High genetic connectivity in a gastropod with long-lived planktonic larvae

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ABSTRACT

Genetic connectivity plays a crucial role in shaping the geographic structure of species. Our aim in this study was to explore the pattern of genetic connectivity in Bursa scrobilator, an iconic marine caenogastropod with long-lived planktonic larvae. Our study was based on the analysis of DNA sequence data for the 658-bp barcoding fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene. This is the largest DNA sequence dataset assembled to date for B. scrobilator. These data confirm that the two recently described subspecies B. scrobilator scrobilator (Linnaeus, 1758), from the Mediterranean and Macaronesia, and B. s. coriacea (Reeve, 1844), from West Africa, constitute two evolutionarily significant units (ESUs). We found that for the nominal subspecies, the variation in morphology (shell, radula and gross anatomy) and DNA sequences was not geographically structured, and this agrees with what we would expect in a species with high connectivity at the larval stage. The divergence between the two subspecies cannot be easily explained by isolation by distance, and we would argue that one or more extrinsic factors may have played a role in isolating the two ESUs and maintaining that isolation.

INTRODUCTION

Connectivity among populations of marine invertebrates is known to have remarkable short-to-medium term effects on genetic variability, genetic structure, range dynamics and persistence, as well as long-term effects on speciation patterns (Lowe & Allendorf, 2010; Castelin et al., 2012 and references therein). For these reasons, connectivity is considered to be a key factor affecting the resilience of species to global change (Mawdsley et al., 2009). In benthic invertebrates, population connectivity is primarily related to dispersal, which tends to occur mostly in the earliest life history stages, the adult stage being only slightly mobile or, in some cases, even sessile (Knowlton & Jackson, 1993; Goven & Sponaugle, 2009; Ellingon & Krug, 2016). The duration of the pelagic larval phase (PLD), that is, the length of time that a larva spends in the water column before settling, is one of the major factors affecting connectivity. Other influential factors (both intrinsic and extrinsic) include habitat characteristics, currents and larval life history traits, such as mortality and settlement competency features. The correlation between PLD and the level of genetic connectivity has been shown by many studies (Modica et al., 2017 and references therein). However, the adequacy of PLD as a predictor of genetic connectivity has been questioned by a few workers (e.g. Shanks, 2009; other factors such as habitat differences (Ayre et al., 2009) and past biogeographical events (Edmands, 2001; Marko, 2004) may have had a substantial impact on connectivity.

In marine gastropods, development may either be entirely intracapular or may include a pelagic phase, during which larvae actively feed on plankton (planktotrophy) or only rely on yolk reserves (lecithotrophy) (Thorson, 1950). Marine gastropods are thus excellent models for testing the influence of PLD and dispersal capacity on genetic structure. An additional advantage is that the developmental type can often be inferred from the morphology of the protoconch, the embryonic and larval shell (i.e. shell developed before metamorphosis or before hatchlings emerge from eggs) that constitutes the initial whorls of the adult shell (Jablonski & Lutz, 1983; Lima & Lutz, 1990). Among gastropods, the Tomnodia (frog shells, tun shells and trumpet shells; Beu, 1997, 1998) are particularly interesting because some species have a long planktonic larval stage (teleplanic larvae) that extends up to several years (e.g. Strathmann & Strathmann, 2007). Only recently has the molecular systematics of the family Bursidae begun to be investigated (Castelin et al., 2012; Sanders et al., 2017; Strong et al., 2019); the same holds true for Bursa scrobilator (Linnaeus, 1758), one of the most iconic marine gastropods of the Eastern Atlantic and Mediterranean. On the basis of extensive shell-based studies and a molecular phyloge-
getic analysis of some Mediterranean and Senegalese specimens, Smriglio et al. (2019) proposed that *B. scrobilator* consists of two subspecies: *B. scrobilator scrobilator* (Linnaeus, 1758) and *B. s. coriacea* (Reeve, 1844). Given that this species has an extended planktonic larval stage, this finding was unexpected and raises questions as to what factors may have contributed to the divergence and isolation of populations in the Mediterranean and West Africa. However, the absence of genetic data from the Macaronesian part of the range hinders any discussion of this issue (Smriglio et al., 2019). This data gap needs to be filled if the systematics and evolution of *B. scrobilator* are to be understood.

Here, we present the first geographically extensive molecular systematic study of *B. scrobilator*. Using an integrative taxonomic approach, our study is based on the phylogenetic analysis of DNA sequence data for the cytochrome *c* oxidase subunit I (COI) barcoding fragment and includes newly generated COI data for samples from Macaronesia and the Mediterranean. We used species delimitation methods to confirm if this expanded COI dataset supports Smriglio et al.’s (2019) hypothesis of two subspecies and to understand the relationship of the Macaronesian specimens to the Mediterranean and West African populations. Our expectation would be that, as a gastropod with a long-lived planktonic larval stage, *B. scrobilator* would show weak or no phylogeographic structure and little or no spatially structured variation in genetic and morphological characters. Thus, in describing the genetic diversity of *B. scrobilator*, we also evaluated if these two predictions are supported by currently available data.

**MATERIAL AND METHODS**

**Samples**

All the Macaronesian specimens of *Bursa scrobilator* used in this study were collected by scuba diving and subsequently fixed in 70–100% ethanol. Due to their extreme rarity in the wild, the Mediterranean specimens used in the study were found through careful searching of local museums and private collections; these samples consisted of two recently collected specimens (MED-PC-1, BAU-3539), which had been fixed in 70–100% ethanol, and three ‘old’ (historical) specimens, of which one was in denatured alcohol (MED-PC-3), one was an ethanol-preserved sample (MOL-652) from the Stazione Zoologica Anton Dohrn (SZN, Naples) and one was a dried sample (MED-PC-4; see Trillo, 2001). Sampled localities are summarized in Table 1 with voucher ID codes.

Radulae were extracted from dissected buccal masses after tissues had been partly dissolved in 10% sodium hydroxide, then rinsed in distilled water and observed under a stereo microscope. Selected radulae and protoconchs were air-dried, mounted on scanning electron microscope (SEM) stubs and gold–palladium-coated in a plasma coater. Radulae and protoconchs were also prepared for scanning electron microscopy (SEM) and observed under a Jeol JSM-6700F SEM. Some of the more recently sampled specimens (e.g. SZN-MOL-0024, BAU-3536.2, BAU-3539) were dissected, and gross anatomy was examined under a stereo microscope. Our samples included one juvenile specimen (BAU-3555.3); the protoconch sculpture of this individual was fully intact, so it could be described in detail.

**DNA extraction, amplification and sequencing**

Samples from museums and those that had been preserved for an extended period were first transferred to fresh absolute ethanol for 1 week. A piece of tissue was dissected from the foot of each specimen, and DNA extraction was performed using a phenol-chloroform protocol after hydration and tissue digestion in proteinase K (see Oliverio & Mariotti, 2001). The 658-bp DNA barcode fragment of the mitochondrial COI gene was amplified using Folmer et al.’s (1994) universal primers, LCO1490 and HCO2198. PCR amplification conditions were as follows: initial denaturation at 95°C for 3 min; 35 cycles at 94°C for 30 s, 48°C for 30 s and 72°C for 60 s; and a final extension step of 72°C at 10 min. PCR products were purified with the Sigma-Aldrich GenElute Gel Extraction Kit following the manufacturer’s instructions, and the amplicons were sequenced using the Folmer primers at the Molecular Biology and Sequencing Service of SZN, Naples.

**Species delimitation and phylogenetic analyses**

Sequences were retrieved from the GenBank and Barcode of Life Data System (BOLD) databases by searching for DNA barcodes of specimens belonging to the genus *Bursa*; the taxonomic identifications in the two databases were cross-checked with the most recent taxonomic literature on the family *Bursidae* (Castellini et al., 2012; Sanders et al., 2017; Smriglio et al., 2019; Strong et al., 2019). The ingroup consisted of 13 species of Bursa: *B. scrobilator*; *B. affinis* (Broderip, 1833); *B. avatici Ray, 1949; B. byfonia* (Gmelin, 1791); *B. cubaniana* (d’Orbigny, 1841); *B. elisabettae* Nappo, Pellegrini & Bonomolo, 2014; *B. fijimensis* (R. Watson, 1881); *B. fosteri* Beu, 1987; *B. granularis* (Rodin, 1798); *B. larvacki* (Deshayes, 1853); *B. quahong Beu, 1987; B. rosa* (Perry, 1811); and *B. tueroesissima* (Reeve, 1814). The outgroup comprised *Tonna galea* (Linnaeus, 1758); *Ranella olearium* (Linnaeus, 1758) and *Galeodea echinophora* (Linnaeus, 1758), with trees rooted on *T. galea*. COI sequences were aligned manually; the alignments are available from the authors on request.

Species delimitation analyses were performed using an integrative taxonomic approach, where species are considered as hypotheses to be tested by independent evidence. Every sequenced specimen was identified to species level based on morphology, following which the COI barcode dataset (in-group only) was analysed using two methods, the distance-based automatic barcode gap discovery (ABGD) (Puillandre et al., 2012) and the monophyly-based Poisson tree process (bPTP) (Zhang et al., 2013). The ABGD analysis was run on the online ABGD web server (http://wwwabi.sw.jussieu.fr/public/abgd/abgdweb.html) using the K2P substitution model, a relative gap width (X) of 1, a divergence of intraspecific divergence of 0.0001 and 0.1 and with Nn bins set at 20. The bPTP analysis was done using the bPTP web server (https://species.h-its.org/); we ran the analysis for 200,000 Markov chain Monte Carlo (MCMC) generations with thinning set at 100 and burn-in at 0.1.

The software ModelTest 2 v. 0.1.7 (Darriba et al., 2012) was used to select the best-fitting evolutionary model; model selection was based on the Akaake information criteria (Akaake, 1974), and the three codon positions of COI were analysed separately. Maximum likelihood (ML) analysis was performed using RAxML v. 0.6.0 (Kozlov et al., 2019), with 1,000 bootstrap replicates (Felsenstein, 1985). Bayesian inference (BI) was performed using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). The MrBayes analysis involved two independent MCMC runs (with four chains per run) for 10 million generations, sampling every 100 generations. Branches were considered to be strongly (well) supported if bootstrap (BS) values ≥ 70 (Hillis & Bull, 1993) and Bayesian posterior probabilities (PP) ≥ 0.95 (Alfaro & Holder, 2006).

**Spatial distribution of genetic diversity**

Relationships between haplotypes were investigated using a median joining (MJ) approach (Bandelt et al., 1999), as implemented in PopART (http://popart.otago.ac.nz; Leigh & Bryant, 2015). Phylogenetic network analyses may perform better than traditional tree-based phylogenetic methods when genetic divergence is low because they take into account population-level phenomena such as multifurcations and reticulations (Posada & Crandall, 2001). MJ, in particular, combines minimum spanning trees within a single network and adds median vectors (representing missing intermediates) to the network using a parsimony criterion.
Table 1. Localities of *Bursa scrobilator* sampled for this study, along with associated collection data, voucher numbers and GenBank accession numbers for COI sequences. All localities are in the East Atlantic–Mediterranean area and belong to the following regions: AZ, Azores Islands; CI, Canary Islands; M, Mediterranean Sea; WA, West Africa.

| Region | Sampling locality, depth and collector | Coordinates (latitude, longitude) | Source | Voucher ID code | GenBank acc. no. for COI data |
|--------|----------------------------------------|-----------------------------------|--------|----------------|-----------------------------|
| AZ     | Fajã Grande, Flores Island, 12 m (leg. M.O., Aug. 2008) | 39.4569, −31.2649 | This study | BAU-3535.1 | MN263830 |
| AZ     | Caloura, São Miguel Island, 8 m (leg. M.O., Sep. 2008) | 37.70669, −25.508 | This study | BAU-3536.1 | - |
| AZ     | Fajã Grande, Flores Island, 12 m (leg. M.O., Aug. 2008) | 39.4569, −31.2649 | This study | BAU-3535.2 | MN263831 |
| AZ     | Fajã Grande, Flores Island, 12 m (leg. M.O., Aug. 2008) | 39.4569, −31.2649 | This study | BAU-3535.3 | - |
| AZ     | Caloura, São Miguel Island, 8 m (leg. M.O., Sep. 2008) | 37.70669, −25.508 | This study | BAU-3536.2 | MN263832 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0021 | MN263833 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0022 | MN263834 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0023 | MN263835 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0024 | MN263836 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0025 | MN263837 |
| CI     | Las Eras, Tenerife Island, ~ 20 m (leg. J.M. Barrios, Feb. 2010) | 28.19796, −16.4194 | This study | SZN-MOL-0026 | MN263838 |
| CI     | Punta de Teno, Tenerife Island, ~ 11 m (leg. M.O., Jul. 1996) | 28.34053, −16.9197 | This study | BAU-3537 | MN263839 |
| CI     | Puerto del Carmen, Lanzarote Island, ~ 18 m (leg. M.O., Feb. 1995) | 28.91814, −13.6693 | This study | BAU-3538 | MN263840 |
| M      | Reggio Calabria, Altafiumara, 5 m (leg. F.C, AV, W. Renda, Aug. 2009) | 38.24446, 15.67524 | This study | MED-PC-1 | MN263841 |
| M      | Maretttino, Punta Bassana, 11 m (leg. M.O., Jul. 2011) | 37.94907, 12.09144 | This study | BAU-3539 | MN263842 |
| M      | Messina, Pace, 5–7 m (leg. P. Micali, c. 1980) | 38.23237, 15.57216 | This study | MED-PC-3 | MN263843 |
| M      | Naples (leg. C.P. Franceschini, May 1911) | 40.81908, 14.22441 | This study | MOL-052 | MN263844 |
| M      | Ustica, 10 m (leg. P. Trillò, Aug. 1999) | 38.71542, 13.15555 | This study | MOL-PC-4 | - |
| M      | Linosa Island, Italy | 35.86805, 12.88163 | Smriglio et al. (2019) | MOL-RM3.1 | LS483261 |
| M      | Naples, Italy | 40.63015, 14.36087 | Smriglio et al. (2019) | MOL-RM3.2 | LS483262 |
| M      | Blue Grotto, Malta | 35.8208, 14.45682 | Smriglio et al. (2019) | MOL-RM3.3 | LS483263 |
| WA     | Dakar, Senegal | 14.75057, −17.5274 | Smriglio et al. (2019) | MNHN-IM-2009-11 908 | LS483264 |
| WA     | Dakar, Senegal | 14.75057, −17.5274 | Smriglio et al. (2019) | MNHN-IM-2009-11 909 | LS483265 |
| WA     | Dakar, Senegal | 14.75057, −17.5274 | Smriglio et al. (2019) | MNHN-IM-2009-11 911 | LS483266 |

To investigate the spatial distribution of genetic diversity within each species, we carried out both an isolation by distance (IBD) analysis and a spatial principal component analysis (sPCA). IBD, as proposed by Wright (1940), is defined as a decrease in the genetic similarity among populations as the geographic distance between them increases. The presence of an IBD pattern can be detected by a nonparametric Mantel test, which is commonly used to test for nonrandom associations between a matrix of genetic distances and a matrix of geographical distances. The genetic distance matrix consisted of K2P pairwise intraspecific genetic distances for the COI alignment, which we computed using MEGA v. 6.0 (Tamura et al., 2013). For the geographical distance matrix, we calculated the shortest marine distance between all pairs of collecting points using Google Earth v. 7.1.2.2041. Both matrices were used as input for IBDW v. 2.0 (Jensen et al., 2005) and 30,000 randomizations. Despite the widespread use of Mantel tests to identify IBD patterns, theoretical reasons and the fact that these tests can be biased by spatial autocorrelation have led their reliability to be questioned (e.g. Meirmans, 2012). Therefore, to avoid misinterpretation of the potential correlations between genetic diversity and spatial distribution, we integrated the Mantel test with a sPCA, as implemented in the R package adegenet v. 2.1.0 (Jombart, 2008). This spatially explicit approach takes into account the genetic variance among studied samples, as well as their spatial autocorrelation (Jombart et al., 2008). The detection of spatial features in the input data is carried out by incorporating Moran’s I statistics (Moran, 1948, 1950) into the georeferenced genetic data. Moran’s I ranges from −1 to +1, with values close to +1 indicating clustering and values close to −1 indicating dispersion. To define neighbours for calculation of Moran’s I, a Gabriel graph for individual sample locations was
generated. Global and local tests based on 99,999 Monte Carlo permutations were used to interpret global and local components of the sPCA. The presence of significant global structuring may reflect patterns of spatial genetic structure (such as IBD), whereas significant local structuring may indicate strong differences between local neighbourhoods (repulsion) (Jombart et al., 2008).

We calculated the number of haplotypes, nucleotide diversity (π) and haplotype diversity (HD). An analysis of molecular variance (AMOVA) was performed to compare intra- and inter-population genetic diversity when the specimens of B. scrobilator were grouped into three (Azores Islands, Canary Islands and Mediterranean) or two (Atlantic and Mediterranean) geographical clusters; a hierarchical analysis was performed, with statistical significance assessed by 1,000 permutations of the original data matrix followed by a Bonferroni adjustment (Rice, 1995). A mismatch analysis was also performed to estimate the most likely demographic dynamics of B. scrobilator; analyses were done using DnaSP v. 5.10 (Librado & Rozas, 2009) and Arlequin v. 3.5.1.3 (Excoffier et al., 2005).

RESULTS

Morphology of Bursa scrobilator

No major differences were observed between the Macaronesian and Mediterranean specimens of Bursa scrobilator, and the description that follows applies to all examined specimens from the Canary Islands, the Azores and the Mediterranean.

Conchology

Shell thick and solid, moderately large for the genus (5–9 cm) (Fig. 1). Larval shell elongate-mammillate, light brown in colour, c. 2.5 in maximum diameter and consisting of 3.75 convex whorls (Fig. 2A–C); protoconch almost invariably corroded in adults, showing ~15% variation in size between different geographic areas, as well as among specimens within the same area. Protoconch I 0.5 whorl, with densely pitted sculpture ending in three to four axial ribs (Fig. 2D). Protoconch II 2.25 whorls, with cancellate sculpture of axial ribs and three spiral cords (Fig. 2E). Teleoconch five to six whorls, sculptured by four major spiral cords, usually knobbed; large varices sculptured with knobs at intersections with spirals. A few specimens devoid of obvious sculpture, with nodules just visible on penultimate varix and on peristome. Aperture large and cocolumellar callus with 12 to 18 small folds. Colour of shell fawn, with whitish and reddish marble and four reddish brown spiral lines overlapping the spiral cords. Light brown operculum (Fig. 1A), with anteriorly terminal nucleus (Fig. 1G, H).

Gross anatomy

Body massive, with irregular white, brown, yellow and blue patches and small yellowish-orange speckles (Fig. 1A). A pair of tentacles with small, dark and round eyes on swellings at their outer bases; tentacles marked with dark annular stripes and sparse yellow-orange speckles (Fig. 1A). Proboscis short and wide, pleurembolic, flattened elliptical and strongly muscularized; dark blue, with an electric-blue ring at tip (Fig. 1B). Jaw plates missing. Radula taenioglossate (formula 2.1.1.1.2; Fig. 3A–C), remarkably variable between and within individuals. Rachidian teeth broad, usually with long, slender and elongated median cusp and two shorter lateral cusps (Fig. 3D–I); median cusp with variable number of secondary basal denticles (from two to four, varying even in same tooth (Fig. 3D–I); lateral cusps with basal triangular processes interlocking with column of central teeth. Lateral tooth large and sickle-shaped, with broad base, one–two denticles on inner edge and two–five denticles on outer edge (Fig. 3J–K). Sickle-shaped marginal teeth, with one or two denticles on inner edge (Fig. 3M–O). One specimen with very large radular ribbon (nearly twice the size of other specimens examined, as is clear from scale bars in Figure 3), thick central rachidian tooth with no secondary denticles, thick lateral teeth with no inner denticles and thick lateral teeth lacking denticles on both inner and outer edges of radial ribbon along its entire length (Fig. 3I, L, P). Three accessory salivary glands present. Digestive tract consisting of solid foregut (forming a C-shaped structure) with terminal caecum, followed by short thin duct and muscular stomach (Fig. 1G, H) that was empty in all the samples examined; short intestine leading to the rectum, which opens into the right side of the mantle cavity. Mantine cavity with large ctenidium, small biplicate oesophagus and hypobranchial gland (Fig. 1G). Males with large, muscular, tongue-shaped penis, deeply wrinkled on dorsal side, just behind right cephalic tentacle (Fig. 1H); seminal groove closed; large testis (flattened and disk-shaped) compacted over posterior part of stomach, with long and convoluted vas deferens that runs mostly along the body’s right side (Fig. 1H). Female gonad compacted in posterior part of the body, just over the muscular stomach (Fig. 1G); a small ovary with a swollen pallial oviduct was found in one apparently immature female (Fig. 1G).

Species delimitation for the genus Bursa and for Bursa scrobilator

Critical study of identifications for all COI sequences sourced from GenBank and BOLD revealed that, in several cases, sequences from the same specimens were assigned to different taxa in the two databases, with preliminary identifications having not been updated in GenBank/BOLD after publication of the relevant papers. Strong et al. (2019) have corrected almost all misidentifications or mismatches. Altogether, our data mining yielded COI sequences belonging to 13 bursid taxa (Tables 1 and 2). We generated COI sequence data for an additional 15 samples of B. scrobilator (Table 1). These samples comprised three individuals from the Azores, eight from the Canary Islands and four from the Mediterranean Sea. Whereas the new sequences were trimmed to 566 bp following their alignment with the GenBank sequences, sequence variation was analysed on the basis of the full-length (i.e. untrimmed) sequences.

The ABGD analysis yielded 13 putative species, which were largely congruent with the a priori morphology-based identifications of bursid species from across the world (Tables 1 and 2; Fig. 4). The bPTP analysis yielded 14 putative species, with B. scrobilator being split into two species; these two species, however, were not strongly supported (B. scrobilator: PP = 0.65; B. s. coriacea: PP = 0.71) (Fig. 4). With B. quirihorai and B. fijiensis treated as a single species, intraspecific genetic divergence for the bursid dataset, excluding B. scrobilator, ranged from 0 to 2.9% (Table 3); the minimum interspecific distance was 6.6%. When B. quirihorai and B. fijiensis were treated as distinct species (as in Castelin et al., 2012), intraspecific genetic divergences ranged from 0 to 2.2%, and the minimum interspecific divergence was 2.9%. Both the bPTP analysis and the ABGD one showed the Mediterranean and Macaronesian sequences of B. scrobilator (altogether corresponding in morphology to the subspecies B. s. scrobilator) to belong to a single putative species. The very low K2P distances between specimens in the Mediterranean-Macaronesian clade (0–0.9%) (Fig. 4) agree with this finding. While the ABDG analysis showed the three Senegalese specimens (corresponding morphologically to the subspecies B. s. coriacea) to belong to the same species as the Mediterranean and Macaronesian specimens, in the bPTP analysis, they formed a distinct species (intragroup K2P divergence 0–5%). We note that genetic distances between the Senegalese specimens, on the one hand, and the Macaronesian–Mediterranean clade, on the other, were relatively high (1.6–2.7%).

Bayesian and ML phylogenies showed five well-supported clades, each comprising between one and four nominal species (Fig. 4). Clade I (PP = 0.99, BS = 80%) consisted of four species, B. avatti, B. fosteri, B. quirihorai and B. fijiensis, with strong support for the sister-group relationship of B. avatti and B. fosteri (PP = 0.99, BS = 83) and...
Figure 1. Shells and gross anatomy of *Bursa scrobilator*. A. Living individual from Altafiumara, Mediterranean (MED-PC-1). B. The same specimen with protruded proboscis. C-F. Representative specimens of different growth stages, as examined in this study. G. BAU-3535.3, Flores I., Azores, (shell height 0.9 cm). D. SZN-MOL-0026, Tenerife I., Canary Islands (shell height 3.2 cm). E. MED-PC-1, Altafiumara, Mediterranean (shell height 7 cm). F. SZN-MOL-0025, Tenerife I., Canary Islands (shell height 6.2 cm). G. Gross anatomy. G. SZN-MOL-0024, female (shell height 75 mm), with digestive tract removed along with the pedal ganglion. H. BAU-3539, male (shell height 79 mm), with digestive tract removed (see bottom right). Abbreviations: E, eye; Fg, foregut; Ft, foot; G, gill; Hd, head; He, digestive gland; I, intestine; Mf, mantle flap; O, operculum; Ov, ovary; Pe, penis; Pg, pedal ganglion; Pr, proboscis; R, rectum; S, inhalant siphon; St, stomach; T, testis; Tn, tentacle; and Vd, vas deferens.

of *B. quirihorai* and *B. fijiensis* (PP = 1, BS = 99%). Clade II, which was strongly supported only in the Bayesian analysis (PP = 0.96), also comprised four species, the relationships among which were unresolved. These were *B. lamarckii, B. rosa, B. tuberosissima* and *B. bufonia*. Clades III and IV were each composed of a pair of sister species; clade III was strongly supported only in the Bayesian analysis (PP = 0.98), whereas clade IV was strongly supported in both Bayesian and ML analyses (PP = 0.99, BS = 89%). Clade III consisted of the sister taxa *B. elisabettae* and *B. cubanana* and clade IV comprised the sisters *B. granularis* and *B. affinis*. Clade V, *B. scrobilator*, was strongly supported (PP = 1, BS = 100%) and consisted of two strongly supported constituent clades corresponding to *B. s.
**Phylogeographic structure and spatial distribution of genetic diversity in Bursa scrobilator**

Nucleotide diversity was broadly similar at all locations, and haplotypic diversity was of the same magnitude at all hierarchical levels (Table 4). In the MJ network analysis, 17 polymorphic sites defined 11 haplotypes in *B. scrobilator*, with two major haploclades corresponding to the two ESUs *B. s. scrobilator* (eight haplotypes; \( \pi = 0.004 \)) and *B. s. coriacea* (two haplotypes; \( \pi = 0.013 \)) (Table 4; Fig. 5). Only the genetic diversity of the ESU corresponding to *B. s. scrobilator* was analysed; the ESU representing *B. s. coriacea* was represented by just three specimens from a single locality, so it could not be analysed. Nucleotide diversity was relatively low in *B. s. scrobilator*, with inter-population variance explaining only 2% of total variance (regardless of whether two or three geographical clusters were considered, both F-st and \( \Phi_{st} \)-st were not significant). Mean intraspecific K2P distances for *B. scrobilator* were comparable to those of other bursids, but the maximum intraspecific K2P distance between *B. s. scrobilator* and *B. s. coriacea* was greater than the maximum intraspecific K2P distances estimated for the other species. The mismatch analysis, in which Macaronesian and...
Figure 3. Features of the radula of *Bursa scrobilator*. A–C. Taenioglossate radula. A. BAU-3535.2, Flores I., Azores. B. SZN-MOL-0026, Tenerife I., Canary Islands. C. BAU-3539, Marettimo I., Mediterranean. D–I. Rachidian teeth. D. BAU-3535.2, Flores I., Azores. E. SZN-MOL-0025, Tenerife I., Canary Islands. F. SZN-MOL-0026, Tenerife I., Canary Islands. G. BAU-3539, Marettimo I., Mediterranean. H. MED-PC-3, Messina, Mediterranean. I. MED-PC-4, Ustica I., Mediterranean. J–L. Lateral teeth. J. SZN-MOL-0025, Tenerife I., Canary Islands. K. SZN-MOL-0026, Tenerife I., Canary Islands. L. MED-PC-4, Ustica I., Mediterranean. M–O. Marginal teeth. M. SZN-MOL-0025, Tenerife I., Canary Islands. N. SZN-MOL-0026, Tenerife I., Canary Islands. O. MED-PC-3, Marettimo I., Mediterranean. P. MED-PC-4, Ustica I., Mediterranean.
Table 2. GenBank and Barcode of Life Data System COI sequences used in the molecular systematic analyses and associated specimen data.

| Strong species identifications | GenBank | As published in relevant molecular systematic study | Voucher ID code | BOLD ID | GenBank acc. no. for COI | Locality (from databases or relevant paper/s) |
|-------------------------------|---------|----------------------------------------------------|----------------|---------|-------------------------|---------------------------------------------|
| Galeodea echinophora          | MNHN-IM-2009-7834 | MHNIM22-18 | TONO226-18 | MHS81337 | France (Rungis International Market, Paris) |
| Ranella olearium              | MNHN-IM-2007-36318 | TONO231-18 | MHS81358 | Mozambique Channel |
| Tonna galea                   | MNHN-IM-2013-56652 | TONO278-18 | MHS81377 | French Guiana |
| Tonna galea                   | MO77867 | BIN225-13 |                        | Israel |
| B. affinis                    | MNHN-IM-2013-11041 | TONO128-17 | MF124209 | Papua New Guinea |
| B. affinis                    | MNHN-IM-2013-52997 | TONO145-17 | MF124220 | French Polynesia (Marquesas Islands) |
| B. affinis                    | MNHN-IM-2007-43060 | TONO098-12 | JX241367 | Vanuatu (Espiritu Santo Island) |
| B. affinis                    | TONO231-18 | TONO158-17 | MF124232 | Marshall Islands (Enek Channel, Majuro Atoll) |
| B. affinis                    | TONO278-18 | TONO168-17 | MF124189 | Guam (Tepungan Channel, Piti) |
| B. affinis                    | TONO226-18 | TONO169-17 | MF124221 | Japan (Okinawa Island, Okinawa Prefecture) |
| B. affinis                    | TONO231-18 | TONO177-17 | MF124218 | New Caledonia (Huon Atoll) |
| B. affinis                    | TONO278-18 | TONO178-17 | MF124214 | Taiwan (Kenting National Park) |
| B. affinis                    | MNHN-IM-2013-56652 | TONO226-18 | MF124212 | Florida (Monroe county) |
| B. affinis                    | MNHN-IM-2013-56652 | TONO226-18 | MF124212 | France (Rungis International Market, Paris) |
| B. affinis                    | MNHN-IM-2013-56652 | TONO226-18 | MF124212 | Mozambique Channel |
| B. affinis                    | MNHN-IM-2013-56652 | TONO226-18 | MF124212 | French Guiana |
| B. affinis                    | MO77867 | BIN225-13 |                        | Israel |
| B. affinis                    | MNHN-IM-2013-11041 | TONO128-17 | MF124209 | Papua New Guinea |
| B. affinis                    | MNHN-IM-2013-52997 | TONO145-17 | MF124220 | French Polynesia (Marquesas Islands) |
| B. affinis                    | MNHN-IM-2007-43060 | TONO098-12 | JX241367 | Vanuatu (Espiritu Santo Island) |
| B. affinis                    | TONO231-18 | TONO158-17 | MF124232 | Marshall Islands (Enek Channel, Majuro Atoll) |
| B. affinis                    | TONO278-18 | TONO168-17 | MF124189 | Guam (Tepungan Channel, Piti) |
| B. affinis                    | TONO226-18 | TONO169-17 | MF124221 | Japan (Okinawa Island, Okinawa Prefecture) |
| B. affinis                    | TONO231-18 | TONO177-17 | MF124218 | New Caledonia (Huon Atoll) |
| B. affinis                    | TONO278-18 | TONO178-17 | MF124214 | Taiwan (Kenting National Park) |
| B. affinis                    | MNHN-IM-2013-56652 | TONO226-18 | MF124212 | Florida (Monroe county) |

Other than Strong et al. (2019), published identifications were obtained from Castelin et al. (2012) and Sanders et al. (2017), which are denoted by the numbers ‘1’ and ‘2’, respectively.
Figure 4. Phylogenetic relationships in the genus *Bursa*, based on COI sequence data. **A.** Part of ML tree showing relationships in the *Bursa scrobilator* clade (see clade V in B). **B.** Tree for the genus *Bursa* (the substitution models used for the first, second and third codon positions are TrN1+I+G, F81 and TPM3uf+G, respectively). Numbers along branches indicate Bayesian posterior probabilities and ML bootstrap values, respectively. Roman numerals I–V denote the five major clades. Putative species delimited by bPTP and ABGD methods are colour-coded. **C.** Histograms of the ranges of intraspecific genetic distances (K2P). *Bursa quirihorai* and *B. fijiensis* are considered first as separate ESUs and then as forming one ESU; *B. s. scrobilator* and *B. s. coriacea* are also shown in the same way.
Table 3. Range in intraspecific COI sequence divergence (% K2P) for species of *Bursa.*

| Species                      | Minimum K2P | Maximum K2P |
|------------------------------|-------------|-------------|
| *Bursa awati*                | 0.000       | 0.005       |
| *B. cubaniana*               |             | 0.005       |
| *B. elisabettae*             |             | 0.005       |
| *B. granularis*              | 0.002       | 0.008       |
| *B. affinis*                 | 0.000       | 0.009       |
| *B. fijensis*                | 0.000       | 0.009       | 0.029*     |
| *B. quirihorai*              | 0.000       | 0.022       |
| *B. fosteri*                 |             | 0.007       |
| *B. lamarckii*               | 0.005       | 0.016       |
| *B. scrobilator scrobilator* | 0.000       | 0.009       | 0.027**    |
| *B. scrobilator coriacea*    |             | 0.000       | 0.005      |

A single asterisk indicates the maximum value when *B. fijensis* and *B. quirihorai* are a single ESU. Two asterisks denote the maximum value when *B. s. scrobilator* and *B. s. coriacea* are a single ESU.

Table 4. Nucleotide diversity (π) and haplotype diversity (HD) for the two ESUs of *Bursa scrobilator,* based on the COI alignment (n indicates the sample size).

| ESU                  | Group | Subgroup | n  | π     | HD   |
|----------------------|-------|----------|----|-------|------|
| *B. s. scrobilator*  | Atlantic | Azores  | 11 | 0.004 | 0.911 |
|                      |        | Canary I. | 3  | 0.004 | 0.667 |
| *B. s. coriacea*     | Mediterranean | Senegal   | 7  | 0.003 | 0.714 |

DISCUSSION

We found that for *Bursa scrobilator scrobilator* genetic connectivity over long distances (i.e. virtually across the whole range) was high, and we found no evidence of geographic structure at both local and global spatial scales. These results agree with what we would expect for a species with long-lived pelagic larvae. Our findings are also consistent with the little that is known on other species of *Bursa* (Castelin et al., 2010) and with available data on other organisms with long-lived planktotrophic larvae (e.g. *Sasia renensa,* Castelin et al., 2010; see also Staton & Rice, 1999). The pattern of genetic connectivity in *B. scrobilator* is relevant for several aspects of its biology; particularly important aspects in this respect are its taxonomic status and its capacity to respond to climate change.

As is common for most mollusc species, the taxonomy of *B. scrobilator* has long been focused solely on shell characters. Smriglio et al. (2019), in an in-depth morphological survey of Recent and fossil shells, published the first molecular systematic analysis for this species, showing that there was a deep genetic divergence between three Senegalese samples and three Mediterranean samples. This analysis formed part of a shell-based assessment of the taxonomic status of the West African morphotype (with finely granulose sculpture and fine to prominent shoulder nodules, corresponding to the nominal taxon *Ranella coriacea* Reeve, 1844) and the typical Mediterranean morphotype (corresponding to the nominal taxon *Murex scrobilator* Linnaeus, 1758). On the basis of the consistent, yet subtle shell morphological differences between Senegalese and Mediterranean samples (see also Cossignani, 1994; Ardovini & Cossignani, 2004; Beu, 2010), and supplementary genetic data, Smriglio et al. (2019) proposed two subspecies: *B. s. scrobilator* from the Mediterranean (probably including the Macaronesian populations) and *B. s. coriacea* (Reeve, 1844) from West Africa. By generating COI barcode data for specimens from the Azores and Canary Islands and for additional specimens from the Mediterranean region, we have substantially added to the genetic data available for this species. In our analyses, we found strong evidence for the Macaronesian and Mediterranean specimens forming a single clade and this clade being sister to a clade of exclusively Senegalese samples. This confirms the general morphological pattern first observed by Smriglio et al. (2019). For the purposes of the present study, we followed Smriglio et al. (2019) and treated the two constituent ESUs of *B. scrobilator* as distinct subspecies. However, we would argue that the status of the West African populations needs to be carefully assessed; their divergence from the Mediterranean–Macaronesian populations is relatively deep (1.6–2.7%, mean 2.1%) and comparable to the divergence between the sister species *B. quirihorai* and *B. fijensis* (1.5–2.3%).

We found that for *B. s. scrobilator* the Mantel test did not support any correlation between geographical distances and genetic structure (Fig. 7). A single asterisk indicates the maximum value when *B. fijensis* and *B. quirihorai* are a single ESU. Two asterisks denote the maximum value when *B. s. scrobilator* and *B. s. coriacea* are a single ESU.

### Table 4. Nucleotide diversity (π) and haplotype diversity (HD) for the two ESUs of *Bursa scrobilator,* based on the COI alignment (n indicates the sample size).

| ESU                  | Group | Subgroup | n  | π     | HD   |
|----------------------|-------|----------|----|-------|------|
| *B. s. scrobilator*  | Atlantic | Azores  | 11 | 0.004 | 0.911 |
|                      |        | Canary I. | 3  | 0.004 | 0.667 |
| *B. s. coriacea*     | Mediterranean | Senegal   | 7  | 0.003 | 0.714 |

Mediterranean *B. s. scrobilator* were treated as one geographical cluster, showed a bimodal distribution (Fig. 6), suggesting demographic stability in recent times; regardless of whether analyses were based on two or three geographical clusters, the Tajima’s *D* test was consistently not significant (*P* > 0.10). The *B. s. scrobilator* clade did not show any geographical structure, and constituent clades contained specimens from all sampled areas (Fig. 4). The Mantel test did not support any correlation between geographical and genetic distance (0.142 ≤ *P* ≤ 0.493). The sPCA showed that there was neither global (*P* = 0.809) nor local (*P* = 0.115) spatial structure in the genetic data (Fig. 7).
Figure 5. MJ network for the haplotypes of *Bursa scrobilator*. The length of the branches connecting the different haplotypes is proportional to the number of mutations. Small segments along the connecting branches represent missing intermediate haplotypes (identified using a parsimony criterion).

Figure 6. Mismatch distribution showing bimodal distribution.

Figure 7. Spatial distribution of the scores of PC1 obtained from the sPCA for *Bursa scrobilator scrobilator*. Each square corresponds to a sampled locality with the colour of the square indicating the score of the relevant haplotype (positive if black, negative if white).

The gross anatomy of the Mediterranean–Macaronesian *B. s. scrobilator* agrees with the relatively scanty knowledge that we have of the family (Houbrick & Fretter, 1969; Beu, 1981). For their large dataset, Smriglio et al. (2019) observed substantial homogeneity in adult shell morphology, and that is paralleled here—albeit for a small number of samples—by homogeneity in other morphological characters (protoconch, radula and gross anatomy) across the entire range of *B. s. scrobilator*. The well-preserved protoconch observed in one of the juvenile shells studied by us (Fig. 2A, D–E) was reticulately sculptured; this character has not been observed so far, not even in the Pliocene specimen from Estepona (see Landau et al., 2004), which our examinations show has a worn protoconch. The elusive nominal taxon *Talisman parfaiti* de Folin, 1887, the holotype of which we figure for the first time (Fig. 8; see also description by de Folin, 1884), was originally described on the basis of a bursid larval shell (Warén & Bouchet, 1990; Beu, 2010), which was collected from southwestern Europe or West Africa. Based on the strongly reticulate sculpture of protoconch II, we suggest that *Talisman parfaiti* was likely based on a larval shell of *B. scrobilator*.

We found strikingly high intra(sub)specific and intra-individual variation in radular characters, but there was no geographic basis to this variation. The variability in radular characters is also evident in a specimen described by Melone (1975); apart from the radula of MED-PC-4 (Fig. 3L, P), the radula described by Melone differs from all the other radulae examined by us. We can say little about the generality of this variability; only a few bursid radulae have been illustrated to date (Beu, 1981; Ekawa & Toki, 2005; Barkalova et al., 2016), and no published data are available for the family on the intraspecific variation in radular characters. It may be that the Bursidae show plasticity in radular characters. Ontogenetic change and sexual dimorphism are known to be potential modulators of radular
Figure 8. Holotype of *Talisman parfaiti* from the Travailleur and Talisman collection, Muséum national d’Histoire naturelle, Paris (shell height 1.5 mm). 

A–C. Apertural (A), dorsal (B) and apical (C) views of the shell. 

D–E. Original labels.

features in molluscs (e.g. Maes, 1966; Bertsch, 1976; Nybakken & Perron, 1988; Meirelles & Matthews-Cascon, 2003; Mutlu, 2004; Matthews-Cascon *et al.*, 2005; Warén, 2005; Martínez-Pita *et al.*, 2006). However, the variation we observed was not related to sex (discriminated on the basis of the presence/absence of the penis) or age (evaluated on the basis of total shell height). Our observations are paralleled by those of Barkalova *et al.* (2016), who reported two very different types of radula from two species of the bursid genus *Tutufa* Jousseaume, 1881. Whereas the radula of *Tutufa oyamai* was very similar to the typical radula of *Bursa* (this applies also to all but one of the radulae examined in our study), that of *Tutufa rubeta* was extremely fatty and similar to the radula of *B. scrobilator* figured by Melone (1975) and to just one of our specimens (MED-PC-4). No explanation, however, was offered by Barkalova *et al.* (2016) for the differences they observed.

Caution is needed when discussing the results of population genetics and morphological analyses based on small sample sizes. This is especially the case when assessing the effects of patterns of genetic connectivity—likely to be complex in the case of long-lived pelagic larvae—on mutation-drift equilibria (Flowers *et al.*, 2002). The mismatch distribution suggests that the demography of the nominal subspecies of *B. scrobilator* has been relatively stable in recent times. This is interesting because *B. scrobilator* has long been considered to be a rare species in the Mediterranean. However, several anecdotal reports from amateur collectors and marine biologists in the last few years seem to indicate an increase in the number of records from Mediterranean sites. If this increase is confirmed in the future, then it suggests the hypothesis that rising temperatures and, more generally, climate change, which are favouring thermophilic species in the Mediterranean Sea (e.g. Moullec *et al.*, 2016 and references therein), may also affect the distribution and population biology of *B. scrobilator*.

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