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Tracking the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*

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Abstract

The spread of nonindigenous species into new habitats is having a drastic effect on natural ecosystems and represents an increasing threat to global biodiversity. In the marine environment, where data on the movement of invasive species is scarce, the spread of alien seaweeds represents a particular problem. We have employed a combination of plastid microsatellite markers and DNA sequence data from three regions of the plastid genome to trace the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*. Extremely low levels of genetic variation were detected, with only four haplotypes present in the species' native range in Japan and only two of these found in introduced populations. These invasive populations displayed a high level of geographical structuring of haplotypes, with one haplotype localized in the Mediterranean and the other found in Northwest Atlantic, northern European and South Pacific populations. Consequently, we postulate that there have been at least two separate introductions of *C. fragile* ssp. *tomentosoides* from its native range in the North Pacific.

Keywords: *Codium fragile*, invasive species, phylogeography

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Introduction

Exotic invasive species pose one of the greatest contemporary threats to global biodiversity and are ranked second only to habitat destruction in terms of potential ecological disaster (Wilcove *et al*. 1998). The introduction of alien species can have serious and long-lasting effects on established communities and may ultimately result in a drastic decrease in the biodiversity of impacted ecosystems. Marine systems are particularly threatened by invasive species but, to date, studies on marine invasions have been vastly outnumbered by those focusing on terrestrial and freshwater habitats (Grosholz 2002). In the marine environment, it is estimated that approximately 10 000 species are transported around the globe daily in the ballast water of ships and reports of the appearance of exotic seaweeds are increasing (Carlton 1999, 2000).

Codium fragile (Suringar) Hariot ssp. *tomentosoides* (van Goor) Silva is a large, dichotomously branched green alga documented to be one of four species of seaweed that have spread dramatically during the last century, not only between ocean basins but also between hemispheres (Trowbridge 1998, 2001). Considered as native to Japan, it first appeared on the shores of Holland shortly before 1900 and subsequently spread throughout Europe, rapidly colonizing the Mediterranean Sea (Silva 1955). It was first noticed in the western North Atlantic in 1957 (Bouck & Morgan 1957) and has since become a problem species along the east coast of America, growing at densities of up to 170 thalli m^{-2} and damaging the natural kelp (*Laminaria* spp.) forests which provide fish nurseries (Trowbridge 1995). *C. fragile* ssp. *tomentosoides* has also been found on the Pacific coast of North America (Silva 1979; Dawson & Foster 1982; Carlton & Scanlon 1985) and is now spreading along the South American Pacific coast of Chile as well as being reported recently in South Africa, Australia and New Zealand (Dromgoole 1982; Chapman 1999). In addition to the ecological impact of its rapid spread, the species also has serious economic implications for aquaculture industries. Indeed, the tendency of ssp. *tomentosoides* to overgrow and smother oyster beds has earned it the nickname 'oyster thief' (Naylor *et al*. 2001).

A crucial factor in the management and control of invasive species is to determine the frequency with which a species is introduced into an area, the size of the introduction and the subsequent pattern of spread (Wadsworth *et al*. 2000). In practice, however, the assessment of these phenomena is extremely difficult in the field as ecological surveys based on observational methods are unable to

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identify cryptogenic taxa, source populations or multiple introductions and cannot quantify levels of genetic diversity (Holland 2000). In recent years, the analysis of molecular genetic data has provided new insights into the population biology of invasive species. The analysis of patterns of genetic variation observed in chloroplast genomes is now routinely used to trace the biogeographical history of many invasive plants and algae (McIvor *et al*. 2001; Schaal *et al*. 2003). The main drawback of using markers specific to plant and algal organelles, however, has been the conservative mutation rates associated with chloroplast and mitochondrial genomes (Wolfe *et al*. 1987) and, consequently, many such studies have been unable to differentiate multiple, cryptic introductions, particularly in algae where the range of available markers is limited (for review see Provan *et al*. 2001).

The discovery of length polymorphism at mononucleotide repeats in the chloroplast genomes of plants has provided a powerful approach to the high-resolution analysis of levels of cytoplasmic variation, particularly below the species level (Powell *et al*. 1995). These chloroplast microsatellites have proved informative in a wide range of plant species but, to date, have not been utilized to study chloroplast genetic variation in algae (Powell *et al*. 1996; Provan *et al*. 2001). In the present study, we have employed high-resolution chloroplast markers for the first time in seaweeds in an attempt to elucidate the levels and patterns of genetic diversity present in populations of *C. fragile* ssp. *tomentosoides* from the species' native and non-native ranges. Two scenarios were tested: i) that there was a single introduction from Japan to Europe followed by subsequent spread from the source population throughout its introduced range, or ii) that there have been multiple introductions around the world and *C. fragile* ssp. *tomentosoides* is spreading to new locations idiosyncratically from various source populations in its native range. The former scenario would result in genetic uniformity of all introduced populations while in the latter scenario, different geographically localized genotypes corresponding to separate introductions would be observed. The ultimate goal of this study was to provide data needed to inform international responses to the increasing transport of marine organisms.

Materials and methods

Sampling and DNA isolation

Samples of *Codium fragile* ssp. *tomentosoides* were collected from introduced populations in the North Atlantic and northern Europe, Mediterranean and South Pacific as well

Table 1 *Codium fragile* ssp. *tomentosoides* samples used in this study

Status	Region	Country	Location	Collector*	*N*
Native	North Pacific	Japan	Nakagi, Honshu	CT	8
			Shirahama, Honshu	CT	8
			Moroiso Bay, Honshu	CT	8
			Awaji Island, Honshu	SS	12
			Toyo, Honshu	SS	8
			Kochi, Shikoku, Honshu	IM	7
			Oshoro Bay, Hokkaido	CT	10
			Sagami Bay, Honshu	CT	10
Introduced	North Atlantic	USA	Wrightsville Beach, North Carolina	CAM	18
			Isles of Shoals, New Hampshire	CAM	18
		Ireland	Fanad, Co. Donegal	CAM	24
			Finavarra, Co. Clare	FR	3
			Spiddal, Co. Clare	FR	2
		UK	Jersey	CT	8
		Wales	Broad Haven, Dyfed	CAM	8
		Netherlands	Bruinisse	HS	16
		Spain	Vidiago	JR	16
	Mediterranean	France	Thau Lagoon	MV	24
			Gulf of Hyères	MV	24
		Slovenia	Izola Bay	CB	15
			Adriatic	CB	20
		Greece	Poros	DEM	2
	South Pacific	Chile	Caldera Bay	JC	8

*CT – Cynthia Trowbridge; SS – Satoshi Shimada; IM – Ichiro Mine; CAM – Christine A. Maggs; FR – Fabio Rindi; HS – Herre Stegenga; JR – José Rico; MV – Marc Verlaque; CB – Claudio Batelli; DEM – D. E. Maggs; JC – Juan Correa.

Table 2 *Codium* chloroplast microsatellite primers

Locus	Repeat	Location	Primers (5′–3′)	T_m	Size
CFCPSSR1	$(T)_8$	*rbc*L intron	TTTGACAAATGAGAGTTTGG TTTTCGAACTCGTTTTTCA	50 °C	117 bp
CFCPSSR2	$(A)_{12}$	*rbc*L intron	TTTTATTGAAAAACGAGTTCG TCGAATAGAGTGACTTTCTAAA	54 °C	116 bp
CFCPSSR3	$(T)_8ACT(A)_{12}(T)_{10}$	*rbc*L intron	CGATTATTTTCTATTAAAACCA TCATAATATTCCAAAGAAATGG	54 °C	122 bp
CFCPSSR4	$(T)_9$	*rbc*L intron	ATTGCGGCTTTACAATTT AGAATGTGTTTCTGTGTAATCC	48 °C	146 bp
CFCPSSR5	$(T)_{11}$	*rbc*L intron	TTCGAAAATGGAATCTTTTTTT TTTCGCGTTGTGCATATCTC	54 °C	108 bp
CFCPSSR6	$(T)_9$	*rbc*L intron	TTTTGGAGATCTCAAAACAGGG CCCCCCTAAGAACCATACGT	60 °C	104 bp
CFCPSSR7	$(A)_{14}$	Upstream of *trn*G(UCC)	CATTTATTTCAATTAATTTAATTG GTAAAAGCAGTACTGGTG	52 °C	124 bp

Table 3 Primers used for sequencing

Locus	Primers (5′–3′)	T_m	Source
*trn*G(UCC)-5S	AGCAGTACAGGGGAATCGAA GAATTCAGTGTAAAACTAGTAATA	60 °C	GenBank accession number U10630
*psb*J-*psb*L	GTWGTWCCAGTATTRGACAT AACCRAATCCNAAYAAACAA	50 °C	This study
*rpl*16-*rps*3	CCMGAHCCCATHCGDTTTTC GGBMGHTTWAATGGHGCHGAWATT	56 °C	UCP6 from Provan *et al*. (2004)

as from eight populations in the native range in Japan (Table 1). They were identified initially by a diagnostic morphological feature, the presence of numerous pointed spines on the surface of the alga, and identification was confirmed by DNA sequencing. Total genomic DNA was extracted from silica gel-dried individual thalli using the Qiagen DNeasy Plant Mini Kit, quantified visually on 1% agarose gels stained with ethidium bromide and diluted to a final concentration of 50 ng/μL for subsequent PCR (polymerase chain reaction) analysis.

Chloroplast microsatellite analysis

Partial *C. fragile* chloroplast sequences in the EMBL database were searched for all mononucleotide repeats of eight bases or more using the STRINGSEARCH and FINDPATTERNS programs (Genetics Computer Group). Primers were designed to amplify the seven mononucleotide repeats found in noncoding regions using the program, PRIMER (V0.5; Table 2). PCR was carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C, annealing at [T_m] °C for 1 min (see Table 2 for T_m values), extension at 72 °C for 1 min and a final extension of 5 min at 72 °C. PCR was carried out in a total volume of 10 μL containing 100 ng genomic DNA, 5 pmol of ^{32}P-end labelled forward primer, 5 pmol of reverse primer, 1 × PCR reaction buffer (5 mM Tris-HCl [pH 9.1], 1.6 mM $[NH_4]_2SO_4$, 15 μg/mL BSA), 2.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase (Genetix). Products were resolved on 6% denaturing polyacrylamide gels containing 1× TBE and 8 M urea after addition of 10 μL of 95% formamide loading buffer. Gels were run at 70 W constant power for 2 h, transferred to 3 mm Whatman blotting paper and exposed to X-ray film for 1 h at –20 °C. In all cases, previously analysed samples were included as controls to compare product sizes across gels.

Chloroplast sequencing analysis

Three regions of the chloroplast genome were sequenced: one using species-specific primers designed from a *C. fragile* sequence in GenBank (*trn*G[UCC]-5S) and two using universal chlorophyte primers (*psb*J-*psb*L and *rpl*16-*rps*3). Primer sequences and sources are given in Table 3. PCR was carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C, annealing at [T_m] °C for 1 min (see Table 3 for T_m values), extension at 72 °C for 1 min and a final extension of 5 min

at 72 °C. PCR was carried out in a total volume of 20 µL containing 200 ng genomic DNA, 10 pmol of forward primer, 10 pmol of reverse primer, 1 × PCR reaction buffer (5 mM Tris-HCl [pH 9.1], 1.6 mM $[NH_4]_2SO_4$, 15 µg/mL BSA), 2.5 mM $MgCl_2$ and 1.0 U *Taq* polymerase (Genetix). 10 µL PCR product was resolved on 2% agarose gels, visualized by ethidium bromide staining, and the remaining 10 µL sequenced commercially (Macrogen, Korea). Sequences were aligned using the CLUSTALW program in the BioEdit software package.

Results

A total of 277 individuals of *Codium fragile* ssp. *tomentosoides* from eight native and 15 introduced populations were genotyped at seven chloroplast microsatellite loci. Only one of these loci (CFCPSSR7) was polymorphic, with all North Atlantic populations and the Chilean population being fixed for the 123 bp allele and all Mediterranean populations being fixed for the 124 bp allele. Within the Japanese populations, only the Tokyo population displayed any intrapopulation variation. The other populations had either the 123 bp allele (Shirahama, Moroiso Bay, Awaji Island and Oshoro Bay) or the 124 bp allele (Nakagi, Kochi and Sagami Bay).

Four individuals from each population (with the exception of Finavarra, Spiddal and Poros, which were only represented in the study by three, two and two individuals, respectively) were sequenced at three plastid loci (5S-*trn*G, *psb*J-*psb*L, *rpl*16-*rps*3). All 87 individuals were monomorphic at the *psb*J-*psb*L (154 bp) and the *rpl*16-*rps*3 (405 bp) loci. Only the 5S-*trn*G locus (244 bp) displayed any variation, with a single C to G transversion at position 77 being observed in one individual from the Kochi population and in two individuals from the Oshoro Bay population.

Combining the sequencing data with the chloroplast microsatellite data from the same 87 individuals gave a total of four haplotypes. The distribution of haplotypes across the populations studied is shown in Fig. 1. All four haplotypes were found in the Japanese populations, with three of these populations (Toyo, Kochi and Oshoro Bay) displaying intrapopulation variation. All introduced populations exhibited either Haplotype 1 or Haplotype 2. The North Atlantic, Northern European and Chilean populations were fixed for Haplotype 1, while the Mediterranean populations were fixed for Haplotype 2.

Discussion

Using a combination of chloroplast microsatellite variation and nucleotide substitutions, we have been able to identify two major introduction events in the biogeographical history of invasive *Codium fragile* ssp. *tomentosoides*. Previous attempts to track the invasion of *C. fragile* ssp. *tomentosoides*

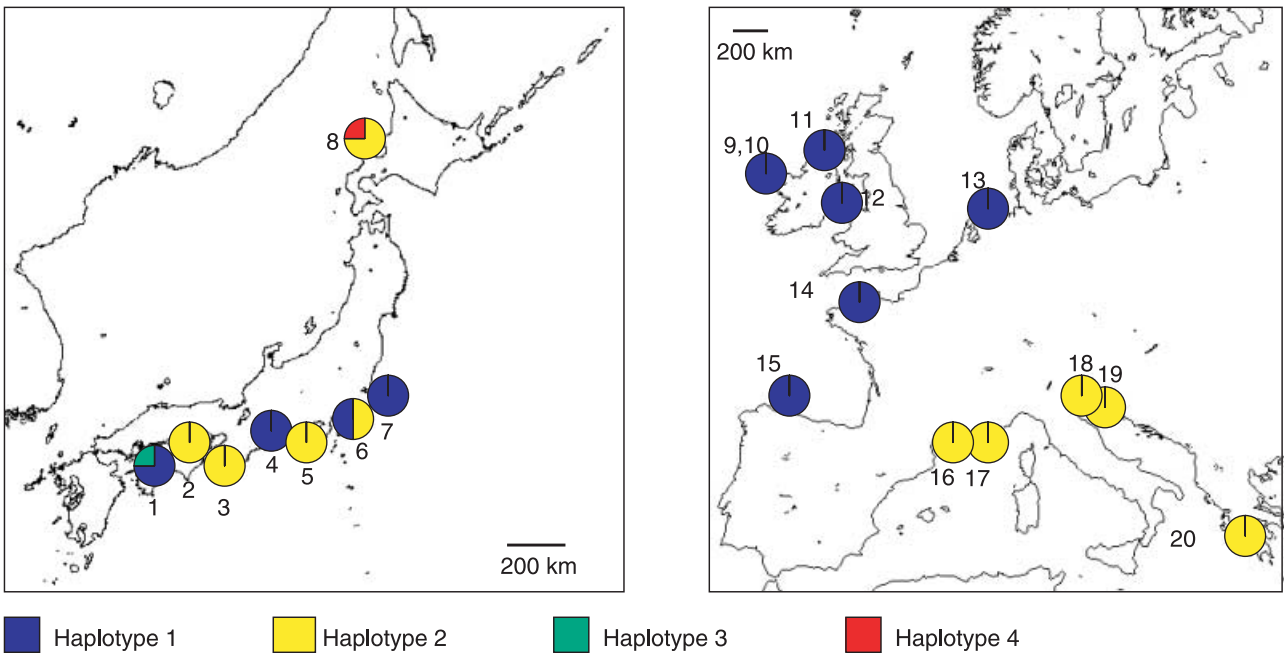

Fig. 1 Haplotype distribution in native Japanese (left) and introduced European (right) populations. Population numbers: 1 — Kochi; 2 — Shirahama; 3 — Moroiso Bay; 4 — Sagami Bay; 5 — Awaji Island; 6 — Toyo; 7 — Nakagi; 8 — Oshoro Bay; 9 — Finavarra; 10 — Spiddal; 11 — Fanad; 12 — Broad Haven; 13 — Bruinisse; 14 — Jersey; 15 — Vidiago; 16 — Thau Lagoon; 17 — Gulf of Hyères; 18 — Izola Bay; 19 — Adriatic; 20 — Poros. Not shown: North Carolina and New Hampshire, USA and Caldera Bay, Chile (all Haplotype 1). The four haplotypes are defined by the following pairs of polymorphisms: 1—124 bp (CFCPSSR7)/C (5 s-*trn*G); 2–123 bp/C; 3–124 bp/G; 4–123 bp — G.

have been frustrated by a lack of genetic variation. Goff *et al*. (1992) detected no variation at the intraspecific level in a range of *C. fragile* samples (including ssp. *tomentosoides*) and similar results were found by Coleman (1996). As discussed later, we also found very low levels of variation but some basic conclusions on large-scale invasion patterns in *C. fragile* ssp. *tomentosoides* can be drawn.

Our analysis revealed the existence of two dominant haplotypes that exhibited a very high degree of geographical structuring in invasive populations, suggesting distinct introductions into the Mediterranean and the North Atlantic from native populations in Japan. A previous study by Feldmann (1956) observed apparent life history differences in French Mediterranean populations, where reproduction was parthenogenetic whereas populations found elsewhere were sexual. It was claimed that this could be due either to the introduction of a parthenogenetic strain into the Mediterranean or to adaptation to local ecological conditions. Our results suggest strongly that these differences were because of the French populations studied were the result of a separate introduction. Trowbridge (1998) has highlighted the difficulty in identifying secondary and/or tertiary introductions but the findings of the present study are not consistent with the northern European and other North Atlantic populations being a secondary introduction from the Mediterranean or vice versa. Although a lack of exhaustive sampling within the native range precludes assignment of introduced populations to definite source populations, it can be hypothesized that the Mediterranean populations were probably not introduced from either the Nakagi, Kochi or Sagami Bay populations because none of these displayed Haplotype 2. By the same reasoning, the North Atlantic and Chilean populations, which all displayed haplotype 1, probably did not originate from either the Shirahama, Moroiso Bay, Awaji Island, Toyo or Oshoro Bay populations. Given the low levels of variation detected overall (see succeeding discussion), it is possible that multiple introductions of the same genotype from different source populations may have taken place but the high degree of geographical structuring of haplotype distribution in the introduced range suggests that this unlikely.

Despite the use of high-resolution genetic markers, this paper revealed extremely low levels of genetic variation in *C. fragile* ssp. *tomentosoides* both in introduced populations and within its native range. Genetic depauperacy as a result of founder effects has been revealed in populations of introduced species in a wide variety of environments, including marine algae (Kooistra *et al*. 1992; Jousson *et al*. 1998; McIvor *et al*. 2001; Fama *et al*. 2002; Marston & Villalard-Bohnsack 2002) but the levels of genetic diversity found in the species native range, while higher than those detected in introduced populations, were surprisingly low. Although this paper represents the first use of chloroplast microsatellites in a nonplant taxon, it is unlikely that the low levels of variation detected are a result of the techniques employed being unable to detect any variation present. Three of the monomorphic loci (CFCPSSR2, CFCPSSR3 and CFCPSSR5) were microsatellites of more than 10 repeats, which regularly exhibit substantial levels of intraspecific variation in plants (Provan *et al*. 2001). A notable exception to this has been described in *Pinus torreyana*, where all 12 loci studied were monomorphic resulting from a well-documented, severe bottleneck in the species' history (Provan *et al*. 1999). Furthermore, all three of the regions sequenced in this paper revealed intraspecific and intrapopulation variation in other *Codium* species (Provan *et al*. unpublished). Together, these observations suggest that the low levels of genetic variation detected in *C. fragile* ssp. *tomentosoides* have a biological cause (i.e. a genetic bottleneck resulting from a founder effect), rather then being the result of a technical shortcoming of the markers employed. It may be possible that, as well as displacing native *Codium* species such as *C. tomentosum* (in Europe) and *C. fragile* ssp. *fragile* (in Pacific North America) in their introduced range (Farnham 1980), one or more particularly invasive strains of ssp. *tomentosoides* has also come to dominate the native populations of the same subspecies in Japan. Indeed, the taxonomic validity of the various subspecies of *C. fragile* in both the native and introduced ranges has been a subject of much debate, particularly with respect to legislation concerning invasive taxa (Goff *et al*. 1992; Trowbridge 1998). Using the same genetic markers as those utilized in this paper, we have been able to consistently differentiate between the major subspecies of *C. fragile* (Provan *et al*. unpublished).

In summary, despite the low levels of genetic variation characteristic of invasive species and because of genetic bottlenecks, we have been able to demonstrate at least two invasions of *C. fragile* ssp. *tomentosoides*. The observation of more than one apparent introduction into Europe is contrary to the widely held belief that the species was only introduced to this region once (Silva 1955) and highlights the fact that the levels of anthropogenically-mediated introduction of exotic species may have been underestimated in marine ecosystems in general. Such additional knowledge of the patterns and modes of introduction of invasive species revealed by high-resolution molecular analysis will provide an important basis for risk assessment programs and should ultimately form an integral component of attempts to reduce the increasingly problematic impacts of marine invasions.

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