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Contrasting population genetic structures in *Amphipholis squamata*, a complex of brooding, self-reproducing sister species sharing life history traits

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ABSTRACT: Life history traits are among the major forces influencing the spatial organisation of biodiversity. Brooding species, lacking a planktonic larval phase, have a weak potential for dispersal and are prone to displaying strong spatial genetic structures of their populations. Self-reproduction allows a single individual to establish a new population. Using nuclear markers to assign specimens to species (1004 specimens) and sequences of the 16S mitochondrial gene (for a subset of 479 specimens) to estimate genetic differentiation, we analyzed spatial and bathymetric sampling of 14 locations along the French Mediterranean coast in order to investigate the genetic effects of brooding and self-reproduction on the *Amphipholis squamata* species complex. The spatial organisation of the complex appeared chaotic, illustrating the random nature of dispersal in these brooding organisms. Bayesian dating confirmed the old age of the species complex (approximately 10 million yr). The different species displayed contrasted levels of genetic diversity and differentiation, despite their similar and extreme self-reproduction rates. This study illustrates the role of stochastic dispersal on species assemblages and genetic structure, and suggests a strong influence of past demographic history on population genetic structure of co-distributed species.

KEY WORDS: Bayesian dating · Demographic history · Dispersal · Mediterranean Sea · Spatial genetic structure · Species complex

INTRODUCTION

There are strong links between biodiversity and ecosystem functioning (Hooper et al. 2005, 2012). In the current context of global change, it is of primary importance to better understand the processes underlying biodiversity. Intra-specific genetic diversity represents the fundamental level of biodiversity, and yet has been less studied than species and ecosystem levels (Pauls et al. 2013). Among the driving factors of genetic diversity, life history traits (e.g. reproduction and developmental modes) and the associated potential of dispersal are known to greatly influence population genetic structure (Palumbi 1994, Tarnowska et al. 2012, Dawson 2014, Haye et al. 2014, Romiguier et al. 2014). In marine organisms, depending on the presence, absence or duration of a pelagic larval phase, species show contrasting genetic patterns (Kelly & Palumbi 2010, Boissin et al. 2011, Higgins et al. 2013, Haye et al. 2014). The low dispersal capacities of brooding species (lacking a free larval stage) may lead to strong population genetic differentiation and/or numerous local speciation events (Kelly & Palumbi 2010, Boissin et al. 2011). Self-reproduction (selfing) also strongly affects population genetic structure, allowing the establishment of a new population from a single successful migrant (Baker 1955, 1967, Pannell & Dorken 2006).

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Owing to the absence of obvious barriers to gene flow in the marine environment, populations of marine organisms were long thought to be mostly panmictic. Yet, the existence of globally distributed species with low dispersal abilities is particularly intriguing (Perez-Portela et al. 2013). However, many species once thought to be widespread were revealed to be composed of several cryptic species with more restricted geographic ranges (Knowlton 1993, Pfenninger & Schwenk 2007, Hoareau et al. 2013). Such species complexes are powerful models to inform about the processes governing speciation and species distribution (Boissin et al. 2008a, Boissin et al. 2011). Additionally, when they are sympatric, complexes of cryptic species allow comparative population genetics analyses between samples from the same set of localities. They also allow the illustration of the putative roles of contingency and/or historical demography among closely related species.

The brittlestar *Amphipholis squamata* (Delle Chiaje, 1928) is a simultaneous hermaphrodite capable of selfing and brooding (Fell 1946), features that confer a weak potential for dispersal (Poulin et al. 1999, Boissin et al. 2008b). Paradoxically, *A. squamata* exhibits a wide geographic range, being found in all oceans except the polar ones (Gage et al. 1983). However, it was revealed to be a complex of numerous cryptic species, some of which have a wide distribution range (Sponer & Roy 2002, Boissin 2008, Boissin et al. 2008a). The 4 species present in the Mediterranean Sea reproduce mainly by selfing (Boissin et al. 2008b). French populations from the Atlantic Ocean and the Mediterranean Sea form separate monophyletic groups (within a previously recognized lineage), suggesting a relatively ancient separation (Le Gac et al. 2004). However, some mitochondrial haplotypes are shared over long distances (2000 km, Boissin 2008; 1000 km, Sponer & Roy 2002). For low dispersers, alternative ways of dispersal exist, such as rafting on floating substrates (Highsmith 1985, Thiel & Haye 2006) or even on jellyfish (Marsh 1998). *A. squamata* has been found on mats of floating algae (Highsmith 1985), a dispersal means proposed to explain the wide distribution range of the species (Sponer & Roy 2002).

After assigning specimens to species, the first goal of the present study was to test whether dispersal of this brooding organism allows recurrent connectivity among populations on the regional scale of the French Mediterranean coast, or whether it simply results in a wide distribution range consisting of loosely connected populations. In the former case, a geographical pattern, such as a correlation of pairwise population genetic distances and spatial distances, would be expected; in the latter case, strong population genetic differentiations and stochasticity in the spatial organization of this species complex should be observed at the intra- and inter-specific levels. Additionally, the presence of 4 sympatric species in the study region offered a unique opportunity to compare population genetic structure in biological replicates. Therefore, our second goal was to estimate the variability in population structure among species sharing very constraining life history traits, i.e. very low dispersal ability and extreme selfing rates (Boissin et al. 2008b).

MATERIALS AND METHODS

Sampling

A total of 1004 specimens of the *Amphipholis squamata* species complex (hereafter *A. squamata* complex) from 14 localities of the French Mediterranean coast sampled over 8 yr were analyzed in this study (Table 1). Among them, 8 localities were sampled over 1 mo in September/October 2005: Banyuls-sur-Mer, Cap d'Agde, Carro, Les Goudes, Port d'Alon, Giens, Saint-Raphaël and Cap Ferrat (N = 606; see Fig. 1, Table 1 for details). Data from 4 additional samples analyzed in Boissin et al. (2008a) were also added (Banyuls-sur-Mer, Les Goudes, Le Brusc and Scandola; N = 192). Another 2 samples from Frioul (N = 28) and Porquerolles (N = 27) in close proximity to Les Goudes and Giens, respectively, as well as another sample from Scandola (Corsica, N = 24) were collected in 2010. These 877 specimens were collected at less than 1 m depth. Additionally, to investigate the bathymetric organization of the *A. squamata* complex, one location (Riou) was sampled at 4 distinct depths (surface, 10 m, 20 m, 42 m) and a second nearby location (Castelviel) at 16 m (N = 127). In the field, sampling was done by scraping off 15 × 15 cm^2 square patches of seaweeds, which were placed individually in bags of seawater. Back at the laboratory, the brittlestars were carefully extracted from the seaweeds and individually preserved in 95% ethanol.

Molecular analyses

DNA was extracted from pieces of arms following a Chelex procedure (Walsh et al. 1991). Specimens collected in September/October 2005 (N = 606) and bathymetric samples (N = 127) were assigned to a

Table 1. Sampling sites and number of specimens analyzed for nuclear loci for each species (A1, A2, A3, and B) of the *Amphipholis squamata* species complex. Numbers in parentheses correspond to the subset for which 16S DNA sequences were obtained. When no depth is indicated after the locality name, sampling was performed near the surface (less than 1 m). Samples from the bathymetric sampling are listed at the bottom and separated with a line. N: number of specimens; date: sampling date (given as dd/mm/yyyy); **bold**: samples from Boissin et al. (2008a)

Locality	Code	Coordinates	Date	N	A1	A2	A3	B
Banyuls-sur-Mer	BAN	42° 27′ 34″ N, 3° 09′ 28″ E	21/10/2005	71	27	1	37	6
Banyuls-sur-Mer	**BAN**	**42° 27′ 34″ N, 3° 09′ 28″ E**	**23/04/2002**	46 (46)	11 (11)	–	18 (18)	17 (17)
Cap d'Agde	AGD	43° 16′ 37″ N, 3° 30′ 59″ E	07/10/2005	145 (10)	–	–	144 (9)	1 (1)
Carro	CAR	43° 19′ 46″ N, 5° 02′ 11″ E	14/09/2005	34 (31)	4 (4)	2 (2)	22 (19)	6 (6)
Frioul	FRI	43° 16′ 48″ N, 5° 18′ 23″ E	17/03/2010	(28)	(2)	–	(17)	(9)
Les Goudes	GOU	43° 12′ 50″ N, 5° 20′ 15″ E	27/09/2005	131 (8)	59 (3)	33 (5)	19	19
Les Goudes	**GOU**	**43° 12′ 50″ N, 5° 20′ 15″ E**	**23/11/2004**	93 (93)	20 (20)	30 (30)	23 (23)	20 (20)
Riou	RIO	43° 10′ 33″ N, 5° 22′ 59″ E	20/04/2006	16 (2)	6	–	10 (2)	–
Port d'Alon	ALO	43° 08′ 39″ N, 5° 42′ 09″ E	21/09/2005	20 (13)	13 (10)	1 (1)	6 (2)	–
Le Brusc	**BRU**	**43° 04′ 20″ N, 5° 47′ 14″ E**	**13/12/2004**	32 (32)	14 (14)	–	12 (12)	6 (6)
Giens	GIE	43° 02′ 01″ N, 6° 06′ 47″ E	28/09/2005	78 (16)	47 (8)	5	25 (7)	1 (1)
Porquerolles	POR	43° 00′ 12″ N, 6° 12′ 34″ E	18/03/2009	27 (27)	9 (9)	3 (3)	15 (15)	–
Cap Ferrat	FER	43° 40′ 30″ N, 7° 19′ 47″ E	15/09/2005	61 (34)	13 (1)	–	45 (30)	3 (3)
Saint-Raphaël	RAP	43° 24′ 45″ N, 6° 47′ 56″ E	12/10/2005	66 (48)	20 (16)	8 (5)	38 (27)	–
Scandola	**SCA**	**42° 25′ 20″ N, 8° 38′ 26″ E**	**29/05/2006**	21 (21)	11 (11)	3 (3)	5 (5)	2 (2)
Scandola	SCA	42° 25′ 20″ N, 8° 38′ 26″ E	05/03/2010	(24)	(7)	(2)	(14)	(1)
Riou 10 m	RIO	43° 10′ 33″ N, 5° 22′ 59″ E	21/06/2006	67 (32)	6 (6)	–	42 (13)	19 (13)
Riou 20 m	RIO	43° 10′ 33″ N, 5° 22′ 59″ E	09/10/2006	14 (4)	–	–	9 (2)	5 (2)
Riou 42 m	RIO	43° 10′ 33″ N, 5° 22′ 59″ E	29/09/2006	10 (5)	–	2 (2)	–	8 (3)
Castelviel 16 m	CAS	43° 11′ 62″ N, 5° 30′ 20″ E	10/10/2006	20 (5)	–	–	1 (1)	19 (4)

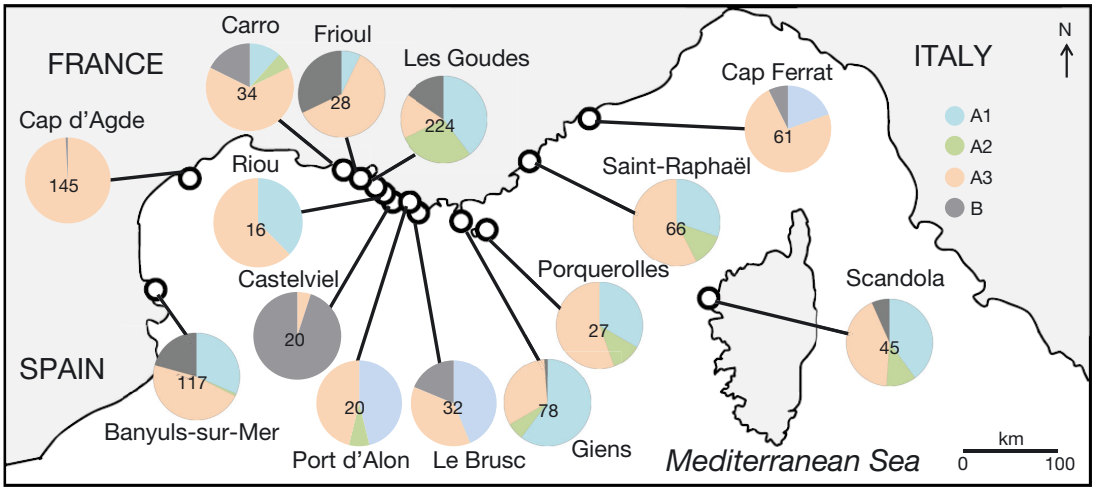

Fig. 1. Map of the sampling localities of the *Amphipholis squamata* species complex along the French Mediterranean coast. Pie charts show the proportion of each species (A1, A2, A3, and B) of the complex at each locality (only the proportion of Riou at the surface is shown, not the proportion of species at other depths). Numbers in pie charts represent the total number of specimens sampled for that locality

species of the *A. squamata* complex (A1, A2, A3 or B) using 2 nuclear markers (Aj9, a microsatellite and Actin2, an intron) following Boissin et al. (2008b) and run on 8% denaturing polyacrylamide gels. PCR cycling and mixture composition were as in Boissin et al. (2008b). Additionally, a portion of the 16S mitochondrial gene was sequenced for subsets of 160/606 and 48/127 specimens, respectively. Specimens from Frioul, Porquerolles and Scandola were all sequenced (N = 79). Amplification was done fol-

lowing Boissin et al. (2008a). PCR products were sent to an academic service center for sequencing (Genomer, Roscoff). Sequences from Boissin et al. (2008a) were also included in the analyses (N = 192; GenBank accession numbers EU251962 to EU251996). Sequences of *Amphipholis pugetana* were used as outgroup (GenBank accession numbers EU251997 to EU251999).

Data analyses

The mitochondrial 16S sequences were aligned using MAFFT online (Katoh et al. 2002). Haplotype and nucleotide diversities were computed per species using DnaSP v.5.10.01 (Librado & Rozas 2009). Neutrality tests were also performed in DnaSP using Fu and Li's F (Fu & Li 1993) and Ramos-Onsins and Rozas' R_2 (Ramos-Onsins & Rozas 2002). Kimura two-parameter (K2P) mean genetic distances within lineages were computed in Mega v.5.05 (Tamura et al. 2011). Phylogenetic reconstructions were performed using (1) neighbor joining (NJ) and maximum likelihood (ML) algorithms in PhyML online (Guindon et al. 2005) and (2) Bayesian inference (BI) in BEAST (Drummond & Rambaut 2007). Support for the nodes in the NJ and ML reconstructions was obtained using a bootstrapping procedure (Felsenstein 1985). MrAIC v.1.4.6 (Nylander 2004) was used to estimate the best fit model of nucleotide evolution. The BI reconstruction was performed running 10 000 000 generations and recording parameters every hundredth generation. Bayes factors were used to decide among the best clock and tree priors (relaxed uncorrelated exponential clock and birth death tree prior). Estimation of the time to the most recent common ancestors (TMRCA) was done for each lineage and group of lineages of interest. As no 16S mutation rate estimate exists for brittlestars and the fossil record is scarce, a broad prior centred at 1.25% per million years (Myr) was used for the molecular clock (normal distribution, 95% highest posterior density [HPD] = 0.5 to 2%), encompassing typically reported rates for 16S ribosomal DNA in several phyla (0.58% per Myr for fishes, 1% per Myr for molluscs, 1.46% per Myr for echinoids and 1.70% per Myr for crustaceans; Lessios 2008). The software TRACER (Rambaut et al. 2014) was used to ensure the reach of 200 for the effective sampling sizes (ESS) and retrieve the estimation of TMRCAs and their 95% HPD.

Haplotype networks were constructed for each lineage using NETWORK (www.fluxus-engineering.com) and the median joining algorithm (Bandelt et al. 1999). Assuming that none of the 16S haplotypes were positively selected for in the past, we investigated past demographic changes in population size for the species showing significant neutrality tests using the Bayesian skyline plot (BSP) framework in BEAST. The main settings were the same as for the TMRCA estimates with a run of 10 000 000 generations and recording parameters every hundredth generation. Similarly, mismatch distributions for each species were computed in DnaSP.

The scale of the spatial connectivity was investigated within each species as follows:

(1) Global and pairwise differentiation estimates were computed in SPADE (Chao & Shen 2010) and ARLEQUIN v.3.5 (Excoffier et al. 2005), respectively. In ARLEQUIN, pairwise F_{ST} estimates between pairs of populations were computed using the matrix of Slatkin's distance and p-values were obtained with 10 000 permutations. The Benjamini & Hochberg (1995) correction for multiple tests was applied. In SPADE, Jost's D-estimators of global differentiation and their confidence intervals (CI) were computed for species A1, A3 and B, for which we compared different shared sets of populations with sample sizes above 9. Pairwise D-values were also estimated for comparison between populations. This parameter, contrary to F_{ST}, is not affected by the level of polymorphism and always reaches a value of 1 when populations do not share any allele. Furthermore, the analysis provides confidence intervals, allowing robust comparisons among species displaying different diversity levels (Chao et al. 2008, Jost 2008). SPADE also yields an adjusted estimator of D, which resulted in exactly the same value as the original estimator in all our analyses (data not shown). Two confidence intervals were computed, the first one based on ±1.96 bootstrap SE and the second one based on an improved bootstrap percentile method, which is recommended when similarity is close to 0 or 1 (Chao et al. 2008).

(2) Principal coordinate analyses (PCoA) were computed in GenAlEx v.6.501 (Peakall & Smouse 2012) for each species to investigate the relationships between populations and search for any groupings.

(3) Isolation by distance (IBD) was tested in GENETIX (Belkhir et al. 2004) using the genetic distances ($F_{ST}/(1 - F_{ST})$) (Rousset 1997) and the log geographical distances (in km) between each pair of localities. The significance was obtained by Mantel test using 5000 permutations in GENETIX.

(4) Analyses of molecular variance (AMOVA) were computed using ARLEQUIN. The groups tested were those revealed by the PCoA analyses (see 'Results').

We also assessed population subdivisions by spatial analysis of molecular variance using SAMOVA v.2.0 (Dupanloup et al. 2002). The software searches spatially homogeneous and maximally differentiated groups (k) of populations without prior grouping assumptions (contrary to the regular AMOVA above). We tested k from 2 to 5 for each species (except for A2, which we tested from 2 to 4 because the number of k tested cannot be equal to the number of populations) and ran the analyses for 10 000 simulated annealing processes. The most likely number of groups (k) is the one resulting in the highest significant Φ_{CT}-value (fixation index among groups) (Dupanloup et al. 2002).

Finally, exact tests were performed on contingency tables built from Table 1, for comparison of species composition on bathymetric and spatial samples, using xlstat (Addinsoft). The null hypothesis tested was that species frequencies are independent of location.

RESULTS

Phylogenetic diversity

The 4 species of the *Amphipholis squamata* complex known from the Atlanto-Mediterranean basin were present along the French Mediterranean coast (Fig. 2). A total of 479 sequences (420 bp) corresponding to 64 distinct haplotypes were used in the analyses (plus 3 sequences of *A. pugetana*; see Table S1 in the Supplement at www.int-res.comarticles/suppl/m539p165_supp.pdf for details; GenBank accession numbers KT780312 to KT780340). All dating estimates had very large confidence intervals, due to the broad prior used for substitution rates. The mean TMRCA of species A and B is approximately 9.93 (95% HPD: 2.69 to 20.68) Myr (Fig. 2), the common ancestor of species A1, A2 and A3 is 4.83 (1.47 to 9.83) Myr and the common ancestors of each species A1, A2, A3 and B are 1.26 (0.33 to 2.57), 1.70 (0.41 to

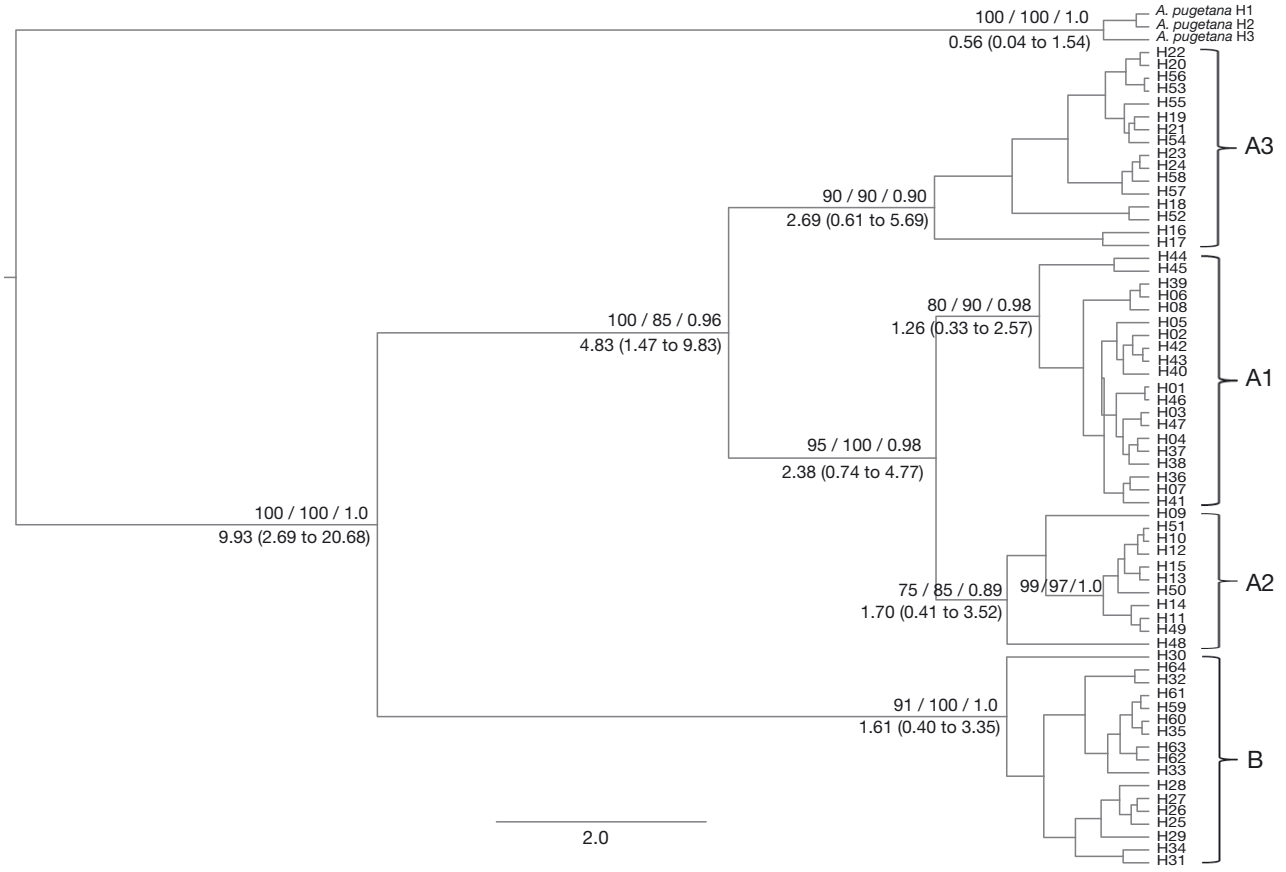

Fig. 2. Bayesian reconstruction of phylogenetic relationships among species (A1, A2, A3, and B) of the *Amphipholis squamata* complex found in the northwestern Mediterranean Sea. The reconstruction is based on the 16S mitochondrial sequences using a relaxed uncorrelated exponential clock and a birth death tree prior. Numbers above the branches show support values (bootstraps from the neighbor joining reconstruction, maximum likelihood reconstruction and Bayesian posterior probability). Numbers below the branches show time to the most recent common ancestors (Myr) with their confidence intervals. *Amphipholis pugetana* was used as an outgroup

Table 2. Summary statistics of 16S sequences of the 479 samples used, pooled by species (A1, A2, A3, and B) of the *Amphipholis squamata* complex. N: number of sequences; H: number of haplotypes; Hd: haplotype diversity with standard deviations in parentheses; Pi: nucleotide diversity with standard deviations in parentheses; neutrality test statistics: F (Fu & Li 1993) and R_2 (Ramos-Onsins & Rozas 2002); K2P: Kimura-two-parameter intra-specific mean genetic distances; ns: not significant; $^{**}p < 0.02$

Species	N	H	Hd	Pi	F	R_2	K2P
A1	122	20	0.465 (0.057)	0.002 (0.0004)	–3.703**	0.024**	0.002 (0.001)
A2	53	11	0.812 (0.059)	0.006 (0.001)	–0.103 ns	0.058 ns	0.006 (0.002)
A3	216	16	0.665 (0.022)	0.0104 (0.0005)	–1.367 ns	0.089 ns	0.011 (0.003)
B	88	17	0.890 (0.014)	0.0115 (0.0004)	–0.504 ns	0.130 ns	0.012 (0.004)

3.52), 2.69 (0.61 to 5.69) and 1.61 (0.40 to 3.35) Myr, respectively. Species B showed the highest genetic diversity (haplotype diversity [Hd] = 0.890) and A1 the lowest (Hd = 0.465) due to the presence of a common haplotype (H1 for 89 specimens of the 122 belonging to species A1; Table S1). Similarly, species A1 showed the lowest nucleotide diversity and species A3 and B the highest, species A2 presenting intermediate values. Similarly, the mean within-lineage K2P genetic distance ranged from 0.002 (for species A1) to 0.012 (for species B; Table 2).

Spatial and bathymetric distribution of the species

The species were present in various proportions and no cline or obvious trend was revealed among locations along the coast (Fig. 1). However, A3 was the dominant lineage at 8 localities: Banyuls-sur-Mer, Cap d'Agde, Carro, Frioul, Porquerolles, Saint-Raphaël, Cap Ferrat and Scandola. A1 was dominant in the other 4 localities. A2 was often rare, except at Les Goudes. B was less common than A1 and A3 but could make up as much as 32% of the community at Frioul and 20% at Banyuls-sur-Mer and Le Brusc. Fisher's exact test did not support any relationships between localities and species composition ($p > 0.05$) (Fig. 1).

For the comparison among depths, all 4 species were present in the bathymetric samples (Table 1). A3 was the dominant species at the surface for Riou. At 10 m and for deeper sites, A3 and B became the dominant species, while A1 and A2 were virtually absent at 16 m and below. The species present at Riou displayed significantly different distributions among the 4 depths (exact tests, $p = 0.0026$).

Spatial scale of connectivity

Some haplotypes were widespread in A1 (H1 shared among 89 specimens from 11 localities) and A3 (H19 and H23 shared among 76 and 99 specimens from 12 and 9 localities, respectively), but most of the haplotypes were represented by only 1 or 2 specimens (48 of the total 64 haplotypes recovered in this study; Fig. 3). There was no geographical clustering of closely related haplotypes (haplogroups). Regarding genetic differentiation, only 4 pairwise comparisons (of 66) and 3 pairwise comparisons (of 21) showed a significant F_{ST}-value in species A1 and species A2, respectively (Table 3). However, in species A3, more than half of the comparisons were significant (50 of 91). Species B showed 23 significant pairwise comparisons (of 76). Pairwise D-values gave similar results to F_{ST}-values (Table 3). In particular, species A1 showed very low values of differentiation ($0.000 < D < 0.209$). Accordingly, with global D estimates, species A1 displayed significantly less global differentiation than species A3 and/or B (no overlap of CI) in comparisons including 2 or 5 populations (Table 4). While species A3 and B displayed similar genetic differentiation in comparisons including 2 populations, species A3 displayed less global differentiation than species B in the comparison involving 4 localities. Therefore, spatial differentiation appeared the lowest in species A1 and the highest in species B, A3 being intermediate.

The PCoA revealed some clustering of populations with no clear geographic grouping, except for some of the remote localities: Banyuls-sur-Mer stood out for species A3 and B; Scandola stood out for species A2 and also for species A3 on axes 2 and 4 (data not shown); and Saint-Raphaël stood out for species A1 (Fig. 4). The groups resulting from the PCoA and further tested in the AMOVA analyses were as follows: for species A1: (1) Giens + Porquerolles + Scandola vs. (2) Saint-Raphaël vs. (3) Carro + Riou + Banyuls-sur-Mer + Les Goudes + Le Brusc + Port d'Alon; for species A2: (1) Les Goudes vs. (2) Scandola vs. (3) Saint-Raphaël vs. (4) Carro + Porquerolles; for species A3: (1) Banyuls-sur-Mer vs. (2) Scandola vs. (3) Cap d'Agde + Cap Ferrat + Carro

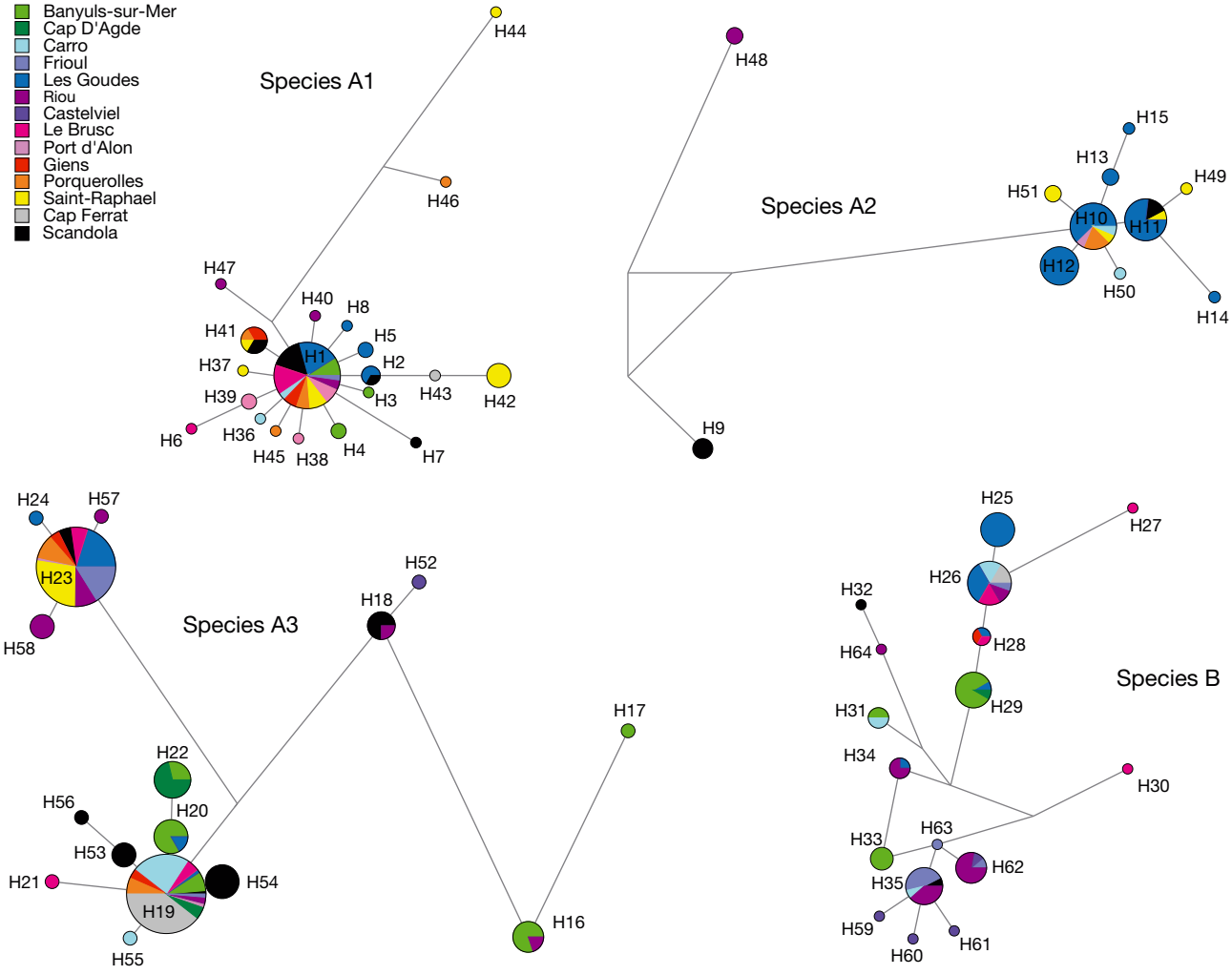

Fig. 3. Haplotype networks for each of the 4 species of the *Amphipholis squamata* complex found in the northwestern Mediterranean Sea. The reconstruction is based on the 16S mitochondrial sequences and the median-joining algorithm. Circle sizes are proportional to the number of specimens showing the given haplotype (the smallest size represents one specimen). Links are proportional to the number of mutations between 2 haplotypes (the shortest link represents one mutation)

vs. (4) Riou + Saint-Raphaël + Les Goudes + Frioul + Giens + Le Brusc + Porquerolles; and for species B: (1) Castelviel + Riou + Frioul vs. (2) Banyuls-sur-Mer vs. (3) Carro + Le Brusc + Cap Ferrat + Les Goudes. These groupings revealed a significant proportion of the genetic variation at the 'among groups' levels in the AMOVA analyses for species A1, A3 and B (Table 5). Further significant partitions of the genetic variation were found at the 'within populations' and 'among populations within groups' levels in species A3 and B, and only at the 'within populations' level for species A1 and A2 (Table 5). The SAMOVA analyses revealed k = 3 groups with the highest and significant Φ_{CT}-values in all 4 species; however, nearly identical Φ_{CT}-values were obtained for alternative values of k for species A3 and B (Table 6a). The groupings roughly corresponded to the PCoA ones, except that Carro clustered with Banyuls-sur-Mer for species B, Scandola clustered with Cap d'Agde + Cap Ferrat + Carro for species A3, and Scandola clustered with Saint-Raphaël for species A2. For species A1, the differentiation of Saint-Raphaël was revealed, but the 2 other groups were not recovered. Therefore, again, no clear geographic grouping was noticeable except for the most remote localities of Saint-Raphaël, Cap Ferrat or Banyuls-sur-Mer, which stood out of the main groups (Table 6b).

Finally, the IBD tests were not significant in any of the 4 species (data not shown).

Table 3. Between-population differentiation for the 4 species (A1, A2, A3, and B) of the *Amphipholis squamata* complex along the French Mediterranean coast. Values below the diagonal line are pairwise F_{ST} estimates (left semi-matrix). Values above the diagonal line are pairwise Jost's D (right semi-matrix). Locality codes are as given in Table 1. Numbers in parentheses are sample sizes. **Bold**: significant p-values after Benjamini & Hochberg (1995) correction at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

A1 (119)	BAN (11)	CAR (4)	GOU (23)	RIO (6)	ALO (10)	BRU (14)	GIE (8)	POR (9)	RAP (16)	SCA (18)
BAN (11)		0.000	0.001	0.000	0.000	0.024	0.007	0.000	0.138	0.000
CAR (4)	0.033		0.000	0.000	0.000	0.000	0.000	0.000	0.082	0.000
GOU (23)	0.059	0.037		0.000	0.005	0.011	0.009	0.000	0.158	0.000
RIO (6)	0.053	–0.030	0.079		0.000	0.015	0.000	0.000	0.070	0.000
ALO (10)	0.071	0.032	0.069	0.051		0.034	0.010	0.000	0.131	0.001
BRU (14)	0.050	0.059	0.016	0.069	–0.006		0.025	0.028	0.209	0.010
GIE (8)	0.099	0.083	0.094	0.068	0.101	0.106		0.000	0.120	0.000
POR (9)	0.029	–0.067	0.062	–0.042	0.027	0.023	–0.028		0.072	0.000
RAP (16)	**0.146***	0.046	**0.177****	0.086	0.068	**0.136***	0.115	0.086		0.138
SCA (18)	0.043	0.004	0.012	0.049	0.050	0.010	–0.035	0.013	**0.153****	

A2 (48)	GOU (35)	POR (3)	RAP (5)	SCA (5)
GOU (35)		0.541	0.338	0.646
POR (3)	–0.034		0.636	1.000
RAP (5)	–0.045	0.032		0.680
SCA (5)	**0.686****	0.364	0.416	

A3 (213)	BAN (18)	AGD (9)	CAR (19)	FRI (17)	GOU (23)	RIO (17)	BRU (12)	GIE (7)	POR (15)	RAP (27)	FER (30)	SCA (19)
BAN (18)		0.358	0.428	0.964	0.945	0.792	0.641	0.552	0.697	1.000	0.449	0.910
AGD (9)	**0.145***		0.371	0.961	0.968	0.858	0.653	0.564	0.694	1.000	0.385	0.925
CAR (19)	**0.233*****	**0.586****		0.937	0.950	0.813	0.516	0.386	0.555	1.000	0.000	0.907
FRI (17)	**0.615*****	**0.880*****	**0.933*****		0.000	0.141	0.119	0.141	0.080	0.000	0.938	0.528
GOU (23)	**0.611*****	**0.832*****	**0.881*****	0.004		0.109	0.101	0.126	0.068	0.007	0.950	0.504
RIO (17)	**0.417*****	**0.607*****	**0.686*****	0.042	0.032		0.010	0.023	0.041	0.182	0.818	0.349
BRU (12)	**0.300*****	**0.494****	**0.589*****	**0.262***	0.221	0.042		0.000	0.000	0.172	0.527	0.421
GIE (7)	**0.264****	**0.526****	**0.669*****	**0.318***	0.252	0.018	–0.123		0.000	0.200	0.400	0.433
POR (15)	**0.377*****	**0.581*****	**0.666*****	0.168	0.129	0.004	–0.061	–0.095		0.125	0.563	0.453
RAP (27)	**0.727*****	**0.967*****	**0.994*****	0.028	0.050	0.166	0.490	**0.619****	**0.381****		1.000	0.554
FER (30)	**0.302*****	**0.714*****	0.025	**0.955*****	**0.910*****	**0.750*****	**0.677*****	**0.764*****	**0.738*****	**1.000*****		0.911
SCA (19)	**0.131****	**0.243****	**0.237*****	**0.486*****	**0.471*****	0.260	0.097	0.057	0.176	**0.628*****	**0.306*****	

B (83)	BAN (17)	CAR (6)	FRI (9)	GOU (20)	RIO (18)	CAS (4)	BRU (6)	FER (3)
BAN (17)		0.884	1.000	0.925	1.000	1.000	1.000	1.000
CAR (6)	0.091		0.512	0.528	0.589	1.000	0.000	0.211
FRI (9)	**0.481*****	0.428		0.915	0.254	0.867	0.820	0.843
GOU (20)	**0.514*****	**0.329****	**0.775*****		0.860	1.000	0.443	0.562
RIO (18)	**0.282*****	**0.278****	0.032	**0.634*****		0.150	0.741	0.819
CAS (4)	**0.542*****	**0.444***	–0.071	**0.831*****	0.092		1.000	1.000
BRU (6)	**0.288****	–0.010	0.593	**0.151***	0.440	0.627		0.167
FER (3)	**0.463****	0.108	**0.759***	0.094	**0.576****	**0.840***	–0.091	

Demographic changes

A1 was the single species showing significant departure from neutrality (both Fu and Li's F and Ramos-Onsins and Rozas' R_2 were significant, Table 2) and the star-like shape of its haplotype network (Fig. 3). The mismatch distribution and BSP plot also suggested an expansion of populations for this species (Fig. S1 in the Supplement).

DISCUSSION

Species composition along the French Mediterranean coast

Although the sample sizes for the bathymetric comparison were low, the proportion of species B seemed to increase with depth. Species B showed the lowest densities near the surface (also noted by Boissin et al.

2008a) and may have been at its range limits. As noticed by Sponer & Roy (2002), this lineage may have a lower temperature preference than the other lineages; however, this needs further investigation.

At superficial localities along the French Mediterranean coast, no geographical trend in the species composition was obvious, probably reflecting the stochastic nature of dispersal in the *Amphipholis squamata* complex. The coexistence of the 4 species in syntopy (i.e. within the same patches) might indeed reflect a stochastic dispersal process. The neutral theory of biodiversity has highlighted the importance of immigration (arrival of new individuals) for the composition of communities compared to post-settlement processes (niche and competition; Hubbell 2001, Rosindell et al. 2011). In particular, the coexistence of species on rocky shores has been used as evidence to support the neutral theory of biodiversity (Shinen & Navarrete 2014). Under the neutral hypothesis, species are equivalent in terms of their abilities to compete, their demographic rates and dispersal potential (Hubbell 2001). A monthly temporal survey at Les Goudes over 2 yr did not reveal distinct recruitment periods or demographic dynamics for the 4 *A. squamata* species (Boissin 2008). Another biological feature estimated for the 4 species, the selfing rate, appeared very similar among species (not distinguishable from 1; Boissin et al. 2008b), supporting the hypothesis of ecological equivalence of species of the *A. squamata* complex (at least for A1, A2 and A3; a potential temperature preference of B was referred to earlier in this section).

Table 4. Global differentiation based on the D-estimator (Jost 2008) for 3 different sets of populations of species A1, A3 and B for which sample sizes were sufficient (≥9) to allow comparison at the same localities. D: estimator of differentiation (Jost 2008); SE: standard error; 95% CI: 95% confidence intervals (2 estimation methods; see 'Materials and methods: Data analyses')

	D (SE)	95% CI (1) min, max	95% CI (2) min, max
2 populations: Banyuls-sur-Mer, Les Goudes (species A1, A3 and B)			
A1	0.001 (0.090)	0.000, 0.177	0.000, 0.278
A3	0.945 (0.047)	0.853, 1.000	0.817, 1.000
B	0.925 (0.071)	0.785, 1.000	0.749, 1.000
5 populations: Banyuls-sur-Mer, Les Goudes, Saint-Raphaël, Porquerolles, Le Brusc (A1 & A3)			
A1	0.056 (0.060)	0.000, 0.173	0.000, 0.192
A3	0.294 (0.037)	0.221, 0.368	0.226, 0.368
4 populations: Banyuls-sur-Mer, Les Goudes, Frioul, Riou (A3 & B)			
A3	0.410 (0.043)	0.326, 0.494	0.341, 0.498
B	0.839 (0.041)	0.759, 0.918	0.751, 0.915

Spatial scale of connectivity

Species of the *A. squamata* complex displayed genetic differentiation along the French Mediterran-

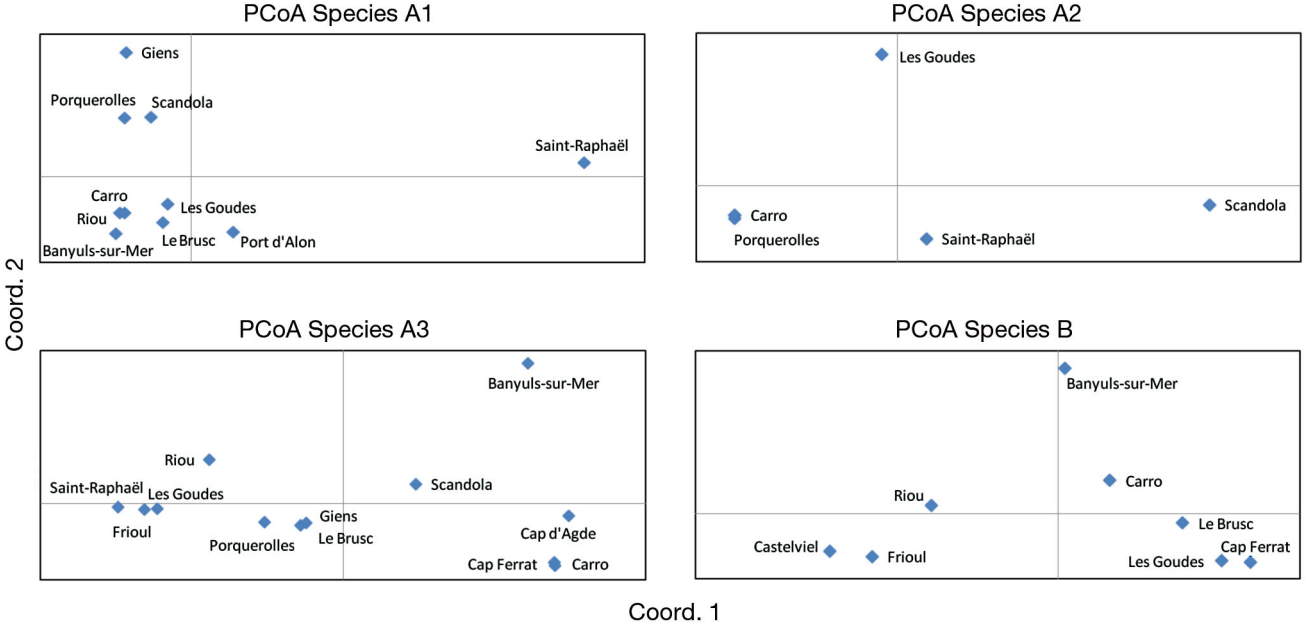

Fig. 4. Principal coordinate analyses representing relationships of populations of the 4 species of the complex *Amphipholis squamata*, based on 16S mitochondrial haplotypes

Table 5. Analyses of molecular variance (AMOVA) for the 4 species of the *Amphipholis squamata* complex along the French Mediterranean coast. Va, Vb, Vc: variance components for groups, populations, and haplotypes within a population within a group, respectively. ns: not significant; df: degrees of freedom; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Species	Source of variation	df	Variance components	% of variation
A1	Among groups	2	0.06 Va	18.31**
	Among populations within groups	7	–0.01 Vb	–1.91 ns
	Within populations	107	0.29 Vc	83.59***
A2	Among groups	3	1.51 Va	76.22 ns
	Among populations within groups	1	–0.94 Vb	–47.56 ns
	Within populations	46	1.42 Vc	71.34*
A3	Among groups	3	1.78 Va	59.50***
	Among populations within groups	8	0.13 Vb	4.35***
	Within populations	201	1.08 Vc	36.14***
B	Among groups	2	1.33 Va	47.29***
	Among populations within groups	5	0.15 Vb	5.35*
	Within populations	75	1.33 Vc	47.36***

ean coast (Tables 3 & 4). However, no clear geographic structure was revealed in the PCoA or the SAMOVA analyses. The AMOVA analyses further revealed significant genetic variation within populations and among populations within groups. Some haplotypes were shared between distant localities separated by more than 450 km, as has already been noticed in a previous work in the study area (Boissin et al. 2008a) or elsewhere on a similar geographic scale (over 1000 km in Sponer & Roy 2002). From these results, the dispersal ability of species of this complex seemed to be regionally restricted but with episodic long-distance dispersal. *A. squamata* has been shown to occur on floating kelps (Highsmith 1985), which may allow for sporadic long-distance dispersal events (Thiel & Haye 2006). As such long-distance migration is probably not recurrent, a successful dispersal event will often lead to a founder effect (i.e. colonization associated with strong demographic bottlenecks). Similarly, founder events linked to brooding have been suggested to trigger strong local genetic structure in another Atlanto-Mediterranean brittlestar species complex, *Ophioderma longicauda* (Boissin et al. 2011). More generally, the effects of limited dispersal linked to brooding ability have been shown in a number of marine invertebrates (Kelly & Palumbi 2010, Haye et al. 2014). In echinoderms, a strong fine-scale genetic structure was indeed recovered in the asterinid seastar *Parvulastra exigua* (Barbosa et al. 2013) and the sea-urchin *Abatus cordatus* (Ledoux et al. 2012), for instance. In *A. squamata*, long-distance dispersal and subsequent founder events likely explain the wide geographic range of the *A. squamata* complex and the numerous cryptic lineages found throughout the range (Sponer & Roy 2002, Le Gac et al. 2004, Boissin et al. 2008a). This is strengthened by the ability of the organism to self-reproduce (the possibility of establishment of a new population from a single migrant) and the old age of the *A. squamata* complex estimated in this study (approximately 10 [95% HPD: 2.69 to 20.68] Myr for TMRCA of species A and B].

Table 6. (a) Spatial analyses of molecular variance (SAMOVA) for the 4 species of the *Amphipholis squamata* complex along the French Mediterranean coast. ϕ_{CT}: fixation index among groups; ns: not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (b) Resulting groupings of SAMOVA

(a)

ϕ_{CT}	$k = 2$	$k = 3$	$k = 4$	$k = 5$
A1	0.28 ns	0.23***	0.19***	0.16**
A2	0.34 ns	0.29**	0.44 ns	–
A3	0.69***	0.70***	0.69***	0.68***
B	0.44*	0.50***	0.49***	0.49***

(b)

Species	Group 1	Group 2	Group 3
A1	Banyuls-sur-Mer Frioul Les Goudes Riou Port d'Alon Le Brusc Giens Porquerolles Scandola	Carro	Saint-Raphaël
A2	Les Goudes	Carro Porquerolles	Saint-Raphaël Scandola
A3	Frioul Les Goudes Riou Le Brusc Giens Porquerolles Saint-Raphaël	Cap d'Agde Carro Cap Ferrat Scandola	Banyuls-sur-Mer
B	Banyuls-sur-Mer Carro	Les Goudes Le Brusc Cap Ferrat	Frioul Riou Castelviel

Comparative population genetics

The 4 species showed contrasting patterns of genetic structure and diversity. Genetic differentiation among localities was observed for species A3

and B but appeared much less pronounced for species A1 and A2 (lower number of significant F_{ST}-values, lower values of D, with non-overlapping CI). There are drawbacks for each method, but the result that species displayed distinct levels of differentiation was not ambiguous. For Jost's D and its CI, the drawback lies in the fact that we compared only a few population pairs, and as dispersal events seem stochastic in the *A. squamata* complex, a few pairs of populations may not be representative of all the populations. The problem in comparing F_{ST}-values across species is that F_{ST}-values are lower when the diversity within population is higher. In our data set, this bias would have produced results opposite to observations, as species B displayed the highest genetic diversity and A1 nearly the lowest, although B was much more differentiated than A1 (Tables 2, 3 & 4). Similarly, haplotype diversity appeared to vary by a factor of 2 between these species, from Hd = 0.45 in species A1 to Hd = 0.9 in species B. Surprisingly, haplotype diversity was the highest in species B, which was not the most abundant in our sample sizes. This suggests that the number of individuals belonging to each of the identified species does not reflect their effective population sizes, because gene diversity is, in theory, positively correlated with effective sizes. This could be owing to the bathymetric preferential distribution of this species (see 'Species composition along the French Mediterranean coast'). The variability in genetic diversity and differentiation among species is striking, given the similar ecology of these cryptic species discussed above (Boissin 2008, Boissin et al. 2008b), and may illustrate the importance of contingency (i.e. distinct events may have impacted species of the *A. squamata* complex differentially) in shaping genetic variation of populations (Palumbi 1994). Such a finding is in contrast with the conclusions of Romiguier et al. (2014) who, comparing polymorphism and substitution rates across a wide range of animal phyla, found that life history traits are good predictors of molecular diversity, whereas species geographic range and contingent aspects of population history play only a secondary role. Species A1 was the only one displaying clear signs of population expansion (Table 2, Fig. 3, Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m539p165_supp.pdf). A different demographic history of species A1 might therefore explain some of the discrepancies observed. Similarly, the effects of historical population genetic processes leading to contrasting phylogeographic patterns among co-distributed species have been evidenced in other marine invertebrates (McGovern et al. 2010). It is also possible that an ecological difference, not yet detected, among these cryptic species, actually confers on them distinct dispersal abilities. A precise knowledge of ecology and life history is very difficult to obtain for marine species, and this study, carried out on a relatively well-studied marine invertebrate, illustrates the range of variability that may be observed within a set of 4 replicate species supposedly sharing the same life history traits. Differentiation (Jost's D) varied by a factor of 2, 6 and up to 900 for a set of 3 populations (Table 4) and haplotype diversity varied by a 2-fold factor (pooled French samples for each species).

To conclude, the selfing and brooding abilities give complex metapopulation dynamics to species of the *A. squamata* complex. However, species do not seem to display the same levels of diversity or spatial genetic structure, probably highlighting the effects of historical demographic processes. Species complexes are interesting models to investigate the link between macro- and micro-evolutionary processes as they facilitate working concomitantly at the inter- and intra-specific levels, giving some insights on both the genetic structure of populations and processes driving community assemblages.

Finally, new techniques such as Approximate Bayesian Computation-based coalescent approaches (Beaumont et al. 2002) can help unravel the micro-evolutionary parameters characterizing the genetic processes in founder events (e.g. number of founding individuals; Boissin et al. 2012). These recent developments open the way to further study the processes involved in the colonization dynamics of widespread species with low dispersal abilities (such as *A. squamata*) and will enhance our understanding of the effects of deviant reproductive traits on population genetic structure.

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