Chapter 13
Comparative Population Genetics of Red Alga Occupying Different Salinity Conditions

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Abstract Osmotic stress is one of the major abiotic stresses in seaweeds, which may physiologically acclimate to changing osmolarity by altering their transcriptome under hyper- or hypo-osmotic conditions. Also local adaptations leading to genetic differentiation between populations may develop. DNA-based genetic markers and sequencing provide the essential tools to investigate genetic diversity and differentiation and signs of selection. Whole-genome or transcriptome sequencing facilitates marker development and allows in-depth investigations on the population genetic structures of organisms. In this chapter, a special attention is paid on a set of genetic studies conducted on the marine red alga *Furcellaria lumbricalis,* including populations in the Atlantic Ocean (35 psu) and the brackish Baltic Sea (3.8-8 psu). The amount of genetic variation did not differ between ocean and brackish populations, but differences were observed between marker types. The expressed sequence tag (EST)—derived microsatellites possessed less variation and showed greater differentiation than the putatively neutral microsatellites, whereas the EST-derived SNP markers contained even less variation and showed even more differentiation. Yet, the most distinct result was that *F. lumbricalis* showed definite differentiation between the ocean and brackish populations in expressed genomic regions, while such differentiation was not detected by presumably neutral loci. Thus, suboptimal salinity is a stress factor that affects population genetic structures. However, such differentiation would have been missed if the investigations on *F. lumbricalis* had relied on the analysis of only neutral markers.

Keywords *Furcellaria* · Microsatellites · Population genetics · Salinity tolerance · SNP markers · Transcription profiling

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13.1 Introduction

High tolerance, phenotypic plasticity and local adaptations may allow species to occur across a range of environments. Adaptation to a combination of environmental factors presumes genetic differentiation, but tolerance depends on physiological responses that usually limit productivity outside the optimal environment (Kamer and Fong 2000; Oetjen and Reusch 2007). Osmotic stress is one of the major abiotic stresses to which many seaweeds are exposed. Yet, osmoregulatory processes are important to all living organisms, since the maintenance of intracellular osmotic pressure or chemical potential of metabolites is of fundamental importance for cell survival (Hoffmann 1987).

High osmolarity caused by elevated salinity lowers the external water potential. To cope with hyperosmolarity, seaweeds may increase the uptake of ions (K^+ and Cl^-), removal of Na^+, water loss, and the synthesis of osmotically active carbohydrates (Karsten et al. 1992; Mostaert et al. 1995). The amount of soluble carbohydrates can be adjusted to maintain osmobalance in cells during salinity stress (Lüning et al. 1990). Hypo-osmotic stress due to decreased salinity increases the external water potential, which may induce water uptake that results in increased cell volume and turgor pressure and in the loss of ions and organic solutes, thus leading to osmotic adjustment. Species exposed to such stresses may show lowered performance due to inefficient cellular metabolism, changed cellular ultrastructure and defective ion metabolites in the cell (Kirst 1990). Furthermore, osmotic stress can induce intracellular generation of reactive oxygen species (ROS) that cause oxidative damage to lipids, proteins, and nucleic acids (Teo et al. 2009).

In seaweeds, adaptation and acclimation to osmotic stresses are of key importance, and it is important to understand the specific mechanisms by which osmotic stress impacts those organisms and what kind of stress-related consequences arise in nature. The distribution of algal species under suboptimal salinity may also be influenced by the impairment of the reproductive system due to reduced performance of gametes or unsuccessful fertilization (Raven 1999; Serrão et al. 1999). It has been shown that extended exposure to higher or lower than optimal salinities inhibits cell division and may result in stunted growth (Graham and Wilcox 2000). It has been discovered that many marine species occurring along the salinity gradient of the brackish Baltic Sea in the northern Europe show at least some degree of physiological adaptation to the brackish water conditions (Kristiansen et al. 1994; Düwel 2001; Bergström and Kautsky 2005). Furthermore, some marine species show marked genetic differentiation between populations living in the Baltic Sea and in marine habitats with higher salinity levels (Luttikhuizen et al. 2003; Olsen et al. 2004; Johannesson and André 2006; Kostamo et al. 2012; Olsson and Korpelainen 2013), although in some cases the diversity and differentiation may be the result of invasions by different evolutionary lineages instead of local adaptation processes (Röhner et al. 1997; Väinölä 2003; Nikula et al. 2007).

13.2 Life Histories and Reproduction of Red Algae

A combination of sexual and asexual modes of reproduction is common in red algae, and asexual reproduction tends to be prevalent in the marginal regions of distribution (Hawkes 1990; Maggs 1998). The occurrence of asexual reproduction has been discovered in the hyposaline waters of the Baltic Sea in several macroalgal species (Bergström et al. 2005; Tatarenkov et al. 2005). Since red algae lack motile sperm, they rely on water currents to transport gametes to female organs or on vegetative fragments to propagate new locations (Lee 2008). Red algae display an alternation of generations. In addition to the gametophyte generation, many red algae have two sporophyte generations, (i) carposporophytes that produce carpospores, which germinate into (ii) tetrasporophytes, which then produce tetraspores that germinate into gametophytes (Lee 2008). Carpospores may also germinate directly into thalloid gametophytes, or carposporophytes may produce tetraspores without going through the tetrasporophyte phase. Thus, the life histories of red algae are complex and varied.

For instance, the marine red alga *Furcellaria lumbricalis* (Hudson) Lamoroux has a triphasic life cycle consisting of a haploid sexual phase (the gametophyte) and two diploid phases: the carposporophyte, which grows parasitically on the female gametophyte, and the tetrasporophyte (Austin 1960a, b). In the Atlantic Ocean, *F. lumbricalis* grows in subtidal habitats on sheltered to moderately expose rocky shores (Schwenke 1971) where its distribution depends on water turbidity, competition and the presence of a suitable growing substrate (Taylor 1975; Holmsgaard et al. 1981). In the low-salinity conditions of the brackish Baltic Sea—a marginal habitat for the species—*F. lumbricalis* grows under the brown alga *Fucus vesiculosus* in the bladder wrack belt, but also in the red algal belt among other red algae (Rosenvinge 1917; Wærn 1952: Mäkinen et al. 1988). Kostamo and Mäkinen (2006) have shown that *F. lumbricalis* is unlikely to reproduce sexually in the northern and eastern parts of the hyposaline Baltic Sea where the salinity is below 7 psu and that the spores produced in the Baltic Sea populations are smaller and more often deformed than those in the Atlantic Ocean populations. This finding indicates that low salinity creates a stressful environment and suggests that there may be consequences on the genetic structure of populations.

13.3 Genetic Tools for Population Genetic Analyses on Red Algae

DNA-based genetic markers and sequencing provide essential tools to measure genetic diversity within and differentiation among populations of red algae. Recent developments in high-throughput sequencing technologies enable the effective discovery of single nucleotide polymorphisms (SNPs, i.e., variation occurring when a single nucleotide, A, T, C, or G, differs) and other polymorphic DNA markers,

such as microsatellites (i.e., repeats of 1–6 base pair long sequences. Consequently, it has become easier to develop markers for, e.g., molecular ecological and conservation genetic research on natural populations. Along with better availability of markers, there has been an increasing interest to compare information of EST (expressed sequence tag)—derived markers that represent portions of expressed genes with data based on noncoding markers. Studies on expressed genomic regions allow the discovery of variation involved in adaptation and make it possible to link patterns of adaptive variation to environmental factors. The presence of adaptive genetic variation is necessary for the survival of organisms when exposed to environmental changes.

Microsatellite repeat sequences are known to be ubiquitous in prokaryotic and eukaryotic genomes and present in both coding and noncoding regions. However, the distribution of microsatellites is not homogeneous within a genome, and the frequency of microsatellite sequences also varies across taxa, in terms of both absolute numbers of microsatellite loci and repeat motifs (Hancock 1999). The results of Korpelainen et al. (2007) suggest that fewer microsatellite regions are present in the red algal genome than in land plants. Even so, microsatellite markers have been successfully developed for many red algal species (Wattier et al. 1997; Luo et al. 1999; Guillemin et al. 2005; Andreakis et al. 2007; Xie et al. 2009, 2013; Krueger-Hadfield et al. 2011, 2013; Kostamo et al. 2012; Choi et al. 2013). In addition, some genetic studies on red algae have successfully used mitochondrial markers, such as cytochrome oxidase subunit 1 (*cox*1) and intergenic spacer between the cytochrome oxidase subunits 2 and 3 (*cox*2-3 spacer) (Yow et al. 2013).

Although whole-genome or transcriptome sequencing and the utilization of resulting libraries greatly facilitate marker development and allow the effective discovery of both neutral and adaptive genetic markers in any organism, the majority of such studies on plant genomes have concentrated on flowering plants. EST projects on red algae include *Gracilaria gracilis* (Lluisma and Ragan 1997), *Porphyra yezoensis* (Lee et al. 2000; Nikaido et al. 2000; Asamizu et al. 2003; Kitade et al. 2008), *Chondrus crispus* (Collén et al. 2006), *Saccharina japonica* (Liu et al. 2010), *F. lumbricalis* (Kostamo et al. 2011), *Pyropia haitanensis* (Xie et al. 2013) and *Pyropia tenera* (Choi et al. 2013). On average, one SNP can be expected every 500–1000 bp of coding sequence, and the mutation rates of SNPs (often about 10^{-8}–10^{-9}) are low when compared with the mutation rates of microsatellites (10^{-4}) (Brumfield et al. 2003). The results by Olsson and Korpelainen (2013) on the red alga *F. lumbricalis* are congruent with this information on the frequency of SNPs (on average, one SNP per 558 bp within the sequenced genomic region).

Kostamo et al. (2012) and Olsson and Korpelainen (2013) developed both putatively neutral and EST-derived microsatellite markers and SNP markers and used them to conduct comparative population genetic analyses of *F. lumbricalis* populations located in geographical locations with different salinity conditions in Northern and Western Europe. Population genetic information obtained from SNP markers (Olsson and Korpelainen 2013) was compared to the results of analyses of *F. lumbricalis* based on putatively neutral and adaptive (EST-derived) microsatellite

Table 13.1 Mean genetic diversity and genetic differentiation (F_{ST}) among Atlantic Ocean and Baltic Sea populations, and mean F_{ST} values between Atlantic Ocean and Baltic Sea populations of *Furcellaria lumbricalis* based on putatively neutral and EST-derived microsatellite markers, and SNP markers

Variable	Atlantic Ocean	Baltic Sea	Atlantic Ocean versus Baltic Sea
Genetic diversity			
Putatively neutral microsatellites	0.734	0.716	
EST-derived microsatellites	0.285	0.272	
SNP markers	0.132	0.111	
Genetic differentiation (F_{ST})			
Putatively neutral microsatellites	0.158	0.124	0.136
EST-derived microsatellites	0.230	0.108	0.401
SNP markers	0.353	0.278	0.535

Data from Kostamo et al. (2012) and Olsson and Korpelainen (2013). Genetic diversity is measured as expected heterozygosity for microsatellites and as Nei's (1987) gene diversity for SNP markers

markers (Kostamo et al. 2012). Although the new primer pairs were designed for *F. lumbricalis*, the SNP markers may have utility in population genetic and phylogenetic studies across red algal species, since SNP marker regions possess lower levels of variation than typically hypervariable microsatellite regions (Table 13.1).

13.4 Challenges of Red Algal Populations Occupying Different Salinity Conditions

In nature, many plants are adversely affected and challenged by various environmental factors that have negative effects on survival, development, and reproduction. Natural selection in the wild is largely created by environmental stress, which is most intensively evoked in an organism at the edges of its ecological niche. The extent to which an organism is able to deal with stresses determines the limits of its ecological amplitude. The possibility to discover large amounts of expressed (i.e., protein coding) sequence information offers a unique chance to screen and detect molecular variation of genes at a genome-wide level, and to discover the polymorphisms that affect the success of organisms in natural environments.

Osmotic stress is one of the most significant natural abiotic stresses of seaweeds. At suboptimal salinities, the growth of red algae is reduced, as shown, e.g., in *Gracilaria* species (Bird and McLachlan 1986; Choi et al. 2006; Guillemin et al. 2013) and *Dixoniella grisea*, in which also mannitol levels increased considerably when salinity increased from the optimal level of 10–60 psu, indicating its role as an osmolyte (Eggert et al. 2007). Seaweeds may physiologically acclimate to changing osmolarity by altering their transcriptome under hyper- or hypo-osmotic conditions, as shown, e.g., in *Gracilaria changii* (Teo et al. 2009), *F. lumbricalis*

(Kostamo et al. 2011), *Kappaphycus alvarezii* (Liu et al. 2011) and *Porphyra yezoensis* (Uji et al. 2012), all of which have shown changes in gene expression levels or in the proportions of ESTs representing different functional categories.

The Baltic Sea provides a unique model system for the study of genetic effects of postglacial colonization followed by ecological differentiation. The entire Baltic Sea was covered by the Northern European ice cap during the last glaciation (Andersen and Borns 1994). The melting of the continental icecap was followed by several freshwater and marine phases, and resulted in the opening of the current connection to the Atlantic Ocean about 8000 years ago. Consequently, a new colonization route to marine organisms was established (Björck 1995). Since then, the channel connecting the brackish Baltic Sea to the North Sea and the Atlantic Ocean has been reduced, which has resulted in a cline of decreasing salinity toward its inner parts and a relatively stable salinity regime on a local scale (Kullenberg 1981). The present salinity gradient of the Baltic Sea ranges from c. 30 psu in the channel to 2 psu in its most northern and eastern parts. Brackish water is a stressful environment for marine organisms, and only marine species capable of survival and reproduction in reduced salinity can remain in the Baltic Sea, resulting in a reduction in species numbers in all major taxa (Middleboe et al. 1997).

For instance, the red alga *Ceramium tenuicorne* possesses considerable variation in growth and reproduction along the salinity gradient of the Baltic Sea (Gabrielsen et al. 2002) and its genetic variation shows the presence of a continuous cline corresponding the salinity gradient, even though the used marker type (RAPDs) unlikely represents adaptive genetic variation. These results may still be indicative of limited successful migration of genotypes possibly adapted to local salinity conditions. The genetic variation pattern of *C. tenuicorne* also corresponds to the previously demonstrated ecotypic differentiation among its populations sampled along this gradient (Düwel 2001). Another red alga, *F. lumbricalis*, occurs in the cold waters of the North Atlantic and Arctic Ocean (Holmsgaard et al. 1981; Bird et al. 1991). The species is well known among larger red algae for its tolerance of low salinity (Bird et al. 1991). An experimental assessment of the effects of salinity on growth has shown that the increase in the biomass is maximal at the salinity of 20 psu under favorable light and temperature conditions (Bird et al. 1979). The distribution of *F. lumbricalis* in the Baltic Sea extends as far as the 4 psu isohaline regions of the Gulf of Bothnia and Gulf of Finland (Zenkevitch 1963; Bergström and Bergström 1999). Only recently, extensive genetic studies have been conducted on *F. lumbricalis* (Kostamo et al. 2011, 2012; Olsson and Korpelainen 2013).

13.5 Genetic Diversity and Differentiation of Red Algal Populations Occupying Different Salinity Conditions

Understanding how environmental factors influence the spatial distribution of genetic variation provides insight into microevolutionary processes. Through studies on *F. lumbricalis*, Kostamo et al. (2011, 2012) and Olsson and Korpelainen

(2013) have tried to develop a better understanding of the genetic adaptation potential of red algae living on the edge of their habitat range in the brackish Baltic Sea, which provides a unique, evolutionary relatively young environment. Genetic studies on *F. lumbricalis* may aid in the planning of conservation measures for also other species living in this vulnerable ecosystem. Thus far, only a low number of marine organisms have successfully adapted to the low-salinity waters of the Baltic Sea during the postglacial period (Middleboe et al. 1997).

13.5.1 Gene Expression Patterns

In order to identify genes with potential roles in the salinity tolerance and other stress responses, and to gain knowledge for further studies on the mechanisms behind the physiological and ecological stress responses of *F. lumbricalis*, Kostamo et al. (2011) conducted a small-scale transcriptome analysis and constructed an expressed sequence tag (EST) library for algal material originating from the marine environment along the coast of Northern Ireland (35 psu) when subjected to extremely low salinity (6 psu). These sequences were compared with EST sequences originating from algal material growing naturally at 6-psu salinity in the northern Baltic Sea along the southern coast of Finland in order to generate new markers for population genetic and stress tolerance adaptation studies. In all, 28 % of annotated ESTs (26 and 30 % in the Atlantic Ocean and Baltic Sea, respectively) played a role in general or specific abiotic stress responses, while 4.3 % of annotated ESTs were similar to genes with known roles in specific salinity stress responses (4.9 and 3.8 % in the Atlantic Ocean and Baltic Sea samples, respectively). Some differences between the two sequence datasets were observed in the proportions of ESTs representing different functional categories indicating moderate functional divergence between the ocean and brackish water populations of *F. lumbricalis* (Kostamo et al. 2011).

13.5.2 Genetic Diversity Patterns

Genetic diversity patterns of *F. lumbricalis* populations occurring in different salinity conditions, ranging from 35 psu of the Atlantic regions to 3.6 psu in eastern Gulf of Finland in the Baltic Sea, were investigated with three types of markers: putatively neutral microsatellites, and possibly adaptive microsatellite and SNP markers developed from EST library sequences (Kostamo et al. 2011, 2012; Olsson and Korpelainen 2013). Information from presumably neutral microsatellite markers was compared with data of EST-derived microsatellite and SNP markers to reveal genetic variation patterns in genomic regions potentially subjected to different evolutionary forces. The hypothesis was that the divergence pattern among *F. lumbricalis* populations differs between the putatively neutral and adaptive

markers and that greater amounts of differentiation will be detected based on EST-derived and SNP markers.

Despite presumed asexual propagation in the Baltic Sea populations, the amount of genetic variation did not differ between the Atlantic Ocean and Baltic Sea populations, e.g., expected heterozygosities equalled 0.734 and 0.716, respectively, based on the neutral microsatellites, 0.285 and 0.272, respectively, based on EST-derived microsatellites, and 0.132 and 0.111, respectively, based on SNP markers (Table 13.1). The sole transition zone population from Sweden living at mid-range but variable salinity conditions possessed the highest amount of variability at EST-derived marker loci (0.420), which may be due to migration from multiple directions or adaptation to a wide range of environmental conditions (i.e., salinity). No evidence of recent bottlenecks was found in the combined Atlantic Ocean samples or in the combined Baltic Sea samples. Although marginal habitats, such as the brackish Baltic Sea, are often expected to have reduced population sizes Primary>Population size that would result in some loss of diversity over time through genetic drift (Johannesson and André 2006), there was no such evidence in relation to *F. lumbricalis*. The question of strict asexuality in *F. lumbricalis* in low-salinity conditions still remains open, as no multilocus genotypes (MLG) were shared by more than one sample in each population. In general, asexual species or populations often possess considerable amounts of genetic variation (Ellstrand and Roose 1987; Bengtson 2003), resulting from new mutations as well as from remnant sexuality and/or multiple origins. Therefore, populations with a predominantly asexual mode of population regeneration can display almost any pattern of genotypic structure.

Although the amount of genetic variation did not differ between the ocean and brackish populations, there was a great difference in variability between the marker types, EST-derived markers possessing considerably less variation than neutral microsatellites, and SNP markers showing even less variation (Table 13.1). It is evident that the expressed regions are subject to selection, unlike neutral microsatellite regions, and that leads to lower amounts of variability. However, genetic drift, gene flow and reproductive patterns affect genetic variation at all loci to the same extent. The influence of drift and/or reproductive patterns was visible as frequent heterozygote deficiencies were detected at neutral microsatellite loci: 52 % of tests showed significant heterozygote deficiencies and 19 % excesses (Kostamo et al. 2012). At EST-derived microsatellite loci, selection counteracts the effects of drift and reproductive patterns, and that shows as fewer heterozygote deficiencies (Kostamo et al. 2012).

13.5.3 Differentiation Along a Salinity Gradient

Selection is implicated when alleles (allele frequencies) at a given locus vary along with a specific environmental factor that creates an environmental cline. Therefore, it is justified to presume that genetic characteristics of algae varying along a salinity

gradient would indicate the presence of selection and resulting adaptation. Studies have previously demonstrated that red algal ecotypes react differently to different salinities, as shown, e.g., in *Bostrychia radicans* and *Caloglossa leprieurii* (Yarish et al. 1979), *Ceramium strictum* (Rueness and Kornfeldt 1992) and *Phycodrys rubens* (Van Oppen et al. 1995).

Based on the analysis of molecular variance (AMOVA), the genetic differentiation pattern of *F. lumbricalis* populations varied depending on the marker type (Kostamo et al. 2012; Olsson and Korpelainen 2013). Genetic differentiation (F_{ST}) based on putatively neutral microsatellites showed similar moderate values among both Atlantic Ocean and Baltic Sea populations and between Atlantic Ocean and Baltic Sea populations, 0.158, 0.124, and 0.136, respectively (Table 13.1). A comparable differentiation estimate (0.164) has been obtained for *P. haitanensis* based on putatively neutral microsatellites (Bi et al. 2014). However, the differentiation pattern of *F. lumbricalis* was different based on EST-derived microsatellites (0.230, 0.108, and 0.401, respectively) and SNP markers (0.353, 0.278, and 0.535, respectively) (Table 13.1). Thus, the results for *F. lumbricalis* indicated definite differentiation between the ocean and brackish populations in expressed genomic regions, while such differentiation was not detected with presumably neutral loci.

Besides AMOVA, the Bayesian Structure analysis, when conducted for expressed marker data, clearly resolved two main clusters, the Atlantic Ocean and the Baltic Sea (Fig. 13.1). Indeed, some of the Baltic Sea populations included in the study were from extreme habitats at the edge of the species' distribution range. The only transition zone population from Sweden clustered to the Atlantic Ocean group (Kostamo et al. 2012; Olsson and Korpelainen 2013). Among the brackish populations, there was a clear transition in the genetic pattern from the higher salinity conditions of the western-central Baltic Sea toward the extreme low-salinity conditions of the Gulf of Finland in the eastern Baltic Sea, which was especially distinct based on EST-derived data (Kostamo et al. 2012).

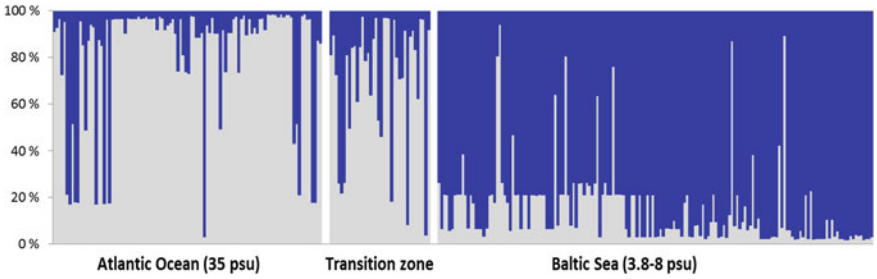

Fig. 13.1 Assignment of individual samples of *Furcellaria lumbricalis* to different pools as inferred by Bayesian clustering analysis based on EST-derived microsatellite markers. Samples represent four populations from the Atlantic Ocean (35 psu), one transition zone population from Sweden (15–30 psu) and six brackish populations from the Baltic Sea (3.8–8 psu) (data from Kostamo et al. 2012). Each color represents one of the two clusters formed in the analysis

Although definite trends were detected in the genetic structure of *F. lumbricalis* in the overall analysis based on EST-derived and SNP markers, it was shown that divergence patterns vary considerably among the loci (Kostamo et al. 2012; Olsson and Korpelainen 2013). Differentiation, apparently resulting from strong selection was most notable at the EST-derived microsatellite loci FI2143 and FI2838, which showed considerable differences in allele frequencies and possessed F_{ST} values equaling 0.389 and 0.354, respectively (for comparison, mean F_{ST} for all SNP markers 0.522, for all EST-derived microsatellite loci 0.267 and for all presumably neutral loci 0.095). These two loci were also among the most variable EST-derived marker loci (PIC values 0.239 and 0.374, respectively). Differentiation reflects differences in allele frequencies, which was especially clear in the frequency of allele 63 at locus FI2143: rare or not detected in the Atlantic Ocean populations but with a frequency range 0.217–0.656 in the Baltic Sea populations (Kostamo et al. 2012). A definite pattern was also detected for allele 213 at locus FI2838: a frequency range of 0.150–196 in the Atlantic Ocean and 0.125–0.978 in the Baltic Sea, the frequency increasing strongly toward extremely low salinity conditions. Although the neutrality tests provided no convincing evidence for selection in the studied genes, it is notable that deviations from neutrality were found only in the brackish Baltic Sea and transition zone populations of *F. lumbricalis*. Low salinity is a stress factor, which appears in the population genetic structures. However, such differentiation would have been missed if the investigations on *F. lumbricalis* had relied on neutral marker information only. Foregoing studies emphasize the importance of studying population genetic structures across geographic gradients using different genetic markers. Contrasts between neutral and adapatively important markers can potentially reveal the effects of natural selection.

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