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SHORT COMMUNICATION

Functional divergence in heat shock response following rapid speciation of *Fucus* spp. in the Baltic Sea

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Abstract In the Baltic Sea, the broadly distributed brown alga *Fucus vesiculosus* coexists in sympatry over part of its range (south west Gulf of Bothnia) with the Baltic endemic *F. radicans* sp. nov, while further north in colder and lower-salinity areas of the Baltic *F. radicans* occurs alone (north west Gulf of Bothnia). *F. radicans* appears to have arisen via rapid speciation from *F. vesiculosus* within the recent history of the Baltic (ca. 7500 BP). Possible functional divergence between the two species was investigated by comparing stress-responsive gene expression in a common-garden experiment. The experiment used two allopatric populations of *Fucus vesiculosus* from the Skagerrak (North Sea) and Central Baltic, as well as *F. radicans* from the same Central Baltic site. The two species in sympatry displayed divergent heat shock responses, while *F. vesiculosus* populations from allopatric sites did not. *F. radicans* was more sensitive to heat shock at 25°C, either alone or together with high irradiance and desiccation, than Baltic or Skagerrak *F. vesiculosus*. The results indicate that rapid functional divergence in the inducible heat shock response has occurred between sympatric species on a timescale of thousands of years.

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Introduction

The brackish Baltic Sea is a marginal environment for marine species, both geographically and environmentally (Johannesson and André 2006). Although its current post-glacial marine-brackish phase is recent (ca. 7500 BP (Ignatius et al. 1981; Donner 1995), isolation near the geographical and physiological limits of a species' distribution may result in periods of accelerated evolution (Orr and Smith 1998; Thompson 1998). These characteristics make the Baltic extremely interesting for the study of adaptive responses of populations to environmental change (Pearson et al. 2000; Gabrielsen et al. 2002; Riginos and Cunningham 2005) and rapid speciation (van Oppen et al. 1995; Pereyra et al. 2009).

A new species, endemic to the Baltic, was recently described (Bergström et al. 2005) as *Fucus radicans*, previously considered a dwarf morphotype of *F. vesiculosus*, together with which it occurs in sympatry in the SW Gulf of Bothnia. Further north in the Gulf of Bothnia (northern Baltic) at salinities of <5 PSU *F. radicans* is the only fucoid present (Tatarenkov et al. 2005; Pereyra et al. 2009). Species status for *F. radicans* is based on genetic and morphological differentiation from sympatric *F. vesiculosus*, and a greater capacity for, and predominance of, clonal reproduction (Bergström et al. 2005; Tatarenkov et al. 2005). Although unresolved by phylogenetic analysis of mitochondrial sequences (Coyer et al. 2006), microsatellite analysis has recently demonstrated reproductive isolation and very recent divergence time estimates between *F. radicans* and *F. vesiculosus*, possibly within hundreds rather than thousands of years BP (Pereyra et al. 2009). Endemic *F. radicans* therefore represents a case of rapid speciation within the Baltic Sea during the recent postglacial Littorina Sea period (ca. 7500 BP) (Ignatius et al. 1981; Russell 1985).

Decreasing sea surface temperature (SST) from the western to the northern Baltic (Siegel et al. 2006) has received less attention than salinity clines, despite the important role of thermal stress in marine biogeography (Somero 2005). While it has been established that extreme low salinity (3–4 PSU in the Gulf of Bothnia) has negative effects on fucoid reproductive ecology (Serrão et al. 1996; Serrão et al. 1999), gradients of both salinity and SST may affect broader distributional patterns of *Fucus* spp. in the Baltic. Divergent physiological tolerances to abiotic stressors (desiccation, freezing) were previously reported between *Fucus vesiculosus* populations from intertidal and Baltic habitats (Pearson et al. 2000). Based on these findings, rapid intraspecific population divergence for adaptive traits has already occurred over the brief evolutionary history of the Baltic Sea.

Here, we test for functional divergence between *F. radicans* and *F. vesiculosus* living in sympatry in the non-tidal central Baltic. A second allopatric population of *F. vesiculosus* from the intertidal North Sea (Skagerrak) was included for comparison of divergence within the *F. vesiculosus* lineage. The heat shock response (HSR) was compared by assessing heat shock protein (Hsp) gene expression following acclimation in a common-garden experiment. A moderate heat shock treatment was applied, and the effects of additional high light and desiccation stressors, more typical of intertidal habitats, were also assessed.

Methods

In the Baltic Sea (SW Gulf of Bothnia; ca. 5.5 psu salinity, no tides), individuals of *F. vesiculosus* and *F. radicans* were collected from shallow sympatric stands (1–2 m depth) at Öregrund (Fig. 1: 60° 20′ N, 18° 26′ E). In the Skagerrak (20–30 PSU, tidal), an intertidal sample of *F. vesiculosus* was collected at Tjärnö on the Swedish west coast (Fig. 1: 58° 52′ N, 11° 10′ E). Samples were collected at the end of June when the SST was similar at both areas, about 18°C.

Growing apices from random samples of ca. 20 individuals per population/species were cultured in salinity-adjusted (5.5 PSU for Baltic populations) or filtered natural seawater (SW; 25–27 PSU) for 14–16 days (15°C, low photon flux density (PPFD) of 50 µmol m^{-2} s^{-1}, 16:8 h light:dark), to provide a common acclimation history (Pearson et al. 2000). Experimental treatments consisted of short-term (30 min) exposure to water and thermal stressors: (1) Control: 15°C and 50 µmol m^{-2} s^{-1}, (2) High light: 15°C and 250 µmol m^{-2} s^{-1}, (3) Heat shock: 25°C and 50 µmol m^{-2} s^{-1}, (4) Heat shock with high light: 25°C and 250 µmol m^{-2} s^{-1}, and (5) Heat shock with high light and desiccation: aerial exposure at 25°C and 250 µmol m^{-2} s^{-1} (final tissue water content 60–70% that of hydrated tissue) Following the treatments, tissue was immediately frozen in liquid nitrogen and stored at −80°C before being lyophilized in preparation for RNA extraction.

Fig. 1 Map of the Baltic–Skagerrak region showing sampling sites for *F. vesiculosus* (filled circles) and *F. radicans* (open circle). Isohalines (*dotted lines*) and salinities are shown

Total RNA was extracted from lyophilized samples following Pearson et al. (2006). RNA samples were treated with DNase I (Qiagen, Hilden, Germany) cleaned using RNeasy (Qiagen, Hilden, Germany), quantified by UV spectrophotometry, checked for integrity by agarose gel electrophoresis, and stored at −80°C. Total RNA (2 µg) from a pool of ca. 20 individuals/treatment was reverse-transcribed with SuperScript III RT (Invitrogen, Carlsbad, CA, USA) and oligo-dT(18) primer (MBI Fermentas, Heidelberg, Germany) in duplicate 20 µL reactions that were then pooled. This was done to minimize possible effects of variations in cDNA synthesis reactions on qPCR. qPCR amplifications were run in triplicate (technical replicates) for each target and reference gene. The efficiency of PCR was calculated for the reference (α-tubulin) and each target gene, from triplicate amplifications of a dilution series (10^{-1}, 10^{-2}, and 10^{-3}), using as template pooled cDNA from all experimental conditions (Pfaffl et al. 2002). Efficiencies were calculated from the slope of the threshold cycle (Ct) versus [cDNA] plot in the standard way from:

$E = 10^{-1/\text{slope}}$

(Pfaffl et al. 2002), where E = amplification efficiency between 1 (minimum) and 2 (theoretical maximum).

The following genes (GenBank accession numbers in parentheses) were selected from *Fucus* EST libraries (Pearson et al. 2009a): Hsp70 (EU780017) and Hsp90 (EU780016), two genes encoding sHsps; sHsp_5 (EU780019) and sHsp_n3 (EU780018), a putative late embryogenesis abundant (LEA) protein (EU780020), and a 14-3-3 protein (EU780021). Primers were designed using Primer3 (http://frodo.wi.mit.edu/) (Tm 68–70°C; amplicon size 100–150 bp) (Table 1). Triplicate PCR per condition was performed in 20 µL using SYBR green-based detection (BioRad, Hercules, California, USA), 1 µL of cDNA (10^{-1} dilution) and 0.5 µM per primer, on an iCycler iQ Detection System (BioRad, Hercules, California, USA). Cycle parameters were 95°C for 2 min and then 40 cycles at 95°C for 10 s and 68°C for 30 s. Differences in relative gene expression between controls (acclimation conditions) and treatment means were assessed using randomization tests implemented with the REST© software of Pfaffl et al. (2002). Randomization tests have a similar power as standard parametric tests, but make no assumptions about the underlying distribution of the data, an advantage with relative gene expression data that are based on ratios.

Results and discussion

A divergent HSR was found for all four Hsp genes tested, with greater expression in *F. radicans* than *F. vesiculosus* (Fig. 2a–d). The results reveal divergence between these species at a functional genomic level, even where they occur in mixed stands in the Central Baltic Sea, reinforcing current evidence for species differentiation (reproductive isolation, clonality, divergent morphology, and reproductive success; (Serrão et al. 1996, 1999; Bergström et al. 2005; Tatarenkov et al. 2005; Pereyra et al. 2009).

The HSR, where present, was broadly similar under heat shock with and without additional light and/or desiccation stressors. High light alone did not induce HSR (see below for sHsp down-regulation), thus the Hsp genes tested responded primarily to thermal stress under our experimental conditions, with no evidence for additive effects of high light and/or desiccation stressors. The heat shock temperature of 25°C was modest, only slightly above the maximum summer temperatures experienced by submerged *Fucus* in the Baltic (Siegel et al. 2006). Exposure time was kept short at 30 min to minimize chronic effects that might obscure regulatory differences in gene expression. Similar temperature conditions are regularly experienced by intertidal fucoid populations, even at these latitudes (Beer and Kautsky 1992), and can be greatly exceeded at other locations within the broad range of *F. vesiculosus* (Pearson et al. 2009b). The limited response of Hsp90 and Hsp70 in intertidal *F. vesiculosus* from the Swedish west coast was therefore unsurprising (Fig. 2a, b). Furthermore, the similarity between the responses of both *F. vesiculosus* populations (Skagerrak and Baltic) suggests that the HSR has probably not undergone evolutionary change at the level of gene expression in Baltic *F. vesiculosus*. However, this is in contrast to physiological responses to emersion-stress tolerance (freezing and

Table 1 Genbank accession number, annotations (Blastx top hit against SwissProt, unless indicated otherwise), and primer sequences used for qPCR of *F. vesiculosus* and *F. radicans* from Öregrund, Baltic Sea (F.ves(B), F.rad(B)), and Tjärnö, Skagerrak (F.ves(SK))

Name	Accession/SP Annotation	Primers 5′–3′	Amplicon (bp)	PCR efficiency (*E*)		
				F.ves(SK)	F.ves(B)	F.rad(B)
Tub	Q40831/Tubulin alpha-1 chain	F: CACGAATTGGATCGTGCGCTTG	119	1.63	1.68	1.62
		R: TACTTGCCGTGCCTAGGGTCGC		(0.109)	(0.172)	(0.155)
Hsp90	EU780016/Heat shock protein 81-2 (HSP81-2)	F: CGTGAAGGGCGTGGTGGACT	140	1.71	1.71	1.65
		R: CGTCCTCGGCAAGCTCGTTG		(0.177)	(0.211)	(0.188)
Hsp70	EU780017/Heat shock 70 kDa protein	F: CGAAGGGCCAGATCCACGAG	101	1.56	1.61	1.61
		R: ACACGGCTCCTTGCCGTTGA		(0.133)	(0.126)	(0.172)
sHsp_n3	EU780018/17.4 kDa class I heat shock protein 4	F: TCCTTGCCTTTTGCGCGTTC	119	1.71	1.80	1.60
		R: ATCCCGTGGTTCCCGTCCTC		(0.206)	(0.250)	(0.140)
sHsp_5	EU780019/18.0 kDa class I heat shock protein	F: CAAGGTGGACATCGACAGCGAGT	112	1.74	1.70	1.69
		R: CACGAAGTGGTACTTGCGCTGCT		(0.175)	(0.162)	(0.086)
14-3-3	EU780021/14-3-3-like protein	F: TCGACGACGCAATCGCAGAG	121	1.85	1.77	1.79
		R: TCCGCCTCTCCCTGGTCTGA		(0.331)	(0.217)	(0.234)
LEA-like	EU780020/[a] plant late embryo abundant related	F: CCGCAGAGGAAGCCGATGAA	147	1.46	1.46	1.55
		R: TGACAGAGTTGGCGGCGTTG		(0.077)	(0.084)	(0.116)

The amplicon size and calculated PCR efficiency (±SE) for each gene are also shown

[a] BLASTO top hit: blast to orthologous groups (Zhou and Landweber 2007)

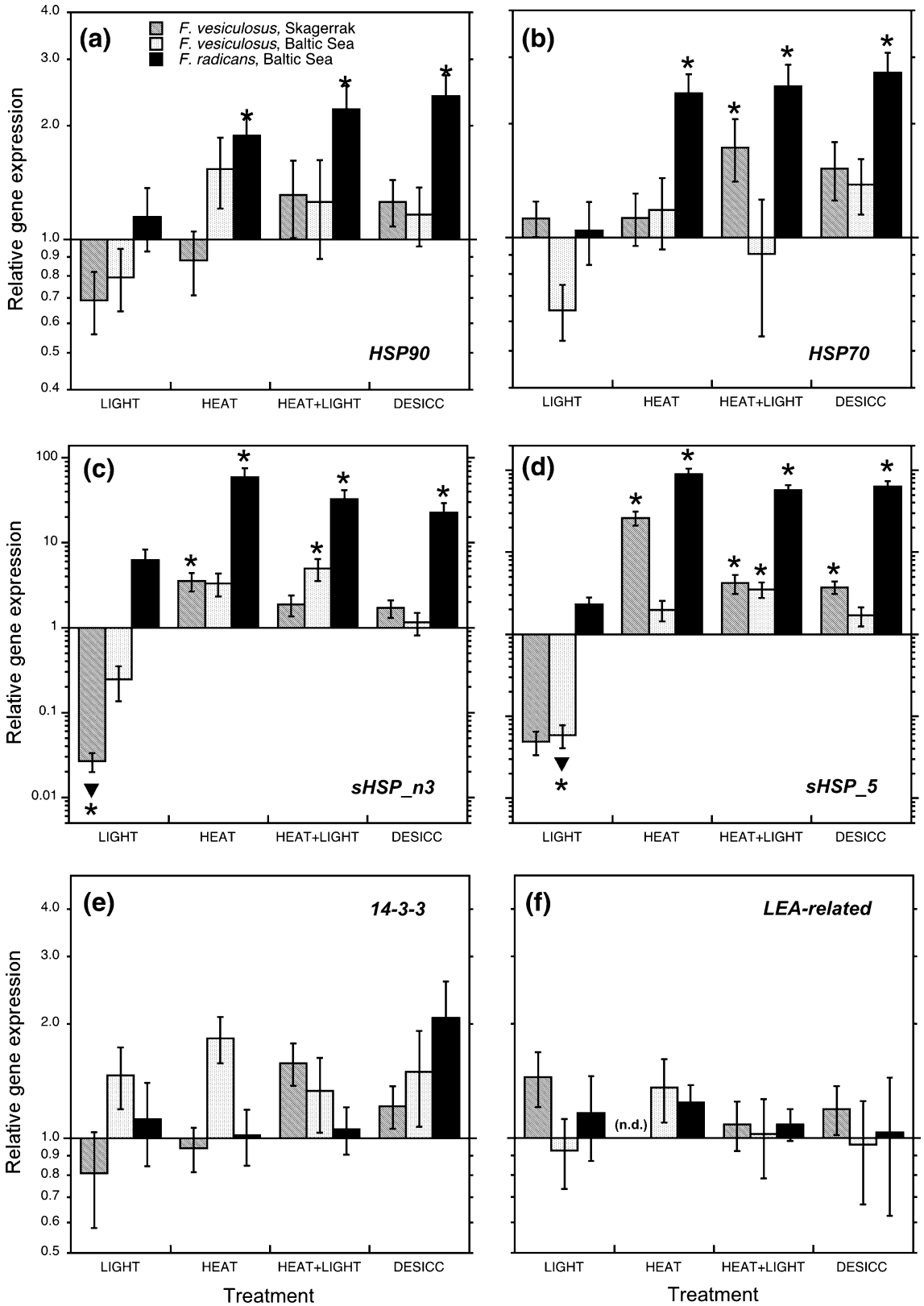

Fig. 2 Relative gene expression (fold change, mean ± SE) after 30 min exposure to high light (LIGHT), heat shock at 25°C (HEAT), combined light and heat shock (HEAT + LIGHT), and desiccation under high light and heat shock (DESICC), in Skagerrak and Baltic *F. vesiculosus*, and Baltic *F. radicans* for: **a** Hsp90, **b** Hsp70, **c** sHsp_3, **d** sHsp_5, **e** 14-3-3, and **f** LEA-like. Reference gene was α-tubulin. Values are means ± SE ($n = 3$). *Asterisks* indicate treatments significantly different ($P < 0.05$) from control (acclimation)

desiccation: Pearson et al. 2000), and longer term and/or exposure to more extreme temperatures will be required to clarify intraspecific variation in HSR.

While members of the Hsp90 and Hsp70 gene families have essential cellular functions and are constitutively expressed (Feder and Hofmann 1999; Frydman 2001), members of the small heat shock protein/α-crystallin family (sHsps) have protective roles (Lee and Vierling 2000; Laksanalamai and Robb 2004) and are transcriptionally regulated in response to HS and other stressors in plants (Waters et al. 1996). Our sHsp data revealed a hypersensitive HSR in *F. radicans* living in sympatry with *F. vesiculosus*. Two sHsp gene products were rapidly and highly over-expressed in *F. radicans*, between >20 and 100-fold within 30 min (Fig. 2c, d). In both *F. vesiculosus* populations (Skagerrak and Baltic), induction remained ca. an order of magnitude lower than for Baltic *F. radicans*. We thus show a clear species-specific difference in HSR. Few differences were detected between *F. vesiculosus* allopatric populations, although the response of sHsp_5 to HS deserves further attention.

The response of individual sHsp family members (>10 in *Fucus* based on EST data—Pearson et al. 2009a) would benefit from a more detailed characterization of expression under a variety of temperature and other abiotic conditions, and over a more extensive time-course. It is unclear why sHsp expression was down-regulated by high light in *F. vesiculosus*; a speculative hypothesis could be that higher photosynthetic rates increase ATP levels allowing (ATP-dependent) Hsp70-mediated refolding of non-native proteins, reducing the need for (ATP-independent) sHsp binding.

Two other transcripts were tested for their response to short-term stress exposure; a member of a multifunctional 14-3-3 protein family, which includes chaperone-type functions (Ferl et al. 2002), and a transcript with limited homology to a late embryogenesis abundant (LEA) protein. Under these experimental conditions, neither transcript significantly changed abundance (Fig. 2e, f).

Species distributions in the Baltic are often considered in the light of salinity gradients, but there is also an important gradient of average SST decreasing from the Gulf of Bothnia (mean SST of 7–8°C at the Central Baltic sympatric site and southern limit of *F. radicans*, Fig. 1) north into the Bothnian Bay (mean SST of <5°C). South of the Central Baltic sympatric site at Öregrund typical mean annual SST is fairly constant at 9–10°C (Siegel et al. 2006). The HSR of *F. radicans* suggests that thermal stress and/or growth temperature requirements could play a role in excluding this species from more southerly parts of the Baltic, restricting it to marginal habitats, where sexual reproduction (particularly male function) is compromised by low salinity conditions (Serrão et al. 1996, 1999), and clonal reproduction is an advantage (Tatarenkov et al. 2005).

Recent support for an evolutionary scenario in which *F. radicans* arose by rapid speciation from Baltic *F. vesiculosus* (Pereyra et al. 2009), as well as the lack of functional divergence between *F. vesiculosus* populations from the Baltic and Skagerrak shown here, implies that rapid evolution of the HSR regulatory machinery has occurred between Baltic *F. vesiculosus* and *F. radicans*. Although our understanding of the HSR in natural populations is limited, in low temperature environments the threshold temperature for heat shock is generally lower in comparison with those from higher or more variable thermal environments (reviewed by Feder and Hofmann 1999). For example, the HSR in Antarctic macroalgae can be induced at temperatures as low as 5°C (Vayda and Yuan 1994). The hypersensitive HSR of *F. radicans* may therefore be a consequence of a more cold-adapted physiology. A second possibility is that reduced tolerance to heat shock is a (non-adaptive) consequence of clonal reproduction and genome-wide loss of genetic diversity near the range edge (Pearson et al. 2009b). Based on the data of Tatarenkov et al. (2005), it is likely that several of the *F. radicans* individuals used in this study were genetically identical clones, while clonality in *F. vesiculosus* is much lower, or absent, in Baltic and Skagerrak populations, respectively. These alternative hypotheses (adaptive divergence versus loss of functional genetic diversity) could be addressed by comparing the relationship between trait means and neutral genetic diversity in less (clonally reproducing) and more (sexually reproducing) genetically diverse populations of *F. radicans* from different areas of the Baltic Sea. Whatever the predominant evolutionary mechanism, the functional divergence of HSR, a fundamental cellular process, between recently diverged species in a marginal marine habitat is a novel observation with potentially significant implications for ecological, evolutionary, and conservation biology.

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