009). These studies suggested that GSD and ESD are not mutually exclusive (Matsuba et al. 2008). A case of complete ESD (i.e., no evidence of GSD) was reported in *Equisetum* sp., a horsetail with haplo-diploid life cycle. Indeed, it was shown that haploid gametophytes were potentially bisexual (Duckett 1977), and that sex expression was, at least partly, determined by environmental factors (Guillon and Fievet 2003). Other mechanisms involved in sex determination evolved in the fern *Ceratopteris richardii*, where male:hermaphrodite ratios are determined epigenetically by an antheridiogen pheromone; thus, male frequencies increased with the density of the population (Tanurdzic and Banks 2004). In this context, evolutionary theory predicts that ESD is an evolutionarily stable strategy, particularly in patchy environments where some patches are more valuable to females and others to males (Charnov and Bull 1977, Bull 1981). Finally, the effect of the environment on sex ratios has been reexamined in the context of global change. Studies in amphibians (Blaustein and Wake 1990), birds (Thomas and Lennon 1999), mammals (Hersteinsson and Macdonald 1992), and butterflies (Parmesan et al. 1999) have highlighted that drastic changes in sex ratio due to a temperature effect could lead to population extinctions (due to the loss of one of

the sexes), and thus to changes in the range of species' distribution. These findings raise the question of whether environmental conditions, occurring in habitats coinciding with the distribution limit for a given species, have an effect on sex ratios. In this context, increased asexual reproduction has been often observed in marginal populations (Eckert 2002), including parthenogenesis, which can modify sex ratio. Additionally, marginal habitats may cause unbalanced sex ratios as a result of: (i) selective mortality of one sex, (ii) evolutionary responses of the mating system, or (iii) a combination of both.

Sex determination is poorly understood in seaweeds. In brown algae (Phaeophyceae), GSD was demonstrated in *Ectocarpus* sp. using crossing experiments (Müller 1967). Sexual reproduction in *Ectocarpus* is isogamous and involves an alternation between diploid sporophytes and haploid, dioecious male and female gametophytes. Sporophytes and gametophytes have a similar morphology, both consisting of branched, uniseriate filaments. The life cycle of kelps (Laminariales), on the other hand, is heteromorphic with an alternation of microscopic haploid dioecious dimorphic gametophytes, and macroscopic diploid sporophytes that produce haploid spores by meiosis. Sexual reproduction is oogamous. Female gametophytes produce eggs that, after fertilized by sperm, produce new diploid sporophytes. A large chromosome was reported in female gametophytes of several kelp species of Laminariaceae, including *Laminaria digitata*, *Laminaria ochroleuca*, *Saccharina latissima* as *Laminaria saccharina*, *Alaria esculenta* and *Chorda filum* (Evans 1963), *Laminaria hyperborea* and *Saccorhiza polyschides* (Evans 1965), and *Laminaria yendoana* (Yasui 1992), suggesting the existence of sexual chromosomes in these species. Recently, a putative sex-determining region was identified in a hybrid of *Laminaria japonica* and *Laminaria longissima* using amplified fragment length polymorphism (AFLP) (Yang et al. 2009). Few studies have reported on sex ratio in culture, most suggesting a similar proportion of males and females (Sauvageau 1918, Schreiber 1930, Cosson 1978). However, it has also been reported that sex ratio can be modified by abiotic stresses such as salinity or temperature (for review, see Bartsch et al. 2008). For example, high temperatures in culture result in a higher proportion of males in *S. latissima* as *L. saccharina* (Lee and Brinkhuis 1988) and in *L. digitata* (Cosson 1978). In contrast, both high and low temperatures resulted in a decrease of males in *Laminaria religiosa* (Funano 1983). More recently, Nelson (2005) demonstrated that high temperature and long days resulted in sex ratio biased toward females in *Lessonia variegata*, suggesting that males were less resistant to stressful conditions. The above evidence suggests that the effect of temperature on sex ratio is variable and species-dependent in kelps. Unfortunately, the effect of temperature on sex ratio in the field is unknown

because gametophytes are microscopic and impossible to observe in nature.

This study focused on the variation of sex ratio in two cryptic species of the *L. nigrescens* complex (Tellier et al. 2009) by comparing individual progenies at three different levels (i.e., within populations, between populations of each species separately, and between species). These two cryptic species were described recently by Tellier et al. (2009), who showed that individuals of this complex were arranged in two highly differentiated lineages with contrasting latitudinal distributions and a contact area at the 29°–30° S biogeographic transition zone (Fig. 1, a and b). The northern species is often exposed to extended periods of warmer waters from the north as a result of El Niño Southern Oscillation events, which, during the 1980s, were associated with massive local population extinctions of this kelp (Castilla and Camus 1992, Martínez et al. 2003). Experimental support to a temperature-related mortality was reported by Martínez (1999), who studied thermal tolerance of young sporophytes of *L. nigrescens* and detected differences between northern and southern populations in both survival and growth rate. We, therefore, specifically tested the hypothesis that the sex ratios of the two cryptic species would be affected differently by temperature.

MATERIALS AND METHODS

Materials. The two cryptic species of *L. nigrescens* dominate the intertidal and shallow-subtidal of wave-exposed areas. They are distributed along the temperate Pacific coasts of South America between 17° and 42° S. One of them is located in the northern region of the Chilean coast, in the biogeographic Peruvian Province (17°37' S–30°14' S), whereas the other is located in the Intermediate Area (29°03' S–41°48' S)

(Camus 2001) (Fig. 1a). The two species (respectively designated as PP and IA species in Tellier et al. 2009 and called here northern and southern species) were never found coexisting in the same location, even within the transition zone between the two range distributions (from 27° S to 30°14' S), where a mosaic of pure populations of the northern or southern species was observed (Tellier et al. 2009) (Fig. 1, b and c).

Sampling and culture conditions. Reproductive fronds from six to 30 mature sporophytic individuals were sampled in 13 locations distributed along the Chilean coast between 20°25' S and 39°46' S (Table 1; Fig. 1). Seven and six locations were chosen for the northern and southern species, respectively, and of these, two populations of the northern species and three populations of the southern species were sampled within the transition zone (Fig. 1c). These latter populations were considered as marginal because geographically they are at the distribution limits of the species. Sea surface temperatures (SST) were estimated from longtime survey data (11 years, 1996–2007) of the Advanced Very-High Resolution Radiometer satellite (Casey and Cornillon 1999). Raw SST corresponded to the 7 d temperature record at each site interpolated to monthly resolution. At the spatial scale of resolution (4 km), all sampling sites were located in different pixels, except two sites (Piqueros and Cerro Elefante). SST ranged from 11.0°C (mean monthly minima) to 15.2°C (mean monthly maxima) in Valdivia, and from 16.2°C to 23.0°C in Los Verdes (Table 1). Temperatures in the transition zone ranged from 13.2°C to 17.9°C.

From each blade, two fragments of equal size (3.8 cm²) with mature sori were placed immediately after washing with running tap water and sterile seawater, in 50 mL Falcon tubes (BD Biosciences, San Jose, CA, USA) containing sterile seawater and a glass slide. These tubes were stored inside a cooler in the dark to avoid germination and at low temperature (average 10°C, minimum 5°C, and maximum 16°C) for their transportation to the lab since kelps are known to be sensitive to higher temperatures. At their arrival in the lab, they were kept in the dark at 10°C. After 12–24 h of incubation, all released spores were attached to the slide but did not germinate as light is required for germination. Just before germination, the sterile seawater was replaced with seawater filtered culture enriched seawater (Correa et al. 1988), and

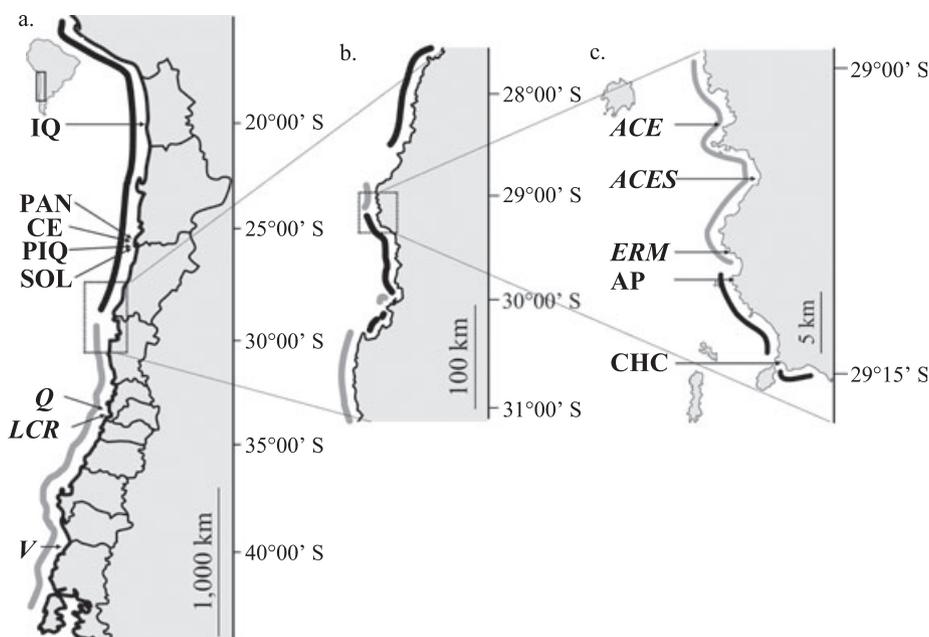


FIG. 1. Distribution of northern and southern cryptic species of *Lessonia nigrescens*: (a) location of central populations along the Chilean coasts, (b) transition zone (28°–31° S), and (c) marginal populations within the transition zone. The southern species is represented in gray (names in italics), and the northern species in black. Species were determined using the mitochondrial marker *atp8/trnS*. See Table 1 for explanation of codes.

TABLE 1. Geographic location and number of studied progenies obtained from sporophytic individuals of each cryptic species sampled in different sites along the Chilean coast.

Site	Code	Species	Latitude (S)	Longitude (W)	Sampling date	Monthly average SST (°C)		<i>n</i>	Sex ratio at 10°C	
						Min.	Max.		Mean	Variance 10 ⁻²
Los Verdes	IQ	Northern	20°25'62	70°12'48	09/04/2007	16.2	23.0	27	0.522	0.267
Pan de Azúcar	PAN	Northern	26°07'58	70°39'14	13/08/2007	14.4	19.9	33	0.521	0.372
Cerro Elefante	CE	Northern	26°08'37	70°40'09	22/01/2007	14.4	19.9	7	0.536	0.133
Piqueros	PIQ	Northern	26°09'12	70°40'09	22/01/2007	14.4	19.9	6	0.476	0.184
Soldado	SOL	Northern	26°09'57	70°40'17	23/01/2007	14.4	19.9	13	0.463	0.583
Chañaral de Aceituno ^a	ACE	Southern	29°04'02	71°29'21	20/02/2007	13.4	17.9	20	0.481	0.432
Aceituno Playa Sur ^a	ACES	Southern	29°06'92	71°28'25	20/02/2007	13.4	17.7	15	0.545	0.709
Ermitaño ^a	ER	Southern	29°08'74	71°30'16	20/02/2007	13.4	17.7	18	0.450	0.830
Apolillado ^a	AP	Northern	29°11'03	71°29'80	21/02/2007	13.3	17.7	21	0.544	0.615
Choros Camping ^a	CHC	Northern	29°15'26	71°27'90	21/02/2007	13.2	17.4	12	0.339	1.828
El Quisco	Q	Southern	33°25'27	71°42'31	20/03/2007	12.5	17.3	24	0.546	0.439
Las Cruces	LCR	Southern	33°30'09	71°38'01	20/03/2007	12.4	16.7	27	0.537	0.268
Valdivia	VAL	Southern	39°46'75	73°23'49	20/03/2007	11.0	15.2	18	0.521	0.227

SST, sea surface temperature; *n*, number of progenies.

^aMarginal populations located in the transition zone.

tubes were placed horizontally in culture chambers under standard culture conditions (10°C, 12:12 light:dark, 25–35 μmol photons · m⁻² · s⁻¹). Culture medium was changed once a week, and observations were done every 3 d. A fragment of each blade was excised and stored in silica gel for molecular identification.

Molecular analyses. In order to verify that sampled plants belonged to either the northern or southern species, five individuals of each population from the northern and southern regions, and 10 individuals from each population within the transition zone, were sequenced and/or analyzed using the single strand conformation polymorphism (SSCP) method as described by Tellier et al. (2009). Total DNA was extracted from ~10 mg of dried material from the meristematic zone of each sporophytic thallus using an extraction buffer that combined a standard cetyl trimethyl ammonium bromide extraction with the addition of polyvinyl pyrrolidone to remove polyphenols (Martínez et al. 2003). Individuals were analyzed using the mitochondrial intergenic markers *atp8/trnS* (Engel et al. 2008), and the PCR conditions were as in Voisin et al. (2005). PCR products were purified and sequenced in an ABI PRISM[®] 3100 Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Sex ratio estimates. Sex ratio was estimated for each progeny after 15 d in culture. Male and female gametophytes were identified according to their morphological characteristics using a Nikon Eclipse TE300 inverted microscope (Nikon Corp., Tokyo, Japan). Female gametophytes are characterized by large cells and filaments with few branches, whereas male gametophytes are smaller and display highly branched filaments formed by small cells. These morphological differences make them unambiguously identifiable under the inverted microscope. The numbers of male and female gametophytes were determined by counting their occurrence in three visual fields per slide using the ×10 objective. Sex ratio was expressed as the frequency of males per progeny, that is, males/(males + females).

Effect of temperature. To test the effect of temperature on sex ratio, additional cultures of gametophytes were established at 14°C using spores from 18 to 30 sporophytic individuals of two northern populations (Los Verdes and Pan de Azúcar) and three populations of the southern species (El Quisco, Las Cruces and Valdivia) (Table 1).

Statistical analyses. The binomial law was used to estimate the probability of detecting sex ratio deviation from $P = 0.5$, and the Bonferoni correction was applied to the tests. In order to

have robust estimates, only replicates with at least 50 gametophytes per progeny were considered. The significance of the differences in sex ratios in progenies was analyzed between species using one-way analysis of variance (ANOVAs), that is, ignoring the population level. Sex ratio variation between and within population was then taken into account to analyze the difference between marginal and central populations in the two species. Differences in sex ratio between populations were tested for each of the studied species. One-way ANOVAs and Levene tests were performed including or excluding marginal populations to test the effect of range limit on variance homogeneity. Sex ratio variations in marginal populations were then compared to central populations of the respective species.

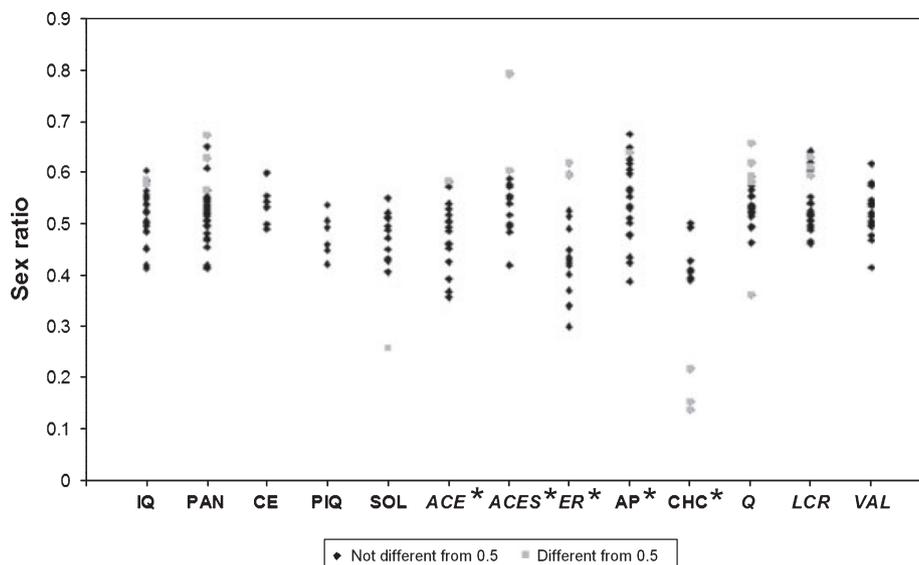
In addition, we examined the effect of environmental factors on sex ratio of both cryptic species: we tested whether there was an effect of an environmental gradient coupled with latitude using regression analyses, and the effect of temperature was tested using a two-way ANOVA with species and temperature as fixed factors. General linear model procedures were used, and Tukey's student range tests were performed for multiple comparisons. Data were transformed when necessary in order to meet the assumption of homogeneity of variance. All statistical analyses (as well variable transformation) were done with MINITAB version 13.2 (State College, PA, USA).

RESULTS

SSCP and sequencing. All 90 parental individuals were unambiguously assigned to either northern or southern species using SSCP and sequencing. Results (summarized in Table 1) verified that individuals from Los Verdes, Pan de Azúcar, Cerro Elefante, Piqueros, and Soldado belonged to the northern species, and those individuals from El Quisco, Las Cruces, and Valdivia corresponded to the southern species. In the contact zone (29°03' S to 30°14' S), individuals from Chañaral de Aceituno, Aceituno Playa Sur, and Ermitaño belonged to the southern species, and the individuals from the populations of Apolillado and Choros Camping to the northern species.

Sex ratio. A total of 241 progenies, 119 from the northern species and 122 from the southern species

FIG. 2. Sex ratio of *Lessonia nigrescens* per progeny (as frequency of males), obtained at 10°C from sporophytes collected in seven populations from the northern species and six populations from the southern species (italics). Each symbol represents one progeny. *Marginal populations located in the transition zone. See Table 1 for explanation of codes.



(corresponding to $\sim 59,000$ gametophytes), were included in the analyses. Sex ratio varied between 0.137 and 0.673 in populations of the northern species and between 0.300 and 0.793 in those of the southern species.

When populations of each species were compared separately, 108 progenies (90.7%) of the northern species showed sex ratios not significantly different from 0.5, whereas 7 (5.9%) presented a significant male bias and 4 (3.4%) a significant female bias (Fig. 2). Similarly, in the southern species, 108 progenies (88.5%) presented sex ratios not different from 0.5, whereas 13 (10.7%) displayed a significant excess of males and 1 (0.8%) a significant excess of females (Fig. 2). Overall, sex ratios of the progenies belonging to the northern species (0.499 ± 0.09 SD) were not significantly different from those displayed by the progenies from the southern species (0.515 ± 0.07) (one-way ANOVA, $F_{1,240} = 2.24$, $P = 0.136$).

The magnitude of sex ratio variation was analyzed within and between populations. In the northern species, the variances were heterogeneous between populations (Levene test, statistic value = 2.698, $P = 0.017$) and ranged from 0.133×10^{-2} (Cerro Elefante) to 1.828×10^{-2} (Choros Camping). In the southern species, the variances were homogeneous (Levene test, statistic value = 1.148, $P = 0.339$) and ranged from 0.227×10^{-2} (Valdivia) to 0.830×10^{-2} (Ermitaño) (Table 1). In both species, the highest values belonged to marginal populations, that is, Choros Camping, Ermitaño, and Aceituno Playa Sur (Table 1). In northern species, when marginal populations were excluded, the variances were homogeneous (Levene test, statistic value = 0.513, $P = 0.73$).

In the northern species, mean proportions of males varied from 0.339 (± 0.135) in Choros Camping to 0.544 (± 0.078) in Apollillado (Fig. 2). Sex ratios were different between populations (one-way

ANOVA, $F_{6,118} = 11.37$, $P < 0.0001$) mainly due to the Choros Camping population (Table 1). In the southern species, mean values of males varied from 0.450 (± 0.091) in Ermitaño to 0.546 (± 0.066) in El Quisco. Sex ratios were different between populations (one-way ANOVA, $F_{5,121} = 6.52$, $P < 0.0001$) mainly due to the Ermitaño and Chañaral de Aceituno populations. In this species, the frequency of females was significantly higher in marginal populations than in central ones (one-way ANOVA, $F_{1,121} = 13.17$, $P < 0.0001$). Sex ratio diminished from the north toward the transition zone (regression analysis, $y = -0.0064x + 0.6598$, $R^2 = 0.0498$; ANOVA, $F = 11.20$, $P = 0.001$) in the progenies of the northern species, and it gradually increased from the transition species toward the south in those from southern species (regression analysis, $y = 0.0052x + 0.3446$, $R^2 = 0.0575$; ANOVA, $F = 8.32$, $P = 0.005$) (Fig. 3).

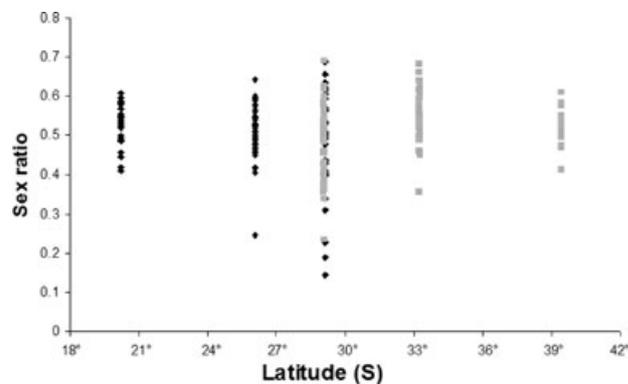


FIG. 3. Sex ratio per progeny according to the latitude of the population sampled. Each symbol represents one progeny. Northern species are represented in black symbols, and southern species in gray symbols.

Temperature effects on sex ratio. Sex ratios in central populations of the northern species (Los Verdes and Pan de Azúcar) increased with temperature (mean 0.526 ± 0.05 at 10°C and 0.551 ± 0.07 at 14°C , respectively; Fig. 4). By contrast, sex ratios of southern species central populations (El Quisco, Las Cruces, and Valdivia) decreased with increasing temperature (mean 0.535 ± 0.06 at 10°C and 0.518 ± 0.05 at 14°C ; Fig. 4). Even though there was no significant effect of temperature and species on sex ratio (Table 2), the interaction of these

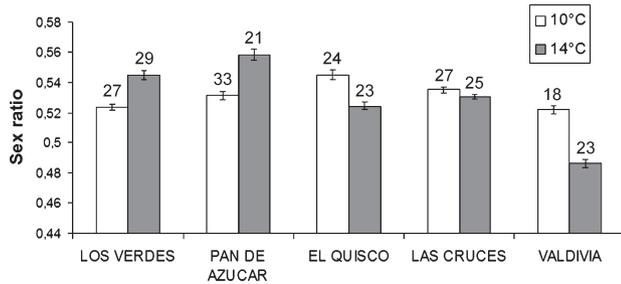


FIG. 4. Effect of temperature on sex ratio. Spores from sporophytes collected in five selected populations were cultivated under two different temperatures (10°C and 14°C). Bars represent mean values, and dispersal corresponds to 1 SE. Number of progenies studied are indicated above bars.

TABLE 2. Effect of the temperature on the sex ratio in the two species: two-way factorial analysis of variance (temperature and species fixed factors).

Source	df	MS	F	P-value
Temperature	1	0.000104	0.03	0.863
Species	1	0.011195	3.20	0.075
Species \times temperature	1	0.028814	8.22	0.005
Error	234	0.003503		

MS, mean squares; df, degrees of freedom.

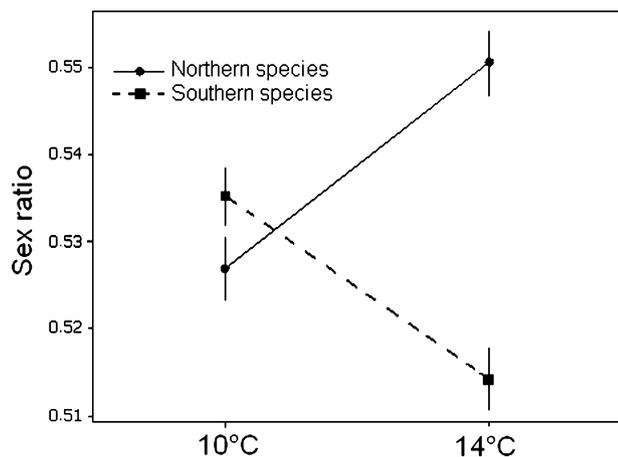


FIG. 5. Interaction plot (fitted means) for frequency of male gametophytes in the two species of *Lessonia nigrescens* for the two temperatures. Temperature and species are the interactive factors.

factors was highly significant (Table 2; Fig. 5). It was also clear that the main deviation from a sex ratio of 0.5 occurred in the northern species at 14°C (Fig. 5).

DISCUSSION

In this study, we first established that males and females generally occurred in equal proportions in natural populations of both cryptic species of *L. nigrescens*. These results suggest that sex determination is likely controlled by one or few genetic loci (i.e., GSD) as shown recently by Yang et al. (2009) in *Laminaria*. We also showed that temperature modulates sex ratio in both species, advocating that there is an interaction between genetic (GSD) and environmental factors (ESD) during the expression of sex. It was further observed that marginal populations of both species displayed significant female excesses as well as the largest variances in sex ratios. Finally, significant differences in sex ratio were revealed between the two cryptic species when exposed to diverse temperature conditions, demonstrating that these two phylogenetic species also correspond to ecological species.

One locus of chromosomal sex determination generally leads to sex ratios close to 0.5 because of the random segregation of sex alleles (or chromosomes) during meiosis (Bull and Charnov 1988). However, deviation from these expected 0.5 values have been often reported in natural populations and explained by interactions with environmental factors (Zaborski et al. 1988, Guillon and Fievet 2003, Nakamura 2009). Many examples of difference in mortality between males and females during their life span have been documented in plants (Delph 1999). Therefore, the resulting estimates of sex ratio have been usually biased in either direction depending on the period of the life history considered to make the observations (De Jong and Klinkhamer 2002). For example, in papayas, the use of molecular genetic markers to distinguish male from female seeds showed that their sex ratio was biased toward males and then changed to equilibrated sex ratios at the adult stage (Parasnis et al. 1999).

In the present study, sex determination was monitored shortly after spore germination, based on the clear dimorphism of the gametophytes. Our results clearly indicate that sex ratio in the two cryptic species of *L. nigrescens* is close to 0.5 and confirm that sex determination is probably governed by a major genetic factor (i.e., sex chromosome) as reported in other kelp species (Yasui 1992). In addition, we demonstrate that sex ratio can be modified by the effect of temperature. The lower frequency of males observed at higher temperature in populations from the southern species is consistent with the observations of *L. variegata* reported by Nelson (2005), showing an increase in female frequency at high

temperature. Temperature-dependent sex ratio was also reported in *L. religiosa* (Funano 1983), where values of 0.5 at optimum temperature shifted toward male-biased sex ratio at both higher and lower temperatures. Similarly, our results showed that an increase in temperature has two opposite effects, depending on the cryptic species of *L. nigrescens*. In populations of the northern species (Los Verdes and Pan de Azúcar), the proportion of males increased with temperature, whereas in populations of the southern species (El Quisco, Las Cruces, and Valdivia), the proportion of males decreased with temperature. These results suggest the occurrence of different temperature optima for each species, probably related to their geographic origin. Indeed, the two temperatures selected for the experimental approach corresponded roughly to the minima and maxima of the monthly mean values to which the southern species is exposed, both being clearly lower than the minimum temperatures encountered by the northernmost population (Los Verdes). Paradoxically, the northern species displayed an equilibrated sex ratio at the lowest temperature, whereas the southern had an equilibrated sex ratio at the highest temperature. To better understand these results, higher temperatures and additional populations must be included in future work. Nevertheless, the differences in responses to temperature observed between the two cryptic species indicate environment–genotype interaction for the expression of this quantitative trait and thus validate that the two genetic entities are ecologically differentiated.

We determined that sex ratios differed greatly among progenies of the same population and among populations of the same cryptic species, suggesting the occurrence of epigenetic factors inducing either segregation distortion during meiosis, or differential mortality between female and male spores, or differences in phenotypic expression, or a combination of these mechanisms. Indeed, most of the studied populations (70%) displayed at least one progeny with a sex ratio significantly different from 0.5. Furthermore, sex ratios were more often biased toward males (22.8% of the progenies) than females (5.8% of the progenies), a phenomenon still in need of explanation.

Interestingly, two populations located in the transition zone (one from the northern species and one from the southern species) presented progenies with an excess of females. The highest proportion of females (85%) was recorded in the progeny from Choros Camping (northern species) and Ermitaño (southern species), located at the southern range limit of the northern species. More broadly, we observed that the frequency of male gametophytes was significantly lower in marginal compared to central populations in both species. Sporophytes from these populations released fewer spores (i.e., lower fertility), suggesting that they might be poorly

adapted to the conditions prevailing at their range limits causing chronic stress (data not shown). This major change in sex ratio, in populations located at the species range limit, indicates that the environmental conditions that are specific of the marginal habitat could affect the sex ratio directly or indirectly. Marginal populations are generally more fragmented and more prone to local extinctions due to environment fluctuations, demographic stochasticity, and edge effects (Kawecki 2008). As a result, changes in the mating system as a response to low fertility success are often observed, in particular an increase of asexual reproduction (Eckert 2002, Kearney 2005). If we had been able to observe progenies exhibiting 100% females, then we would have been able to prove the occurrence of parthenogenesis (as previously observed in this complex species by Oppliger et al. 2007) in the range limit. However, such progenies were never observed in the present study. Rather, the highest sex-ratio variability observed in marginal populations could be due to the increase of the frequency of deleterious (or lethal mutations) driven by genetic drift. Thomas et al. (2003) have reported such increase of lethal alleles in small populations, in the phytopathogenic fungus *Microbotryum violaceum*, in which biased sex ratios are due to the presence of lethal alleles linked to the mating type. The highest proportion of females recorded here, in the transition zone, could thus be the signature of the reduction of genetic variation at the margin compromising its ability to respond to selection (see, e.g., the case of the plant *Mercurialis annua*, Pujol and Pannell 2008).

In conclusion, the sex ratio of gametophytes in *L. nigrescens* seems to be governed first by a major genetic factor (sex chromosomes or a sex locus) and secondarily modulated by environmental factors such as temperature. The two closely related species of *L. nigrescens* present different temperature optima for reproduction. It would be valuable to compare sex ratio under a broader spectrum of temperature conditions to determine the optimum for each species in order to, at least in part, explain the geographic distribution of these two sibling species. Optimum reproduction at range limits could influence the survival of local populations and thus the preservation of a species.

We deeply thank M. Ramirez, G. Peralta, C. Figueroa, C. Chianale, D. Thomas, M.-L. Guillemin, L. Prado, A. Gonzalez, P. von Dassow, and B. Broitman for their help during various stages of the research. V. Oppliger was supported by a CONICYT-FRENCH EMBASSY PhD grant, and F. Tellier by a doctoral grant from the Ministry of Education and Research (MENRT). This study is part of the research programs FONDAP 1501-0001 Program 7, ANR ECOKELP (ANR 06 BDIV 012), Pôle Mer Bretagne, “ARCUS” of the French Ministry of Foreign Affairs, and the Laboratoire International Associé “Dispersal and Adaptation of Marine Species (LIA DIAMS).

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