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Pathways of spread of the introduced ascidian *Styela clava* (Tunicata) in Northern Europe, as revealed by microsatellite markers

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Abstract *Styela clava*, a solitary ascidian native to the NW Pacific, has become a conspicuous member of fouling communities in NW European waters. As its natural dispersal appears to be limited, the wide distribution of *S. clava* along coasts within its introduced range may be attributed to secondary spread assisted by human activities. Here, we used six microsatellite loci to examine the genetic diversity and extent of gene flow among *S. clava* populations in its European introduced range. Samples were collected from 21 populations within Europe ($N = 808$), 4 populations within the USA and two populations within the native range (Japan). Large variation in genetic diversity was observed among the European populations but were not explained either by the geographic distance from the first introduction area (i.e. Plymouth, UK) nor by the time elapsed since the introduction. No founder effect was observed in the introduced populations, except possibly in Puget Sound (USA). At least two different introductions occurred in Europe, identified as distinct genetic clusters: northern Danish populations (resembling one Japanese population), and the rest of Europe; a sample from Shoreham (England) possibly represents a third introduction. In North America, the population from the Atlantic was genetically similar to the majority of European populations, suggesting a European origin for populations on this seaboard, while populations from the Pacific coast were genetically similar to the same Japanese population as the Danish populations.

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Introduction

Introduction of non-indigenous marine species to new geographical areas is a wide-spread phenomenon (Carlton 1996; Vermeij 1996; Ruiz et al. 1997; Castilla et al. 2004) which is often mediated by transport on ships' hulls and in ballast water (Carlton and Geller 1993). Marinas and harbours are thus repeatedly entrance gates for new invasions of exotic fauna and flora (Zibrowius 1991; Goulletquer et al. 2002) and ascidians in particular (Carlisle 1954; Holmes 1968; Brunetti 1979; Monniot 1981; Castilla et al. 2002; Lambert and Lambert 2003; Lambert 2004). Ascidians are now recognized as important invaders, dominating fouling communities and inter-fering with bivalve culture (Osman and Whitlatch 1999; Lambert 2007). Among colonial ascidians, *Didemnum vexillum* is an aggressive invader with rapidly expanding populations on the east and west coasts of North America that reach high abundance in newly colonized areas and affect aquaculture habitats by overgrowing shellfish and infrastructure (Valen-tine et al. 2007). Among solitary species, *Styela clava* is considered to be a serious pest into Prince Edward Island, Canada where it has become a nuisance to mussel culture activities (Bourque et al. 2007 and references therein).

Invasions may constitute rapid evolutionary events in which populations are subjected to founder effects during colonization, followed by a rapid expansion (e.g. Sakai et al. 2001). Introduced populations are thus more likely than native populations to be out of mutation/migration-drift equilibrium (Eckert et al. 1996). In particular, colonization events are predicted to result in reduced genetic diversity, a loss of rare alleles and an increase in population differentiation owing primarily to founder effects and genetic drift (Wright 1943; Nei et al. 1975; Cornuet and Luikart 1996). However, human transport vectors may pro-mote high genetic diversity within introduced popu-lations when independent introductions from different regions of the species' native range merge (Simon-Bouhet et al. 2006). These opposing scenar-ios have important implications for the adaptation, and therefore the successful establishment, spread, and proliferation of exotic species.

Styela clava, a solitary ascidian native to the coasts of Japan, Korea, China and Siberia (Herdman 1882; Abbott and Johnson 1972), has become a conspicuous member of fouling communities in various parts of the world. First reported outside its native range in California in the late 1920s, this large sea squirt has subsequently also colonized NW Europe, eastern and western USA, southern Australia, eastern and western Canada New Zealand and one site in the Mediterranean basin (Abbott and Johnson 1972; Lambert and Lambert 1998; Lützen 1999; Davis and Davis 2006, 2008; Locke et al. 2007). Since its initial discovery in Plymouth (UK) in 1953 (Carlisle 1954), *S. clava* has spread around Britain and also to the Channel Islands, Ireland, Scotland and along the coast of Europe from Denmark to Portugal (Davis and Davis 2004a). This species is mostly confined to sheltered localities free of strong wave action, such as inlets, bays, harbours and marinas. The total time spent as planktonic egg and larva is approximately 24–43 h (see references in Dupont et al. 2009). Adults might also disperse occasionally attached to drifting wood or weed (e.g. *Sargassum muticum*, Lützen 1999; Davis and Davis 2004a). However, as natural dispersal appears to have a limited range, the widespread occurrence of *Styela clava* within its introduced range may be attributed to secondary transportation by human activity. It has been suggested that dispersal could occur as settled juve-niles attached to commercial oysters that have been transported and re-laid (Christiansen and Thomsen 1981; Minchin and Duggan 1988), as mature adults attached to the hulls of ships (Millar 1960; Buizer 1980) or within sea-chests and, finally, as eggs and larvae carried in ballast water (Davis and Davis 2004a).

A recent population genetic study using microsat-ellites at small and intermediate geographical scales highlighted the importance of human-mediated dis-persal in range expansion and occupancy by *S. clava* in south-west England, while the limited dispersal ability of this ascidian resulted in fine-scale popula-tion structure in several marinas (Dupont et al. 2009). In addition, Dupont et al. (2009) showed that enclosed marinas might function as reservoirs of propagules for subsequent spread whereas others might be sinks for migrants.

Here, we used microsatellite nuclear markers to examine the overall connectivity, i.e. genetic rela-tionships, among *S. clava* populations located in its Northern European introduced range. We carried out extensive sampling of *S. clava* along European coasts, with emphasis on English and French coasts, in order to test the hypothesis that Plymouth might have been

the source for secondary introductions in other localities in Europe. Specifically we ask: (1) is there any evidence for founder effects or bottlenecks in the introduced populations? (2) is there evidence for multiple invasion sources or multiple introductions through human-mediated processes? and (3) can the pattern of genetic structure among European populations be used to infer the vectors of introduction and/ or pathways of spread?

Methods

Sampling and DNA isolation

Samples of *Styela clava* were collected from 21 populations within Europe (Fig. 1, details in Table 1) between January 2004 and September 2005. In order to compare genetic diversity indices with populations introduced outside Europe, 4 American populations were added to the dataset. Most of the introduced populations were collected in sites where the date of first observation of *S. clava* was recorded in the literature. Extensive sampling was undertaken along the Channel coasts around Plymouth, the first site where *S. clava* was recorded in Europe. In addition, two populations within the native range (Japan) were

obtained for preliminary comparison of genetic diversity between introduced and native populations. A portion of the branchial sac tissue of each individual was dissected and preserved in 96% ethanol for DNA analysis. Total genomic DNA was extracted using a CTAB method (described in Sambrook and Russell 2001, p. 6.61). Extractions were visually confirmed using 1% agarose gels stained with ethidium bromide.

Microsatellite genotyping

For the samples from Plymouth, Mission Bay and Otsuchi, genotypes were obtained from Dupont et al. (2006) and for Falmouth population, genotypes were obtained from Dupont et al. (2009) with additional genotyping completed for the locus Sc3e1. For the 23 other populations, individuals were genotyped at six microsatellite loci (Sc1b3, Sc1c8, Sc1h1, Sc2b12, Sc2h9 and Sc3e1) following protocols detailed in Dupont et al. (2006). Only loci presenting no or negligible heterozygote deficiency in Dupont et al. (2006) were chosen in order to avoid null-allele biases. In order to limit genotyping errors, all samples presenting new or rare alleles were amplified twice. PCR products were screened on a 6.5% polyacrylamide gel using a Li-Cor NEN Global IR2 DNA sequencer system.

Fig. 1 Location of the study populations. Two clusters identified by BAPS (see text and Fig. 3) were composed of one population (SHO and TSU), one cluster was composed of the populations indicated by *italic font* and the fourth cluster was composed of all other populations
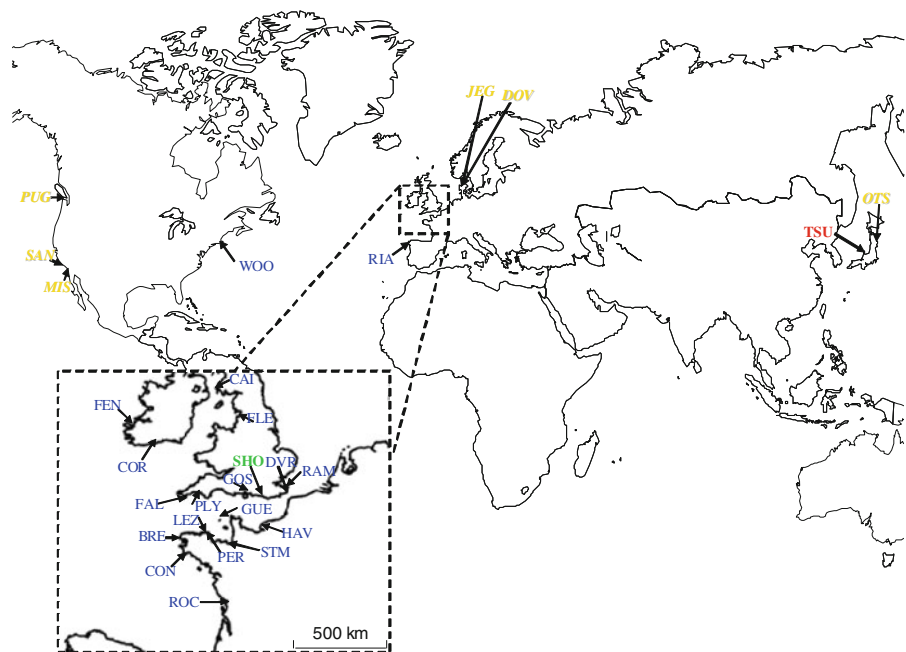
Table 1 Characteristics of sample sites

Countries	Locality	Code	Date	Site	First record	N	N_{all}	A_r	H_e	$\hat{f}$	p_{Wil}
European introduced populations											
Denmark	Jegind	JEG	2004	Jegindø Havn (marina)	c.1965[a]	20	4.17	4.00	0.480	0.063	0.078
	Doverodde	DOV	2004	Doverodde Havn (marina)	c.1965[a]	24	4.33	3.91	0.477	0.114	0.055
Ireland	Fenit	FEN	2004	Fenit Harbour (marina)	2004[b]	17	4.67	4.67	0.604	0.227	0.039
	Cork	COR	2005	East Ferry Marina	1971[c]	50	6.33	4.81	0.560	0.082	0.016
UK	Cairnryan	CAI	2004	Derelict pier (former military wharf)	1987[b]	35	5.50	4.69	0.601	0.131	0.023
	Fleetwood	FLE	2004	Wyre Dock Marina	1983[d]	35	6.00	4.85	0.584	0.039	0.016
	Falmouth	FAL	2004	Falmouth Marina	2003[b]	24	5.17	4.83	0.609	0.043	0.055
	Plymouth	PLY	2005	Plymouth Yacht Haven (marina)	1953[e]	30	5.17	4.63	0.582	0.124	0.219
	Gosport	GOS	2004	Hornet Sailing Club (marina)	1957[d]	29	5.33	4.91	0.546	0.096	0.023
	Shoreham	SHO	2005	Lady Bee Marina, Shoreham Harbour Canal	1963[f]	50	5.33	4.49	0.542	0.080	0.219
	Dover	DVR	2005	Granville Dock Marina	1969[d]	36	5.50	4.77	0.584	0.217	0.016
	Ramsgate	RAM	2005	Ramsgate Marina (inner & eastern sectors)	1989[b]	50	5.67	4.55	0.546	0.165	0.023
	Guernsey	GUE	2005	QEII and Victoria Marinas, St Peter Port	–	60	5.83	4.67	0.605	0.129	0.039
France	Le Havre	HAV	2005	Bassin de la Citadelle & Bassin Vauban	1977[g]	49	5.00	4.41	0.579	0.075	0.078
	St Malo	STM	2005	Port de Plaisance des Sablons (marina)	1971[h]	32	5.33	4.55	0.622	0.004	0.078
	Perros-Guirec	PER	2004	Port de Plaisance de Perros-Guirec (marina)	–	48	5.67	4.62	0.614	0.130	0.039
	Lézardrieux	LEZ	2004	Port de Plaisance de Lezardrieux (marina)	–	49	6.33	5.00	0.622	0.098	0.016
	Brest	BRE	2005	Port de Plaisance du Moulin Blanc (marina)	1973[i]	52	6.67	5.21	0.584	0.168	0.016
	Concarneau	CON	2005	Port de Plaisance de Concarneau (marina)	–	51	6.00	4.64	0.595	0.116	0.039
	La Rochelle	ROC	2005	Port des Minimes (marina)	–	32	5.33	4.34	0.555	0.177	0.078
Spain	Ria de Ferrol	RIA	2005	Puerti Deportivo de La Graña (marina)	1988[j]	35	5.17	4.50	0.566	0.041	0.023
Mean						37.1	5.45	4.62	0.574	0.110	–
SD						13.7	0.63	0.30	0.040	0.059	–
Total						808	8.67	–	0.589	0.127	–
American introduced populations											
East coast	Woods Hole	WOO	2005	Eel Pond	1970[n]	30	5.00	4.58	0.589	0.153	0.055
West coast	Mission Bay	MIS	2005	Seaforth Marina and Dana Landing	1959[k]	41	4.50	4.05	0.564	0.123	0.500
	Santa Barbara	SAN	2005	Santa Harbor Yacht Club (marina)	1970[l]	49	4.67	4.03	0.541	0.039	0.219
	Puget Sound	PUG	2005	Pleasant Harbor Marina (Hood Canal)	1998[m]	32	3.17	2.93	0.449	−0.080	0.500
Mean						38.0	4.34	3.90	0.536	0.059	–
SD						8.8	0.80	0.69	0.061	0.104	–

Table 1 continued

Countries	Locality	Code	Date	Site	First record	N	N_{all}	A_r	H_e	$\hat{f}$	p_{Wil}
Total						152	6.50	–	0.557	*0.099*	–
Native populations											
Japan	Otsuchi Bay	OTS	2004	Aquaculture cages, Ocean Research Inst.	–	50	6.00	4.30	0.556	0.077	*0.016*
	Tsukumo Bay	TSU	2004	Hulls and ropes, Noto Mar. Biol. Station	–	28	5.83	4.91	0.575	0.005	*0.039*
Mean						39.0	5.92	4.61	0.566	0.041	–
SD						15.6	0.12	0.43	0.013	0.051	–
Total						78	7.50	–	0.570	*0.062*	–
Mean						38.4	5.32	4.51	0.568	0.097	–
SD						11.6	0.75	0.45	0.043	0.068	–
Total						1038	10.00	–	0.589	*0.127*	–

For each sample, the following are indicated: code of population name, date of collection, nature of sampling site, date of first documented occurrence of *S. clava* in area, sample size (N), mean number of allele per microsatellite locus (N_{all}), allelic richness (A_r), gene diversity (H_e), estimator of the fixation index F_{IS} ($\hat{f}$) and Wilcoxon test P value for heterozygote deficit compared to expectations at mutation-drift equilibrium (p_{Wil}). Significant values for heterozygote deficiency (after Bonferroni correction) are in italic

[a] Lützen and Sørensen (1993); [b] Davis and Davis (2004b); [c] Guiry and Guiry (1973); [d] Coughlan (1985); [e] Carlisle (1954); [f] Holmes (1968); [g] Breton and Dupont (1973); [h] Huwae and Lavaleye (1975); [i] Minchin and Duggan (1988); [j] Vázquez and Urgorri (1992); [k] Cohen and Carlton (1995); [l] Abbott and Johnson (1972); [m] Cohen et al. (1998); [n] Berman et al. (1992)

Genetic diversity analyses

For each population, the genetic diversity was analyzed by computing allele frequencies, number of alleles (N_{all}) and expected heterozygozity (H_e) using GENETIX v 4.04 (Belkhir et al. 2004). To take into account variation in sample size, allelic richness (A_r; El Mousadik and Petit 1996) was estimated using FSTAT v.2.9.3 (Goudet 1999). The null hypothesis of independence between loci was tested from statistical genotypic disequilibrium analysis using exact tests implemented in the GENEPOP v 3.4 program (Raymond and Rousset 1995).

Analysis of genetic structure within populations

Distribution of the genetic diversity of introduced species can depart from equilibrium models for several reasons, including (1) recent demographic expansion of a local and isolated founding population or (2) recent mixing within a population of a set of genetically differentiated immigrants. We tested for deviation from mutation-drift equilibrium in the study populations using the approach proposed by Cornuet and Luikart (1996) and implemented in their software BOTTLENECK. Using a Wilcoxon test, the observed heterozygosity is compared with the heterozygosity expected under equilibrium considering a two-phase mutation model (TPM) recommended for microsatellite data (Di Rienzo et al. 1994). Recently founded populations were expected to display a transient excess of heterozygosity whereas expanding populations (e.g. recovering from a bottleneck) or populations resulting from immigration from differentiated sources should exhibit the opposite (Cornuet and Luikart 1996).

We used population heterozygote deficiencies to investigate the occurrence of cryptic population structure within populations (i.e. Wahlund effect, see Hartl and Clark 1997). Such a pattern may appear in invasive species because of their regular transportation from one site to another through human-mediated activities (e.g. fouling), generating mixtures between genetically differentiated pools of individuals. We quantified the Walhund effect within each population by calculating the Weir and Cockerham's (1984) $\hat{f}$, a monolocus estimator of the fixation index F_{IS}, with GENEPOP v 3.4. Conformity to Hardy–Weinberg equilibrium (HWE) was assessed with exact tests implemented in the GENEPOP v 3.4 program (Raymond and Rousset

1995) with specified Markov chain parameters of 10,000 dememorization steps, followed by 1,000 batches of 10,000 iterations per batch.

Analysis of genetic structure among populations

In order to investigate the importance of genetic exchanges among populations, exact tests of allelic differentiation were carried out between populations using GENEPOP v.3.4 software. To adjust for multiple comparisons, sequential Bonferroni correction was used. We also used a traditional population differentiation approach based on F_{ST} analysis. Weir and Cockerham's (1984) estimator of the fixation index F_{ST} ($\hat{\theta}$) was calculated with GENEPOP v.3.4. Multidimensional scaling (MDS) plots of the localities were produced from the matrix of pairwise F_{ST} estimates using STATISTICA v.6 software (StatSoft, Inc. www.statsoft.com.).

We used the program BAPS v.4.14 (Corander et al. 2003, 2008) to detect clusters of genetically similar populations in the Northern Hemisphere introduced range of *S. clava* and to estimate individual coefficients of ancestry with regard to the detected clusters. BAPS uses a stochastic optimization to infer the posterior mode of genetic structure which greatly improves the speed of the analysis compared to traditional MCMC-based algorithms (Corander and Marttinen 2006). When testing for population clusters, we ran 5 replicates for $k = 5$, $k = 10$, $k = 15$, $k = 20$, $k = 25$ and $k = 30$, where k is the maximum number of genetically divergent groups (populations). When estimating individual ancestry coefficients via admixture analysis we used recommended values of (1) the number of iterations used to estimate the admixture coefficients for the individuals [100], (2) the number of reference individuals from each population [200] and (3) the number of iterations used to estimate the admixture coefficients for the reference individuals [20].

Results

Genetic diversity

Values of genetic diversity indices were similar when comparing European and American populations (Table 1). For example, H_e ranged from 0.449 to 0.589 (mean = 0.536) in American populations and

from 0.477 to 0.622 (mean = 0.574) in European populations. Values computed in the two Japanese (native) populations were in the same range with H_e estimates of 0.556 and 0.575 (OTS and TSU respectively; mean = 0.566). Among populations in the introduced range, allelic richness and gene diversity were highly variable (Table 1). PUG presented the lowest level of genetic diversity ($A_r = 2.93$; $H_e = 0.449$). In Europe, the two Danish populations (JEG and DOV) presented the lowest levels of genetic diversity ($A_r = 4.00$; $H_e = 0.480$ and $A_r = 3.91$; $H_e = 0.477$ respectively) while two French populations (BRE and LEZ) had high levels of allelic richness (≥ 5). These large variations of genetic diversity among European populations were not explained by the time since the approximate date of introduction (i.e. first report in the literature). For instance, H_e was not correlated with this time since introduction ($r^2 = -0.129$, $P = 0.673$). Under the hypothesis that English populations originated in Plymouth (i.e. the first recorded European population, 1953; Carlisle 1954) and spread progressively from there along the coasts, undergoing successive bottlenecks, we expect a decrease of genetic diversity in English populations with increasing distance from Plymouth, but no significant correlation was observed for H_e ($r^2 = -0.287$, $P = 0.640$) and Ar ($r^2 = -0.506$, $P = 0.385$).

Genetic structure within populations

When testing for deviation from mutation-drift equilibrium in BOTTLENECK, a significant genetic diversity deficit (Wilcoxon test $P < 0.05$) was detected in 62% of European populations and in both Japanese populations. Under a two-phase mutation model (TPM) and in the absence of sub-structure within a population, which is the case for COR, FLE, GOS, RAM, LEZ, CON and RIA and both native populations (See values of $\hat{f}$ in Table 1), such a result supports the hypothesis of population expansion. Alternatively, as genetic structure within a population mimics the effect of demographic expansion (Cornuet and Luikart 1996), the deviation from mutation-drift equilibrium observed in FEN, CAI, DVR, GUE, PER and BRE might be due to sub-structure: significant departures from Hardy–Weinberg equilibrium were indeed observed in these sites (Table 1) (as well as in

ROC, SAN and WOO) suggesting sub-structure (Walhund effect) within these localities.

Genetic structure among populations

F_{ST} analysis showed a significant genetic structure at the level of the whole study (27 populations, $F_{ST} = 0.031$, $P < 0.001$) and when considering only the introduced populations (American and European populations, $F_{ST} = 0.030$, $P < 0.001$) suggesting limited gene exchange among populations and a non-unique source (in time or space) for the introduced populations.

When considering each population pair, 209 values of F_{ST} out of 351 (59%) were associated with a significant exact test after Bonferroni correction (Table 2). When considering only pairs of European populations, 42% of F_{ST} values were significant. These pairwise F_{ST} estimates were pictured with MDS plots which clearly illustrated the genetic isolation of the Danish, Japanese and western American populations from the remaining European populations (Fig. 2a). In agreement with this picture, it is noteworthy that the percentage of significant pairwise F_{ST} values fell to 30% of pairwise comparisons if the Danish populations were excluded from the analysis. In particular DOV was found to be different from every other European population except JEG, the other Danish population studied. Figure 2b illustrates the central position of GUE, FAL, PLY and BRE and the genetic differentiation of STM and SHO from the others among the European populations. This explains the significant F_{ST} value computed among the 21 European sampled sites ($F_{ST} = 0.019$, $P < 0.001$; without Danish populations $F_{ST} = 0.015$, $P < 0.001$), The American introduced populations were also genetically different ($F_{ST} = 0.047$, $P < 0.001$) even when only Pacific populations were considered (i.e. without WOO population $F_{ST} = 0.033$, $P < 0.001$). Moreover, the group of populations from the Pacific Ocean (American and Japanese) were genetically different from the group of populations from the Atlantic (American and European)($F_{ST} = 0.029$, $P < 0.001$). Our study did not aim focus on native populations. However, it is interesting to note that the two sampled Japanese populations also showed significant genetic differences between them ($F_{ST} = 0.026$, $P < 0.001$).
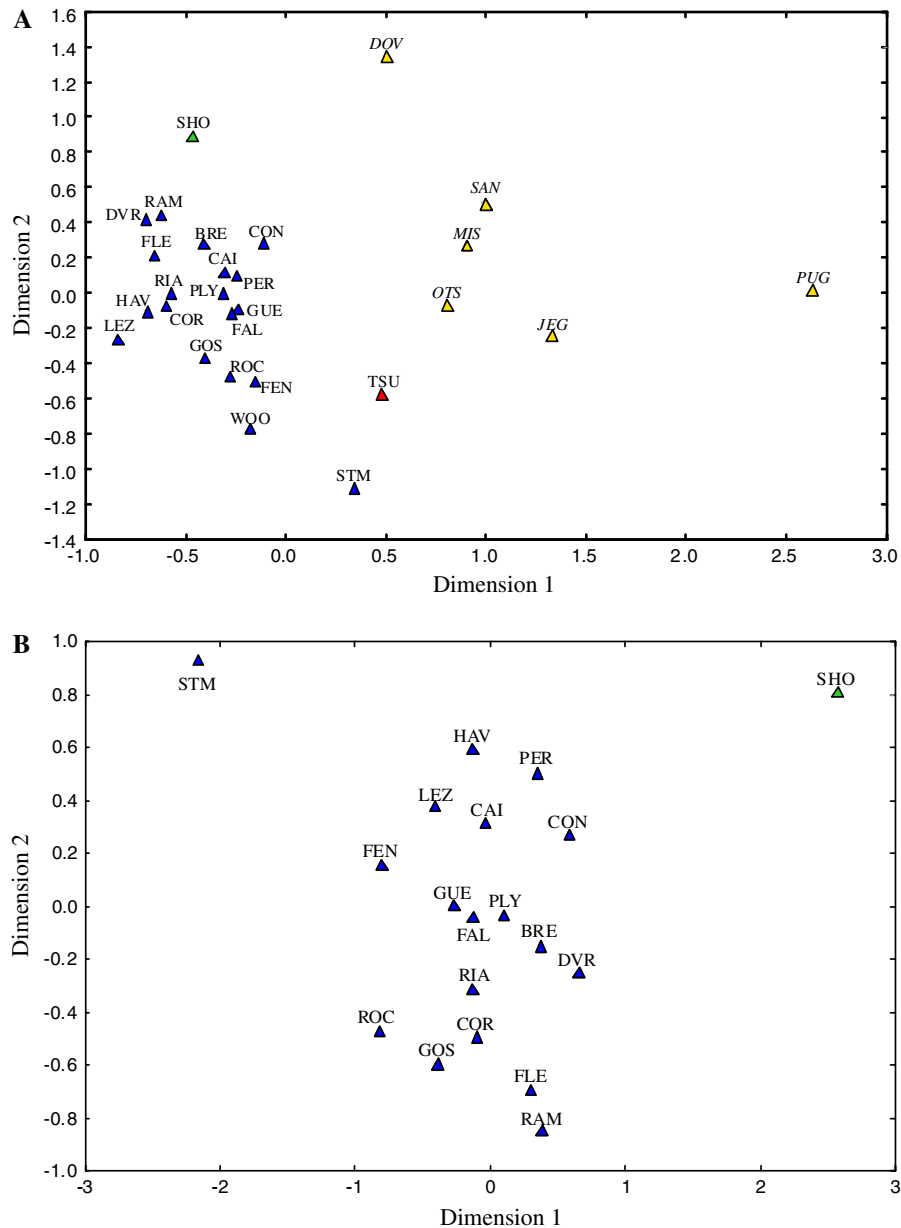
Table 2 Pairwise multi-locus estimates of $\hat{\theta}$

	JEG	DOV	FEN	COR	CAI	FLE	FAL	PLY	GOS	SHO	DVR	RAM	GUE	HAV	STM	PER	LEZ	BRE	CON	ROC	RIA	WOO	MIS	SAN	PUG	OTS
DOV	0.04																									
FEN	0.04	0.07																								
COR	0.05	0.06	0.01																							
CAI	0.05	0.04	0.01	0.01																						
FLE	0.07	0.05	0.03	0.01	0.00																					
FAL	0.05	0.06	−0.01	0.00	0.00	0.01																				
PLY	0.05	0.06	0.00	0.01	0.01	0.01	−0.01																			
GOS	0.04	0.06	0.00	0.01	0.01	0.01	0.00	0.00																		
SHO	0.09	0.06	0.04	0.05	0.05	0.06	0.03	0.03	0.05																	
DVR	0.08	0.06	0.03	0.01	0.01	0.00	0.01	0.00	0.02	0.04																
RAM	0.07	0.05	0.02	0.01	0.01	0.00	0.01	0.01	0.01	0.04	0.00															
GUE	0.05	0.06	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.04	0.01	0.01														
HAV	0.07	0.06	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.05	0.01	0.03	0.01													
STM	0.04	0.06	0.01	0.02	0.02	0.01	0.04	0.03	0.03	0.07	0.05	0.06	0.03	0.03												
PER	0.05	0.06	0.02	0.01	0.01	0.00	0.00	0.02	0.00	0.04	0.02	0.02	0.01	0.01	0.04											
LEZ	0.08	0.07	0.01	0.01	0.00	0.01	0.00	0.02	0.00	0.05	0.02	0.02	0.01	0.00	0.03	0.01										
BRE	0.06	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.03	−0.01	0.00	0.00	0.01	0.04	0.01	0.01									
CON	0.05	0.05	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.00	0.02	0.01	0.00	0.03	0.00	0.02	0.00								
ROC	0.04	0.06	0.00	0.00	0.02	0.02	0.02	0.01	0.01	0.05	0.03	0.02	0.02	0.02	0.02	0.01	0.01	0.02	0.02							
RIA	0.06	0.05	0.00	0.00	0.00	−0.01	0.01	0.00	0.00	0.04	0.01	0.00	0.00	0.01	0.04	0.01	0.01	0.00	0.01	0.00						
WOO	0.06	0.10	0.01	0.03	0.03	0.04	0.01	0.01	0.02	0.06	0.04	0.04	0.01	0.03	0.03	0.02	0.03	0.02	0.02	0.01	0.00					
MIS	0.02	0.05	0.04	0.04	0.04	0.05	0.02	0.02	0.04	0.06	0.05	0.05	0.04	0.05	0.05	0.02	0.06	0.04	0.04	0.04	0.05	0.04				
SAN	0.03	0.05	0.06	0.04	0.04	0.04	0.03	0.03	0.04	0.08	0.04	0.04	0.04	0.05	0.06	0.03	0.06	0.03	0.03	0.05	0.05	0.05	0.00			
PUG	0.03	0.10	0.09	0.13	0.12	0.14	0.11	0.11	0.12	0.12	0.13	0.13	0.11	0.13	0.08	0.11	0.14	0.11	0.09	0.11	0.13	0.11	0.05	0.05		
OTS	0.00	0.03	0.02	0.03	0.03	0.04	0.02	0.02	0.03	0.06	0.04	0.04	0.03	0.04	0.03	0.03	0.04	0.03	0.02	0.03	0.04	0.04	0.00	0.01	0.03	
TSU	0.02	0.06	0.02	0.04	0.04	0.03	0.04	0.02	0.04	0.08	0.05	0.05	0.02	0.04	0.04	0.04	0.05	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.09	0.03

Estimates associated with a significant exact test after sequential Bonferroni correction are in bold. The sub-matrixes between localities in the same ocean (Atlantic or Pacific) are highlighted in italic

Fig. 2 Multidimensional scaling plots constructed using pairwise F_{ST} estimates among **a** all populations (stress = 0.09) and **b** all European populations except Danish ones (stress = 0.15). The same colour code as in Fig. 1 was used

Main results from F_{ST} computations were confirmed with Bayesian analyses. Analyses using BAPS identified 4 genetic clusters overall ($P = 1$; Fig. 3a, b): two clusters were composed of only one population (SHO and TSU), one cluster was composed of all the non-Danish European populations except SHO plus the American population from the Atlantic (WOO), and the fourth cluster was composed of the

Danish populations, the American populations from the Pacific coast and one Japanese population (OTS).

Discussion

None of the studied introduced populations of *Styela clava* in Europe and America displayed evidence of
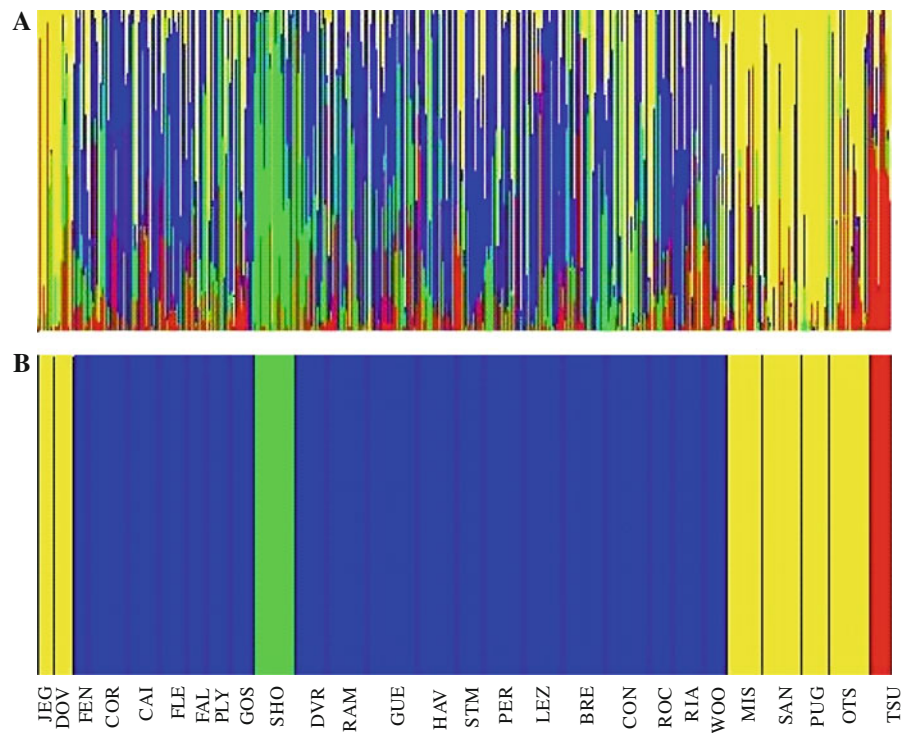
founder effects. Genetic diversity within introduced populations was generally high and similar whatever their geographic origin. In addition, the two samples from Japan, within the native range of *S. clava*, showed genetic diversity of the same level. Altogether, there was little evidence of population bottlenecks with attendant loss of genetic diversity during introduction and secondary spread. Only the sample from Puget Sound (PUG) showed lower, but still appreciable, diversity. No general link was demonstrated between the date of first occurrence of *S. clava* at a locality and the genetic diversity there, although Puget Sound, with relatively low diversity, had also been colonised relatively recently.

The relative genetic distinctness of the two Japanese samples, taken on opposite sides of the main island Honshu, suggests that populations of *S. clava* are genetically differentiated within its native range, although much more extensive sampling is clearly necessary to confirm this. If so, and given sufficiently strong differentiation within the north-west Pacific, independent introductions derived from different parts of the native range would be genetically distinguishable even in the absence of

bottlenecking and related founder effects. Pairwise F_{ST}, MDS and BAPS analyses all pointed out the genetic isolation of two Danish populations (JEG and DOV). Our data thus suggest that (at least) two separate introductions have occurred in Europe, represented by the samples from Denmark and the rest of Europe with the possible exception of Shoreham (SHO). According to the BAPS analysis, the majority of samples from Europe belongs to a single genetic cluster, but differs from the Japanese populations on either side of Honshu. It has been suggested that the introduction of *S. clava* in the early 1950s was linked to the return of warships from the Korean War (1950–1953) to naval bases in southern England (Plymouth, the site of first occurrence, and perhaps also Portsmouth) (Coughlan 1969; Minchin and Duggan 1988), presumably involving hull fouling. The genetic distinctness of the bulk of European populations from the Japanese samples is in keeping with an origin elsewhere in the species' native range, and thus with the hypothesis of a Korean derivation, but more extensive sampling of the native range would again be necessary to test this theory properly.

The Limfjord, a shallow sound in northern Denmark where JEG and DOV populations were collected, has high salinity and high summer temperatures compared to other areas of the region. As a result it holds several southern faunal elements, and has been a recipient of several species of immigrants that originate from widely different regions (Knudsen 1989). Lützen (1999) noted that the first record of *S. clava* in the Limfjord was in an oyster bed to which spat from the English Channel had been transferred as part of commercial operations. He also suggested that many French and some Dutch populations of *S. clava* could be attributed to importation of the Pacific Oyster (*Crassostrea gigas*) from Japan. Our molecular data suggest the reverse relationships: (1) they link the Northern Danish populations with Japan (and/or the west coast of North America), rather than England, thus potentially implicating the importation of oysters from a Pacific site, and (2) indicate a common origin for French and English populations, with the chronology of first occurrences suggesting spread from England to France. The first definite Danish record was in 1980, concerning individuals believed to represent the 1978 year-class (Christiansen and Thomsen 1981), but Lützen and Sørensen (1993) and Lützen (1999) note that the species 'had presumably been present from the mid-1960 s'. The basis for this statement is that a fisherman interviewed by J. Lützen recalled seeing *S. clava* for the first time in the mid-1960 s while operating close to oyster beds in the northern part of Nissum Bredning (Lützen and Sørensen 1993 and J. Lützen *in litt.* to JDDB in 2006); in contrast to the 1978 date, this timing would place the arrival of *S. clava* before the recorded commercial importation of *C. gigas* to Europe had begun. However, the molecular data and a first occurrence in the late 1970 s are in keeping with the suggestion of Christiansen and Thomsen (1981) that the repeated importation of commercial oysters from California to the western Limfjord during the 1970 s may explain the origin of the populations of *S. clava* in northern Denmark.

The existence of a genetically distinct population of *S. clava* at Shoreham (SHO) in SE England was not anticipated, and no particular explanation can be offered. Lady Bee Marina, with berths for 120 vessels, lies within Shoreham Harbour, a nationally significant commercial and fishing port. Of possible relevance are the facts that (1) the sampled site is in the inner part of the harbour in the lock-gated Shoreham Harbour Canal, and thus relatively limited exchange with other populations would be expected, and (2) the harbour was heated by cooling water discharge for most of the twentieth century until the coal-powered Brighton 'B' Power Station closed in 1987. This may have explain why some warm-water species have been recorded in Shoreham Harbour (e.g. the barnacle *Amphibalanus amphitrite* (Bishop 1947) and the polychaete *Hydroides elegans* (Monro 1938, as *H. incrustans*). The new gas-powered Shoreham Power Station, was commissioned in 2000, and uses the same cooling water outfall structures in the Shoreham Harbour Canal as Brighton 'B'; Lady Bee Marina is nearby, on the opposite bank of the canal. The genetic differentiation of the SHO population was supported by four of the six supposedly neutral markers (i.e. microsatellites developed from a conventional enriched library, Dupont et al. 2006) used in this study. Indeed, between 60 and 100% of significant tests were obtained for the loci Sc1b3, Sc1h1, Sc2h9 and Sc3e1 when monolocus exact tests of allelic differentiation were carried out between SHO and the other European population (data not shown). Thus, a hypothesis of selection of some alleles by temperature may be ruled out. The relatively high genetic diversity of the Shoreham population of *S. clava*, comparable to that of the surrounding sites, and the presence of two unique alleles, albeit as single copies in our sample, suggest that this sample may represent a third independent European introduction, reflected in its recognition as a separate BAPS cluster.

The clustering of the sample from Woods Hole (WOO), Massachusetts, with the most widespread European grouping of populations suggests a European origin for the initial colonisation of the eastern coast of North America. The timing of appearance of *S. clava* on the E. coast of the USA is in keeping with this suggestion: the species was first noted off Massachusetts in 1970 (Berman et al. 1992), by which time it had become widespread and locally abundant on the S coast of England and had spread to Wales and mainland Europe. Concerning the western coast of North America, *S. clava* is believed to have been introduced to Californian waters in the 1920s via ships previously docked in Asian ports (Abbott and Johnson 1972). Our findings are in keeping with this hypothesis: the populations from the west coast of the USA are genetically similar to the Otsuchi population (OTS) from Japan.

Table 3 Genetic features of introduced ascidians

Species	Native range	Native pop.	Introduced pop.	Age of introduction (years)	Markers	Genetic diversity in introduced range	Multiple introductions?	Founder effect?	Ref.
Botryllus schlosseri	Presumed Mediterranean origin	N/A	New Zealand	230–150	5 μsats	High but lower than in native range[a]	Yes	Yes	Ben-Shlomo et al. (2001)
		N/A	East coasts USA	>170	4 μsats		No	N/A	Stoner et al. (2002)
		N/A	West coasts USA	~60			No	N/A	
		N/A	Iberian peninsula and Brittany	N/A	4 μsats	High	Gene diversity levels mirrored the movement of the ice front in the last ice age		Ben-Schlomo et al. (2006)
			Germany and England			Intermediate			
			Scotland			Low			
		N/A	Southern Europe	N/A	COI	Low	Yes	Yes	Lopez-Legentil et al. (2006)
Corella eumyota	Southern hemisphere	South Africa New Zealand	France	<10	12 μsats	Extremely low, lower than in native pops	No	Yes	Dupont et al. (2007)
Microcosmus squamiger	Australia	Australia	Indian ocean Pacific ocean Atlantic ocean Mediterranean sea	N/A	COI	High, comparable to native range	Yes (or high number of colonizers)	No	Rius et al. (2008)
Styela clava	Japan, Korea, China and Siberia	Japan	European East coasts USA West coast USA	~50 ~35 ~85	6 μsats	High, comparable to native range	Yes	No	This study

For each genetic study of an ascidian biological invasion, the populations studied, the markers used [microsatellites or mtDNA sequences (*COI* gene)], the presumed age of the introduction (years ago), the genetic diversity in the introduced range, the occurrence of multiple introductions and the possible occurrence of founder effect, as described in the cited paper, are indicated

[a] Comparison with data of genetic diversity obtained using microsatellites in populations from Israel and Croatia representing the presumed source of the Mediterranean populations (Rinkevich et al. 2001; Paz et al. 2003)

Conclusion

The six microsatellite markers used in this study all showed a relatively high degree of polymorphism in the introduced European and North American populations, indicating a lack of strong founder effects. This result adds to the long list of marine invaders not showing the expected founder effects associated with introduction processes (see Roman and Darling 2007 for a review). Similar results have been recorded in other introduced ascidians, namely *Microcosmus squamiger* and, to a lesser extent, *Botryllus schlosseri* (Table 3). These findings corroborate the study by Silva and Smith (2008) using AFLPs that showed that invasive populations of ten different ascidian species (including *S. clava*) are highly polymorphic. In contrast, extremely low genetic diversity due to a combination of founder effects and selfing was observed in introduced populations of the tunicate *Corella eumyota* (Dupont et al. 2007, Table 3).

A more complete survey on the whole distribution range would be necessary to fully understand the pattern of invasion of *Styela clava* in the Northern Hemisphere as well as the pattern of spread of the species at a worldwide level. However, the study dataset helped to substantiate two independent routes of introduction of *S. clava* into Europe by different vectors, in accordance with previous suggestions based on circumstantial evidence. Because of the multiplicity of introduction pathways and vectors (see for example Voisin et al. 2005), cryptic introductions appear to be common in marine biological invasions.

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