Molecular analysis reveals a cryptic species of *Chthamalus* (Crustacea: Cirripedia) in the Cape Verde Islands

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The intertidal barnacle *Chthamalus stellatus* has a broad distribution, occurring in the Mediterranean, the east Atlantic shores and east Atlantic Macaronesian Islands (Madeira, the Canaries and the Azores). Traditionally, based on morphological characters, *Chthamalus* of the Cape Verde Islands were also regarded as *C. stellatus*. However, using a mitochondrial gene and two nuclear genes, we found that although *Chthamalus* from Cape Verde is morphologically similar to *C. stellatus*, there are genetic differences between the two that are larger than those found between different species of *Chthamalus*. We thus claim that these genetic differences justify the assignment of the Cape Verde populations as an evolutionarily significant unit and a sister clade to *C. stellatus*. We also show that the connection between taxonomic units that are close to each other lies not only in the resemblance between DNA sequences. We have found that numerous point mutations characterizing the Cape Verde *Chthamalus* are present as infrequent alleles in *C. stellatus*, indicating that two close taxonomic units can also share polymorphisms present in their common ancestor.

ADDITIONAL KEYWORDS: barnacles – Chthamalidae – *COI* – *EF1* – Macaronesia – *NaKA* – polymorphism – speciation.

INTRODUCTION

In his monumental monograph, Darwin (1854) recognized eight species of the barnacle genus *Chthamalus* Ranzani, 1817 (Chthamaloidea: Chthamalidae), including *Chthamalus stellatus* Poli, 1791, which is of almost worldwide distribution and consists of several races or varieties. Most of the varieties were later recognized as valid species, and in addition, more species of *Chthamalus* were described. O'Riordan *et al.* (2010) counted 24 nominal species of *Chthamalus*. Since then, Chan & Cheang (2015) have added *Chthamalus williamsi* Chan & Cheang, 2015 to this list. Southward (1976) reported that in the north-east Atlantic Ocean and the Mediterranean Sea, *C. stellatus* is composed of two species, *C. stellatus* and *Chthamalus montagui* Southward, 1976. Both species occur intertidally; often their distributions overlap, and a mixed population has been found (Fig. 1A). Although the geographical range of the two species covers the north-east Atlantic coasts, the Mediterranean Sea and the Black Sea (Crisp *et al.*, 1981), on the Atlantic Islands (Madeira, the Canaries and the Azores) only *C. stellatus* is found (Stubbings, 1967; Southward, 1976). The pelagic stage of *C. stellatus* lives longer and disperses further offshore than that of *C. montagui*. This strategy allows *C. stellatus* to maintain populations on oceanic islands further from the continental shelf, where *C. montagui* is usually absent (Crisp *et al.*, 1981). The occurrence of *C. stellatus* in the Cape Verde Islands was already recorded by Darwin (1854: 457). Its presence there was confirmed by Stubbings (1967) and by Southward (1976). Stubbings (1967) stated that there is no record of *C. stellatus* south of the Cape Verde Islands, where it is replaced by *Chthamalus dentatus* Krauss, 1848.

The identification of barnacles in the field is based mainly on the morphology of the opercular valves and shell. This becomes more difficult in locations where sympatric species occur, because they are

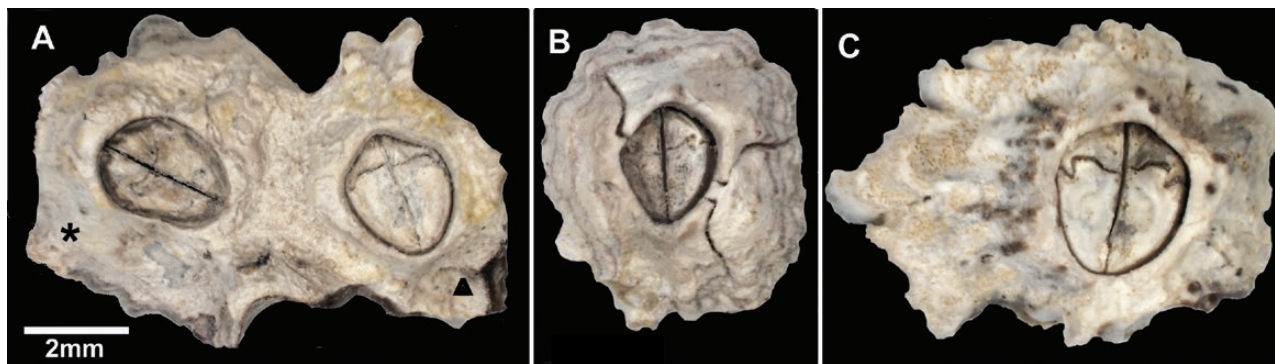*Corresponding author. E-mail: yaront@ruppin.ac.il

Figure 1. *Chthamalus* from three localities. A, *Chthamalus stellatus* (asterix) and *Chthamalus montagui* (triangle) from Boccadasse, Genoa, Italy. B, *C. stellatus* from Las Palmas, Gran Canaria, Canary Islands. C, *Chthamalus* sp. from Praia, Santiago, Cape Verde Islands.

easily confused. In such cases, the morphology of the arthropodial appendages, cirri and trophi are often used for separation of species. The distinctive characters of the '*stellatus*' group is the presence of conical spines on the base of cirrus I (Fig. 2A) and denticulated setae on cirrus II (Figs 2B, 2D). Chan *et al.* (2016) used these criteria for the separation of seven species from the Tropical East Pacific. They used enzyme electrophoresis and the mitochondrial gene *COI* as genetic support to determine distinct species.

In the past three decades, the developing field of molecular biology has become a major component in taxonomic research, including barnacle phylogenetics. Dando & Southward (1980) used morphological characters and enzyme electrophoresis to separate species of *Chthamalus* from the western shores of the Atlantic. They recognized four distinct informal groups of species within the genus *Chthamalus*, namely, the '*challengeri*', '*fissus*', '*stellatus*' and '*malayensis*' groups (see also Southward & Newman, 2003: table 1). The 27 currently known nominal species (Chan *et al.*, 2020) are placed in these informal groups, but not all species can be separated using morphological characters. Based on genetic distance, these can be recognized as a valid species or cryptic species. It is expected that the use of molecular tools will reveal more such cryptic species.

The present study deals with populations of *C. stellatus* that represent a possible cryptic species. Populations of *C. stellatus* were reported throughout the entire Mediterranean and the eastern shores of the East Atlantic Islands, including Madeira, the Canaries and Cape Verde, as shown in morphological studies (Southward, 1976; González *et al.*, 2012). In this study, we compare the *Chthamalus* population from the Cape Verde Islands, a group of volcanic islands in the Atlantic Ocean ~600 km west of the Senegal coast, with the Mediterranean and East Atlantic populations of *C. stellatus*.

MATERIAL AND METHODS

Samples of *Chthamalus* were collected from intertidal rocks by us or were donated by colleagues. Barnacles were fixed and stored in 96% ethanol. The samples used for this study are stored at the Israeli National Natural History Collections at the Hebrew University of Jerusalem (for details, see Supporting Information, Appendix S1).

Samples were examined and selected specimens photographed using an Olympus SZX10 dissecting microscope. Skeletal structures (wall and opercular plates) were separated from individual barnacles, soaked in diluted bleach for a few hours, and then examined under the dissecting microscope; adhering chitin was removed using needles and a fine paintbrush. Dried samples were mounted on brass stubs, coated with gold and examined with a JEOL scanning electron microscope at 25 kV. Images were stored using the Autobeam software.

Images of soft parts, cirri and mouthparts of selected specimens from Genoa (Italy), Biarritz (France), Gran Canaria (Canary Islands, Spain) and Praia (Cape Verde) were acquired with a Zeiss AxioImager Z1 microscope using a colour AxioCam MRc camera and driven by Zeiss AxioVision software.

MOLECULAR ANALYSES

DNA was extracted using the AccuPrep genomic DNA extraction kit (Bioneer, Daejeon, Korea) from soft tissue of 265 specimens of *C. stellatus* that were collected from ten different locations along the Mediterranean and eastern Atlantic coasts (Table 1), plus 44 samples of *Chthamalus* from the Cape Verde Islands.

Three gene segments were amplified using already known primers: the mitochondrial *COI* (Folmer, 1994), the nuclear Na-K-ATPase (*NaKA*) and elongation factor 1α (*EF1*) (Wares *et al.*, 2009). Polymerase chain
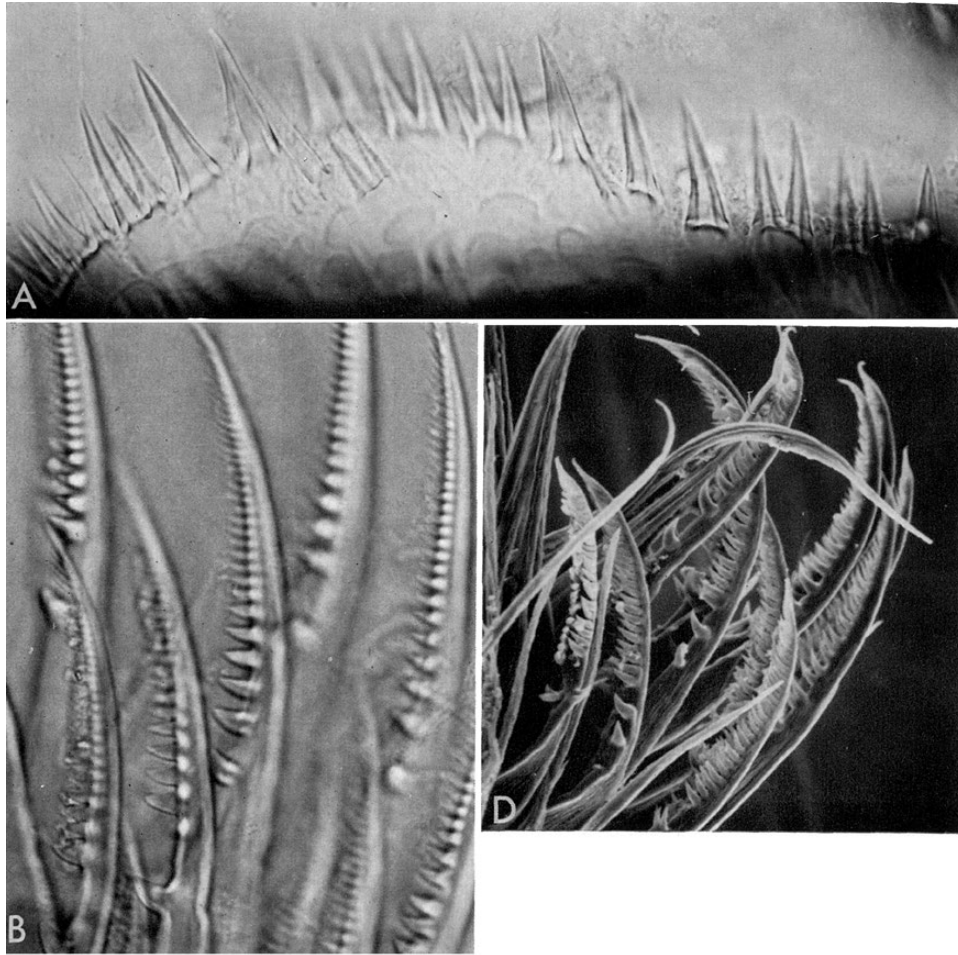
Figure 2. *Chthamalus stellatus* reproduced from Southward (1976: plate II). A, conical spines found at the base of the anterior ramus of cirrus I. B, D, denticulate setae on cirrus II.

Table 1. Number of individuals for each marker

Chthamalus species	*EF1*	*NaKA*	*COI*
C. alani	24	8	19
C. angustitergus	18	7	23
C. dalli	8	8	164
C. fissus	10	6	12
C. hedgecocki	10	8	9
C. panamensis	19	9	33
C. proteus	16	9	22
C. stellatus	236	233	61
Cape Verde population	37	37	12
Total	378	325	355

reactions (PCRs) were carried out in 25 µL reaction volumes containing 1× PCR buffer (including 1.5 mM MgCl$_2$), 0.2 mM of each dNTP, 1 µM of each primer, 1 unit of Super-Term Taq polymerase (Hoffmann-La Roche) and ~100 ng template DNA. The PCRs were processed in an MJ Research thermal cycler with the following thermal regimen: an initial step of 2 min at 95 °C, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 57 °C and 1 min at 72 °C, followed by 3 min at 72 °C and then held at 15 °C. The PCR products were visualized on 1.5% agarose gels and sequenced bidirectionally using the PCR primers on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

Given that the Cape Verde *Chthamalus* sequences were significantly different from all other *Chthamalus* species, we wanted to construct a phylogeny for the genus that includes this new population, based on three different markers. We examined eight species of *Chthamalus*, for which we had at least six sequences for each of the three genes, namely *Chthamalus alani* Chan, 2016, *Chthamalus angustitergum* Pilsbry, 1916, *Chthamalus dalli* Pilsbry, 1916, *Chthamalus fissus* Darwin, 1854, *Chthamalus hedgecocki*

Pitombo & Burton, 2007, *Chthamalus panamensis* Pilsbry, 1916, *Chthamalus proteus* Dando & Southward, 1980 and *C. stellatus* (Table 1). GenBank accession numbers used in our study are given in the Supporting Information (Appendix S1)

After alignment, we discarded the monomorphic nucleotide positions and considered only the polymorphic markers. Thus, we were left with 150 positions for *EF1*, 90 positions for *NaKA* and 330 positions for *COI*. The following analysis was done for each marker separately. For each of the nine populations, we calculated the distribution of the four different nucleotides (A, C, G and T) in each of the nucleotide positions. We then compared, for each position, the distribution of the four different nucleotides between the nine different populations, by using a distance or a similarity metric (see next paragraph). Thus, we obtained, for each position, 36 pairwise distances (or similarities). These distances (or similarities) were averaged over all relevant positions of the marker to obtain the final pairwise distances (or similarities) for the marker. The results were arranged in a 9 × 9 symmetric distance (or similarity) matrix. We then summed the three distance (or similarity) matrices (one for each marker) to obtain the comprehensive distance (or similarity) between the populations. This final matrix served for construction of a population dendrogram.

We used two different similarity coefficients, Pearson's correlation coefficient and the modified Morisita's similarity coefficient, and two different distance measures, the squared Euclidean distance and the Manhattan (or city blocks) distance. If x_1, x_2, x_3 and x_4 are the proportions of A, C, G and T in population 1, and y_1, y_2, y_3 and y_4 are these proportions in population 2, then:

$$\text{Pearson's correlation coefficient} = \frac{\sum\limits_{i=1}^{4}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum\limits_{i=1}^{4}(x_i - \bar{x})^2 \sum\limits_{i=1}^{4}(y_i - \bar{y})^2}},$$

$$\text{modified Morisita's similarity coefficient} = \frac{2\sum\limits_{i=1}^{4}x_i y_i}{\sum\limits_{i=1}^{4}x_i^2 + \sum\limits_{i=1}^{4}y_i^2},$$

$$\text{squared Euclidean distance} = \sum_{i=1}^{4}(x_i - y_i)^2 \text{ and}$$

$$\text{Manhattan distance} = \sum_{i=1}^{4}|x_i - y_i|.$$

The four final matrices are presented in the Supporting Information (Appendix S2).

We used four different amalgamation procedures [UPGMA (unweighted pair group method with arithmetic mean), minimum variance, nearest neighbour and farthest neighbour (using the MVSP software; Kovach Computation Services, 2013)], and thus we could construct 13 different trees (i.e. all different combinations, except that minimum variance is applicable only for the squared Euclidean distance).

Unfortunately, sufficient genetic information for *C. montagui*, a species geographically neighbouring the Cape Verde Islands, was not available for all three genes. Thus, we separately performed cluster analysis on the *COI* gene only, including the nine above-mentioned populations plus a population of 188 individuals of *C. montagui* (Supporting Information, Appendix S2).

RESULTS

MOLECULAR ANALYSIS

In all three gene segments, the sequences obtained for the Cape Verde *Chthamalus* are significantly different from all recorded chthamalid sequences, in both GenBank and our *C. stellatus* samples. All 13 trees exhibit a similar configuration. Three species, *C. alani*, *C. hedgecocki* and *C. panamensis* (which belong to the '*panamensis*' complex; Chan *et al.*, 2016), are in one cluster, and *C. stellatus* and the Cape Verde population are in another cluster. Moreover, the distances within the former cluster are smaller than the distance between *C. stellatus* and the Cape Verde population. For simplicity, we present in Figure 3 only four dendrograms, spanning all four distance/similarity measures and all four amalgamation procedures. A separate *COI*-based dendrogram, constructed by Ward's method (squared Euclidean distance and minimum variance amalgamation), demonstrates the genetic distinction between *C. montagui*, *C. stellatus* and the Cape Verde populations (Supporting Information, Appendix S3).

MORPHOLOGICAL DESCRIPTION

CHTHAMALUS SP.

(FIGS 1C, 4–6)

Material examined: Chthamalus sp. from Praia, Santiago, Cape Verde (14°55′53″N, 23°30′45″W). For comparison, we used samples from Boccadasse Beach, Genoa, Italy (44°23′23″N, 8°58′24″E), Biarritz, France (43°28′48″N, 1°33′20″W) and Las Palmas, Gran Canaria, Spain (28°5′59″N, 15°24′48″W).
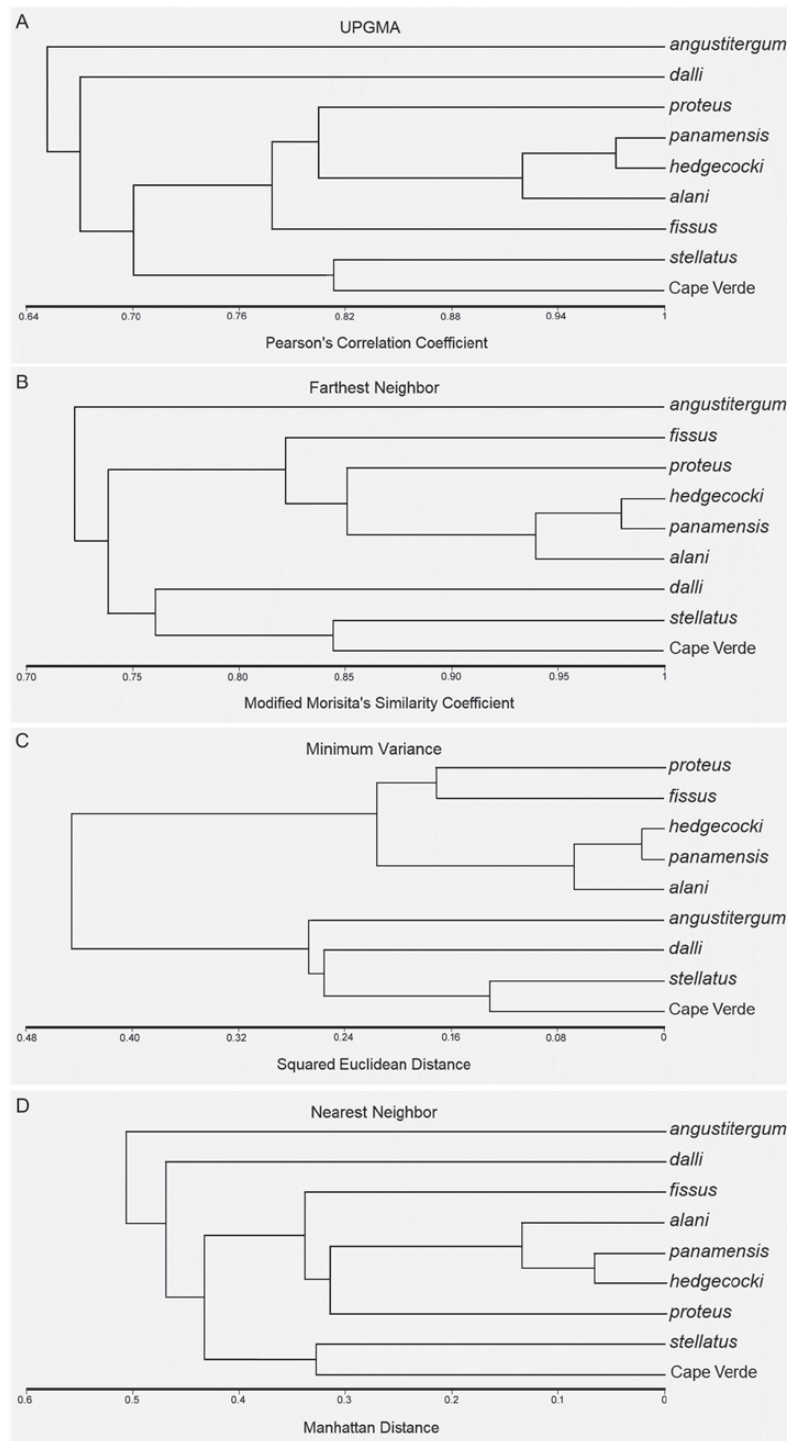
Figure 3. Four dendrograms depicting the hierarchical relationship between the nine *Chthamalus* populations. A, Pearson's correlation coefficient with UPGMA amalgamation. B, modified Morisita's similarity coefficient with farthest neighbour amalgamation. C, squared Euclidean distance with minimum variance amalgamation. D, Manhattan distance with nearest neighbour amalgamation.
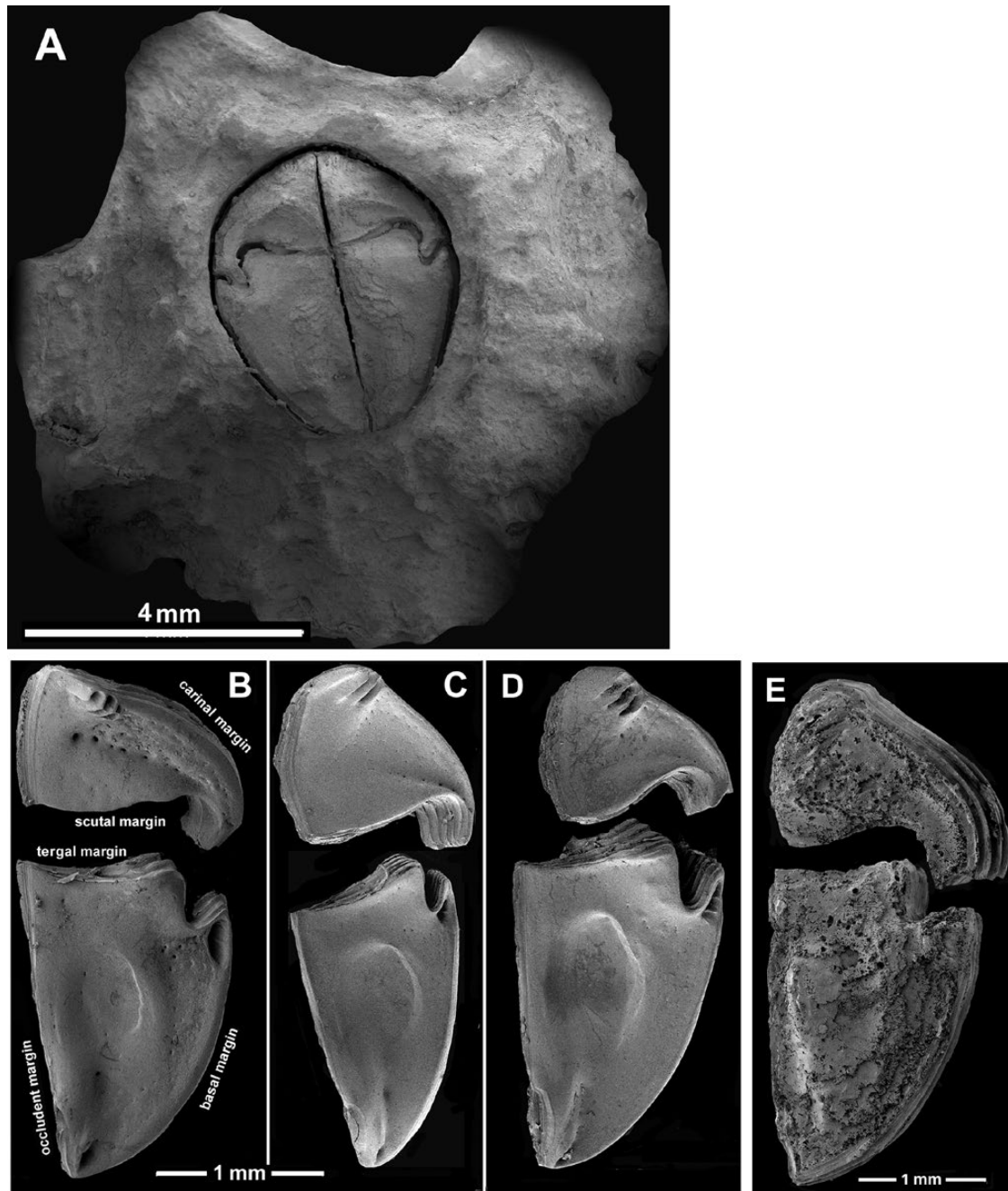
Figure 4. Shell and opercular valves. A, *Chthamalus* sp. from Praia, Cape Verde, top view. B–D, scutum and tergum inner view: B, *Chthamalus* sp. from Praia, Cape Verde; C, *Chthamalus stellatus* from Las Palmas, Gran Canaria, Canary Islands; D, *C. stellatus* from Biarritz, France. E, scutum and tergum outer view, *Chthamalus* sp. from Praia, Cape Verde.

Description: Shell low conic (Figs 1C, 4A); opercular aperture kite shaped in small specimens, circular in big specimens. Scutum (Figs 4B–D) triangular; occludent margin forms a right angle with tergal margin. Deep, round pit for adductor muscle occupies about half of width of scutum; no articular ridge. Small pit for the lateral scutal depressor muscle.

Tergum (Figs 4B–D) triangular; carinal margin arched, with no spur. At the angle between carinal and scutal margins, a projection interlocks with an indentation in the tergal margin of the scutum, close to the basal margin. Three to four small crests for tergal depressor muscle. Suture between scutum and tergum sinusoidal (Figs 1C, 4A).
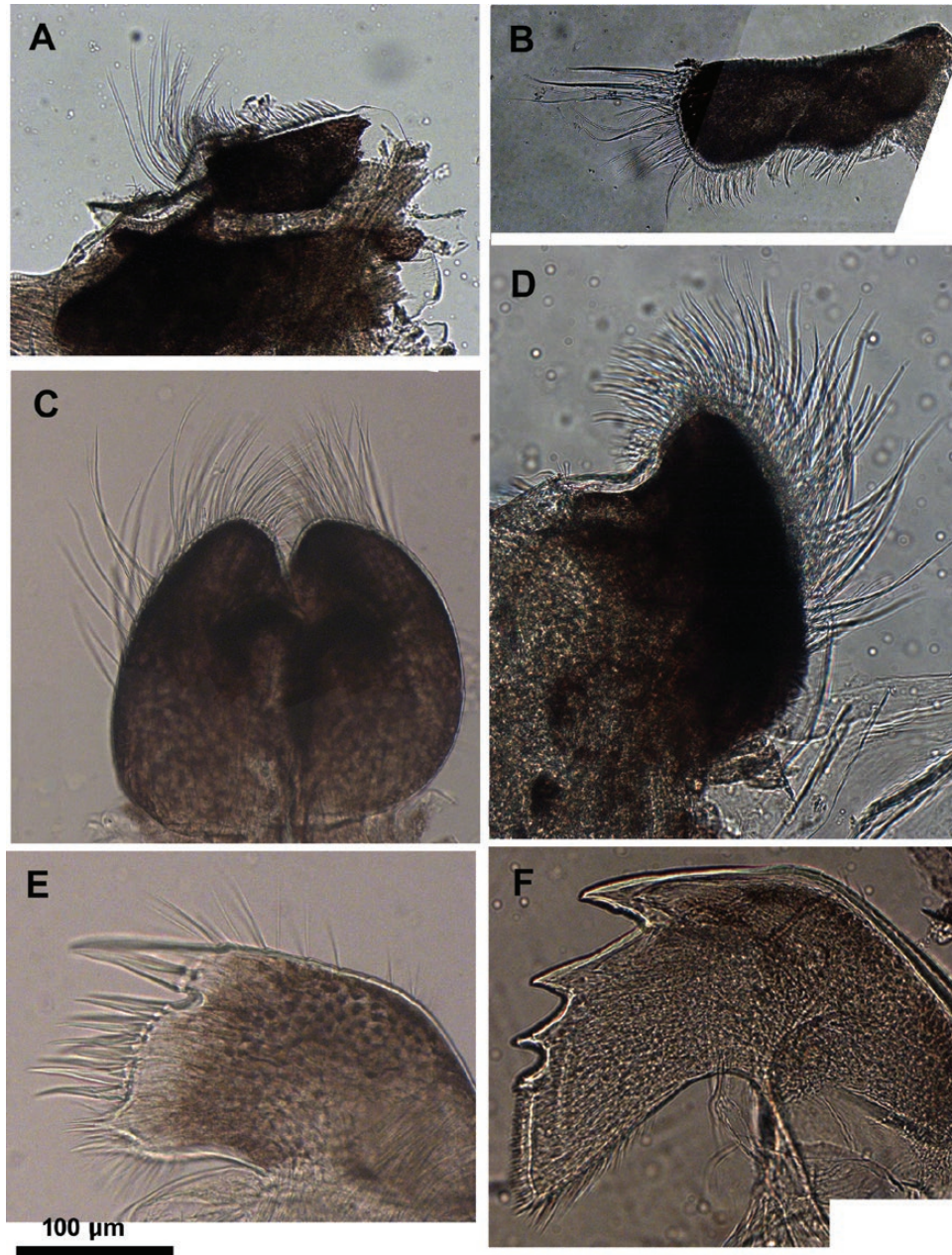
Figure 5. *Chthamalus* sp. from Praia, Cape Verde. Trophi. A, labrum and labial palp. B, Isolated labial palp. C, pair of maxillae. D, isolated maxilla. E, maxillule. F, mandible.

Labrum (Fig. 5A) has slightly concave cutting edge, with small teeth. Palpi club shaped (rectangular), with long simple setae at distal part and short on the upper margin (Fig. 5B).

Maxilla (Fig. 5C, D) bilobed; lobes round, with simple setae along interior margin and distal part. Maxillule (Fig. 5E) with two large spines at distal end followed by a notch and a series of smaller spines; stout setae at lower angle. Short, simple type of setae on surface of maxillule close to cutting edge.

Mandible (Fig. 5F) with four teeth; lower one bidentate. A series of small spines along cutting margin; at edge two bigger spines; long bristles at lower part.

Cirrus I (Fig. 6A) anterior ramus longer than posterior; segments carry simple and plumose setae. Conical spines on two proximal articles of the anterior ramus (Fig. 6F). Cirrus II (Fig. 6B) shorter than cirrus I; terminal articles with bidenticulate setae (Fig. 5G, H); in some setae, pair of 'basal guards' (Fig. 6G). Cirrus III
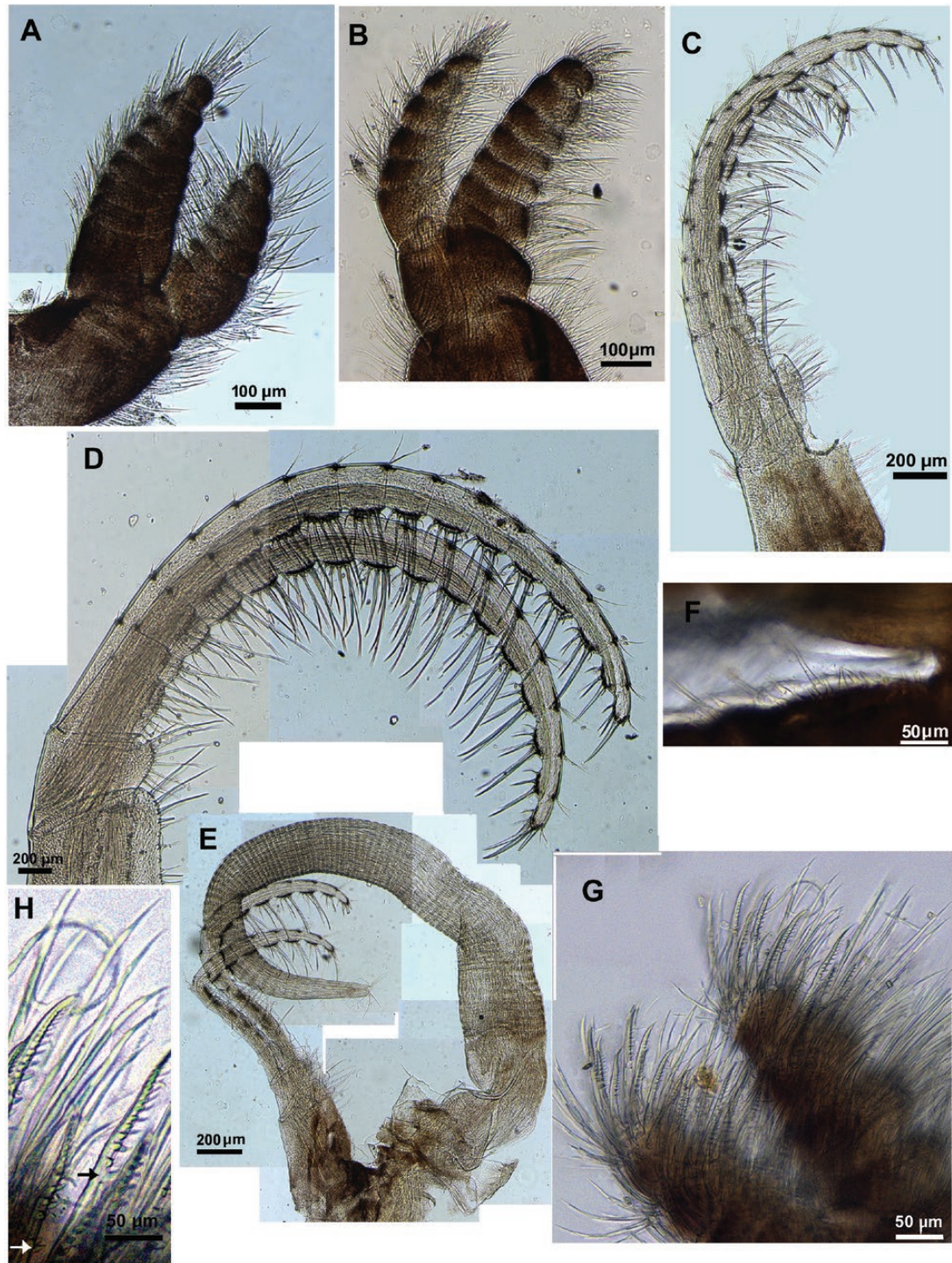
Figure 6. *Chthamalus* sp. from Praia, Cape Verde. Cirri. A, cirrus I. B, cirrus II. C, cirrus III. D, cirrus IV. E, cirrus VI and penis. F, spines on anterior ramus of cirrus I. G, terminal articles of cirrus II. H, enlargement of setae of cirrus II; arrows indicate basal guards.

anterior ramus is longer than posterior (Fig. 6C). Cirri IV–VI are similar; each segment carries four or five spines of gradual size, with rami more or less of same length. Penis annulated, with short setae scattered along the penis.

DISCUSSION

Field examination of the outer features of *Chthamalus* from the Cape Verde Islands (Fig. 1A) and the comparison with specimens known as *C. stellatus* from other localities, and the description and the illustrations by Darwin (1854) (Fig. 7) and Southward (1976) (Fig. 8), reveal that the Cape Verde Islands population should be assigned to *C. stellatus* on morphological grounds. Further examination of opercular valves (Fig. 4) shows that the morphological variability found between specimens from Cape Verde Islands and those from other localities lies within the range of variability of a population and among populations of *C. stellatus* from different localities (Fig. 9), as also presented by Southward (1976). The mouthparts are also identical to those described by Southward (1976) and depicted by Pilsbry (1916) (Fig. 10). However, it was already noted by these authors that the mouthparts are indistinguishable

within the genus *Chthatmalus* and are of limited use in the separation of species of *Chthamalus*. Diagnostic morphological criteria of *C. stellatus* are the presence of conical spines on the anterior ramus of cirrus I, and the bidenticulate setae on the terminal articles of cirrus II. These morphological features are found also in the specimens from the Cape Verde Islands. However, if a molecular analysis is performed, as carried out in the present work, a different systematic situation is revealed.

It appears that the biodiversity of barnacles is underestimated, and many barnacle species might comprise several cryptic species that form genetic lineages with more restricted ranges than the entire complex. Such cryptic species are recognized in cirripedes, including *Chthamalus*. Tsang *et al.* (2008b, 2012b) identified four genetically differentiated allopatric clades within *Chthamalus malayensis* Pilsbry, 1916. Based on differences in zonation patterns and abundances within vertical ranges, they proposed that these clades are distinct species. Comparison of *COI* in *Chthamalus moro* Pilsbry, 1916 from 14 sites in East Asian islands revealed that this nominal species comprises three distinct genetic lineages, which represent three cryptic species (Wu *et al.*, 2015). In a different genus, molecular and morphological studies of the common intertidal barnacle *Tetraclita squamosa*
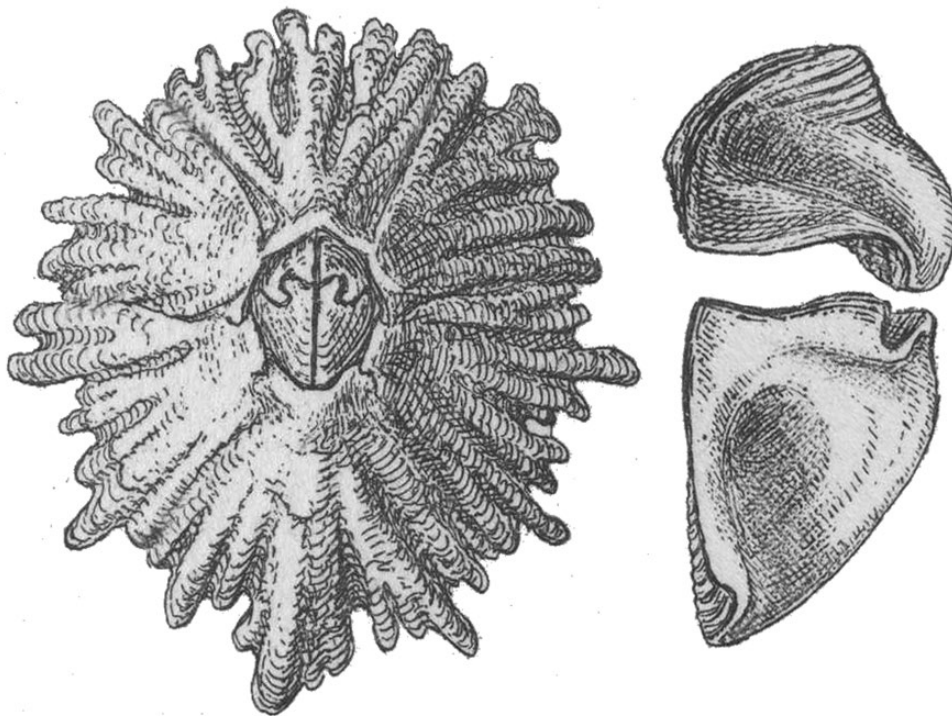

Figure 7. Shell and opercular valves redrawn from Darwin (1854: plate XVIII 1a, 1f).
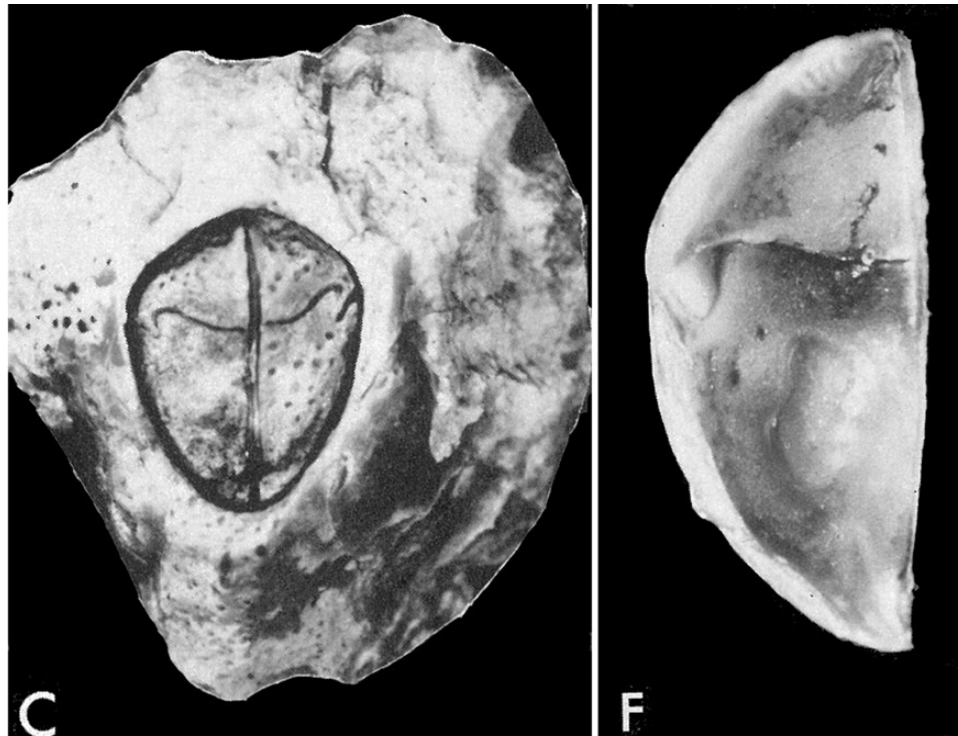
Figure 8. *Chthamalus stellatus* reproduced from Southward (1976: plate I). C, *C. stellatus* from the UK. F, interior of tergum, from the UK.

(Bruguière, 1789) have revealed three distinct species in this complex (Chan *et al.*, 2007a). In the Indo West Pacific, Chan *et al.* (2007b) asserted that the nominal species, *T. squamosa*, is a complex of cryptic species with high morphological and genetic diversity. Based on these parameters, they described one of these species as *Tetraclita singaporensis* Chan, Tsang & Chu, 2007. Likewise, *Tetraclita serrata* Darwin, 1954 consists of two evolutionarily significant units [based on mitochondrial DNA (mtDNA) analysis] along the east to west coast of South Africa. These two evolutionarily significant units could not be distinguished by morphological analysis and nuclear *H3* sequences. An additional taxon of barnacles in which the existence of cryptic species was recorded is *Hexechamaesipho pilsbryi* (Hiro, 1936). The two mitochondrial lineages, from Japan and from Taiwan, represent two cryptic species (Tsang *et al.*, 2013). The similarities in morphology within and between species of *Chthamalus*, in addition to the change of morphology with age and habitat, are a source of confusion in the taxonomy of species of *Chthamalus*. In many cases, it is not possible to separate the species in the field, especially when species are sympatric. This is the case for the three western Pacific species of *Chthamalus*, i.e. *Chthamalus challengeri* Hoek, 1883, *C. malayensis* and *C. moro*. Southward & Newman (2003) used the 'arthropodal' characters, mainly those of the setae of

cirrus II, to separate these species. Chan *et al.* (2016) emphasized the difficulty in identification of species on the shore. They used, in addition to morphology, gel electrophoresis of enzymes and the barcoding marker *COI* to separate seven species of *Chthamalus* from the coasts of the Tropical Eastern Pacific. However, two of the seven species, *C. panamensis* and *C. hedgecocki*, could not be separated by DNA barcoding.

Taxonomic species identification and delimitation have been heavily supported by DNA barcoding, using the *COI* gene, with DNA barcoding being the most important factor in species determination, because conventional morphological taxonomy is sometimes problematic and indecisive. Reliance on a single mitochondrial gene can be insufficient and even misleading. Therefore, a growing number of taxonomic studies have introduced nuclear gene-based identification (Eberle *et al.*, 2020). One limitation of DNA barcoding, namely the intrapopulation variability, can be solved by sampling many individuals. All these considerations were taken into account in our research, because we used one mitochondrial and two nuclear genes for our analysis. The number of specimens from each taxonomic group ranged from six to 236, depending on availability and level of polymorphism. For our main purpose of comparing the Cape Verde Islands *Chthamalus* with *C. stellatus*, we used the highest number of specimens:
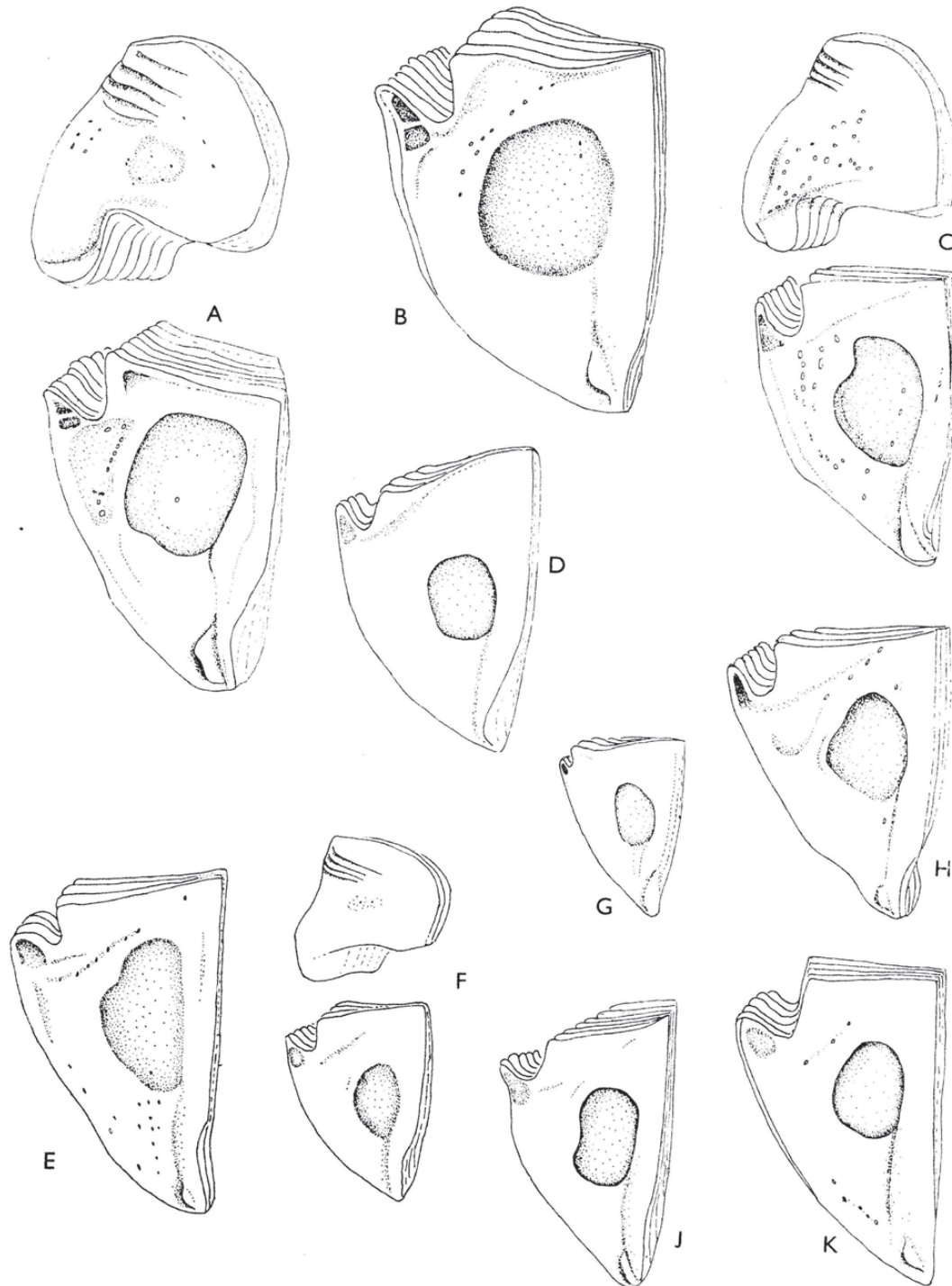
Figure 9. *Chthamalus stellatus* reproduced from Southward (1976: fig. 1). Interior views of opercular plates from different localities in the Mediterranean and East Atlantic. A, Banyuls-sur-Mer, France. B, Tantura, Israel. C, Tinside, Plymouth, UK. D, Azores, Portugal. E, F, Kerry Head, Ireland. G, Cape Verde Islands. H, Cape Cornwall, UK. J, Rame Head, Cornwall, UK. K, Brook, Isle of Wight, UK.

37 and 236 for the nuclear genes and 12 and 61 for the *COI* gene, respectively. Our results demonstrate that the Cape Verde *Chthamalus* is unique and clearly separated from the widespread *C. stellatus* species. Using various clustering algorithms to build phylogenetic trees within the *Chthamalus* genus, we
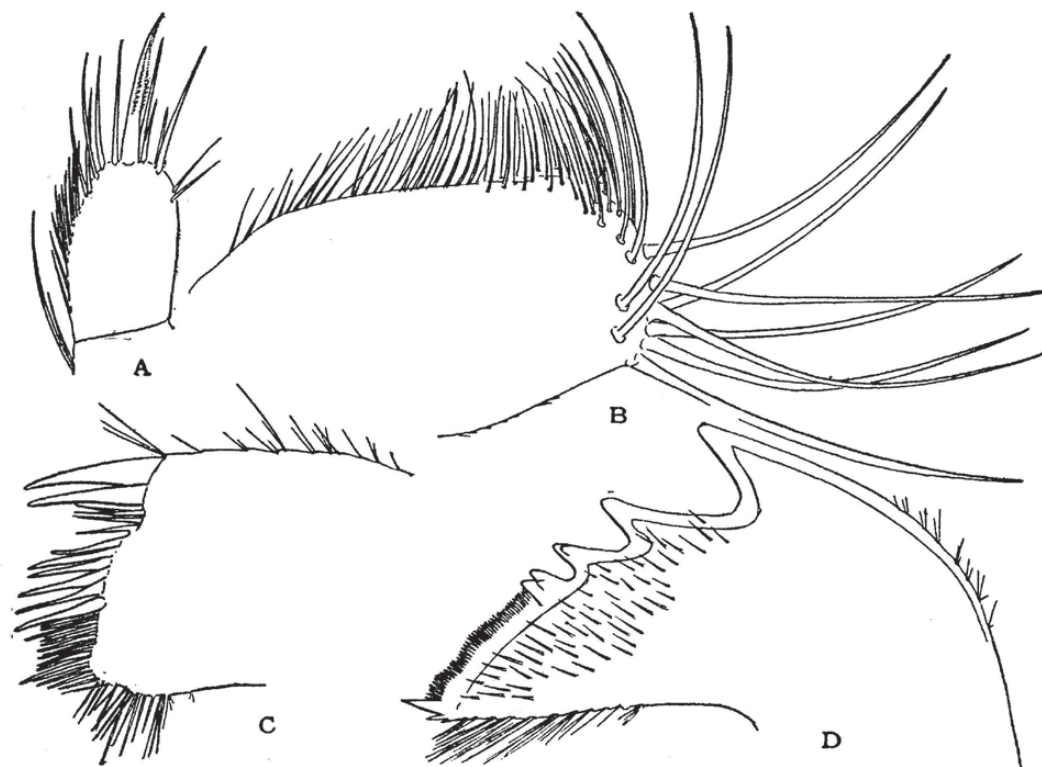
FIG. 84.—CHTHAMALUS STELLATUS, NAPLES. *a,* TERMINAL SEGMENT OF THE SHORTER RAMUS OF CIRRUS II, SHOWING ONE LANCEOLATE, SERRATE SPINE. *b,* PALPUS. *c,* MAXILLA. *d,* MANDIBLE.

Figure 10. *Chthamalus stellatus* reproduced from Pilsbry (1916: fig. 84, p. 303). A, terminal article with denticulate setae of cirrus II. B, mandibular palpus. C, maxillule. D, mandible.

always see that the separation between the Cape Verde Islands population and *C. stellatus* is greater than the differences separating *C. hedgecocki*, *C. panamansis* and *C. alani*, and within the range separating *C. fissus* and *C. proteus* from all other *Chthamalus* species. The evident genetic differences in the three markers leads us to conclude that *Chthamalus* from Cape Verde Islands is an independent evolutionarily significant unit. Given that the consensus sequences of the *COI* mtDNA gene of the Cape Verde *Chthamalus* and *C. stellatus* are 57 point mutations apart, a completely different maternal lineage is apparent.

To exclude the possibility that the Cape Verde *Chthamalus* is merely a unique population of *C. stellatus*, we used the genomic DNA to compare the variability between the Cape Verde population and ten geographically different *C. stellatus* populations from the East Atlantic and the Mediterranean Sea. For each of the two nuclear genes, *EF1* and *NaKA*, we calculated the squared Euclidean pairwise distance between the 11 samples (the Cape Verde and the ten *C. stellatus* populations). We then averaged the distances of the two genes to obtain a single distance matrix

(Supporting Information, Appendix S2) and carried out a principal coordinates analysis (using the MVSP software; Kovach Computation Services, 2013; see Fig. 11). It turns out that the mean distance between the Cape Verde samples and each of the *C. stellatus* samples is 0.2239, whereas the mean pairwise distance among the *C. stellatus* samples is merely 0.0185 (~12 times smaller). Moreover, the minimal distance between Cape Verde and *C. stellatus* is 0.211 (between Cape Verde and Bodrum, Turkey); much larger than the maximal distance among *C. stellatus*, which is 0.0520 (between Madeira and Fažana, Croatia). It is therefore obvious that the Cape Verde *Chthamalus* is a unique taxonomic unit, different from all studied *C. stellatus* populations across the Mediterranean and the relatively neighbouring locations of the Atlantic shores and islands.

The four archipelagos of the Eastern Atlantic Islands (Madeira, the Canaries, the Azores and Cape Verde Islands) are commonly regarded as a single zoogeographical unit, Macaronesia. This unit is known for its extraordinarily high levels of species diversity and endemism in both terrestrial and marine realms.
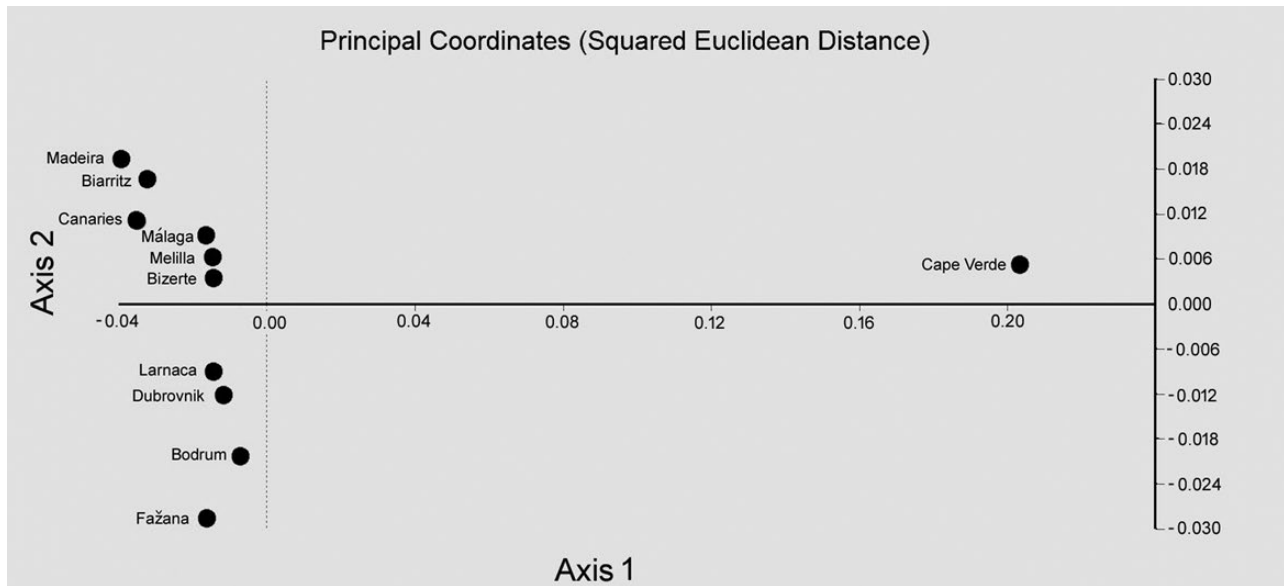
Figure 11. A minimum variance principal coordinates analysis, based on the nuclear *EF1* and *NaKA* genes, demonstrating the relationship between Cape Verde and ten other populations of *Chthamalus stellatus*.

Among the four archipelagos, the Cape Verde Islands has the highest level of zoological endemism. Using molecular markers, Cunha *et al.* (2005) showed that of the 52 species of the gastropod *Conus* Linnaeus, 1758 in Cape Verde Islands, only three are non-endemic. They suggested that in the case of Cape Verdean *Conus*, it is the absence of pelagic larval stages that limits the dispersal and is the major factor contributing to diversification of *Conus* in these islands. Cunha *et al.* (2017) also studied endemism of the keyhole limpets *Fissurella* Reeve, 1849 and *Diodora* Gray, 1857 in the Cape Verde Islands. Mitochondrial and nuclear markers indicate the existence of seven endemic species of *Fissurella* and one of *Diodora*. Freitas *et al.* (2019) used representatives of six marine groups with different dispersal abilities. They concluded that Cape Verde should be excluded from the other Macaronesian Islands and should be given the status of a biogeographical subprovince within the West African Islands. These authors compiled data for 3737 marine species representing six marine groups with different dispersal abilities. They suggest that the patterns of circulation of the tropical East Atlantic currents, particularly the Azores Current and the Madeira Current, provide a plausible sea route for the dispersal of shallow-water marine organisms from the Azores to the Canary Islands. These currents help to elucidate their role as biogeographical filters, because gene flow depends on the dispersal capacity of each organism. Moreover, the Cape Verde Front, located north of the Cape Verde archipelago, functions as an important biogeographical barrier for the dispersal of marine organisms, thus isolating the Cape Verde Islands from the other Macaronesian archipelagos.

Water circulation is an important agent in mixing populations within marine basins, but on the contrary, creates a barrier that contributes to differentiation between populations. A well-documented model of such a barrier is the Almeria–Oran Front, in the Alboran Sea that separates Mediterranean and East Atlantic populations (Patarnello *et al.*, 2007). The Almeria–Oran Front is a transition zone showing step changes of allele frequencies within populations of the same species. Pannacciulli *et al.* (1997) demonstrated that there is a genetic differentiation to Atlantic and Mediterranean populations in both *C. montagui* and *C. stellatus*, but the degree of differentiation is smaller in *C. stellatus* owing to the longer life of the pelagic larvae of this species (~22 days), enabling a larger dispersal range. The larger larval dispersal was also suggested by Pannacciulli *et al.* (2009) in order to explain the higher genetic connectivity found in *C. stellatus* when compared with *Tesseropora atlantica*. In a series of studies, Chan *et al.* (2007a, b) and Tsang *et al.* (2008a, 2012a) demonstrated that the distribution of species of *Tetraclita* depends on oceanographic parameters. In the West Indian Ocean, the allopatric distribution of six species of *Tetraclita* reflects the oceanographic conditions of this area (Tsang *et al.*, 2008a, 2012a). They suggested that the system of currents and the fronts between currents creates barriers that restrict the transfer of the pelagic larvae and, in addition, it facilitates larval transport within each system and is the major factor in maintaining

genetic and phenotypic differentiation between these populations. Likewise, Chan *et al.* (2009) showed that *Tetraclita reni* Chan, Hsu & Tsai, 2009 is restricted to the eastern coast of Madagascar and Mauritius and suggested that larvae of *Tetraclita reni* do not disperse across the Mozambique Channel to the East African coast, owing to the anti-cyclonic eddies of the Agulhas Current in the Mozambique Channel.

We might suggest that the *Chthamalus* population of the Cape Verde Islands is partly isolated from the other Macaronesian populations to the north by the Cape Verde Front, which is part of the Canary Current (Fig. 12). This current branches from the North Atlantic and flows south-west as far as Senegal, where it turns west and later joins the Atlantic North Equatorial Current. Another possible explanation is that the Cape Verde archipelago partly blocks the flow of the Canary Current. This might create a genetic barrier between the *Chthamalus* population of the Cape Verde archipelago and the northern populations, leading to the formation of a new taxonomic unit.

Another possible explanation for the genetic differentiation is that the Cape Verde Islands served as glacial refugia. During the Pleistocene, a series

Figure 12. Scheme illustrating the main geographical features and mean oceanographic surface currents (italics), drawn from Pelegrí & Peña-Izquierdo (2015), © UNESCO, http://hdl.handle.net/1834/9135.

of glacial and interglacial periods, with associated marine regressions and transgressions, affected the area (Blanc, 1968). Atlantic, Mediterranean and offshore island populations of *Chthamalus* spp. might have become physically isolated during these cycles and differentiated genetically (Dando & Southward, 1981). The refugia hypothesis also explains the genetic differentiation in the presently disconnected populations of *C. malayensis* (Tsang *et al.*, 2008b, 2012b) and of *C. moro* (Wu *et al.*, 2015). Nevertheless, in *C. challengeri* there is no significant population differentiation (Cheang *et al.*, 2012), suggesting that this species has effective larval dispersal, which would have facilitated expansion from glacial refugia.

Research into speciation processes has gained practical support from DNA sequencing, changing some of the more established theories, especially when time frames are considered. The variation of a population before splitting into two distinct species seems greatly to influence the predicted mode of speciation and the time scale (Marques *et al.*, 2019). Henning & Meyer (2014) showed that different cichlid fishes, known for their extraordinarily fast speciation, share extensive older DNA polymorphisms. In our study, 18 of the 57 *COI* mtDNA point mutations separating the Cape Verde consensus sequence from *C. stellatus* were found as the minor haplotype in the different *C. stellatus* samples, compared with zero in all other *Chthamalus* species that we have checked. This might reflect not only the close connection between the two taxonomic units, but also the mode of speciation maintaining pre-split polymorphism as variation in both. Given that each female transfers a population of mtDNA molecules to the next generation, and heteroplasmy is common (Tikochinski *et al.*, 2020), the size of the founding group cannot be estimated accurately. Genomic DNA inheritance, in contrast, involves transfer of one molecule from parent to offspring. In the two nuclear markers that we used, we have found 21 point mutations separating the Cape Verde consensus from *C. stellatus*, with only one leading to an amino acid change (glycine to asparagine). Twelve of these null mutations were found as minor alleles in different *C. stellatus* samples. This serves as a better indication of a speciation event starting with an already polymorphic population. In order to obtain a better estimation of the described event and the founding population size, other considerations, such as mutation rate, must be taken into account, and higher numbers of individuals and genetic markers should be used.

On the basis of the morphological characters of both the hard parts and the soft parts, it is not possible to distinguish the *Chthamalus* from the Cape Verde Islands from that of the East Atlantic and the Mediterranean. However, the molecular data indicate
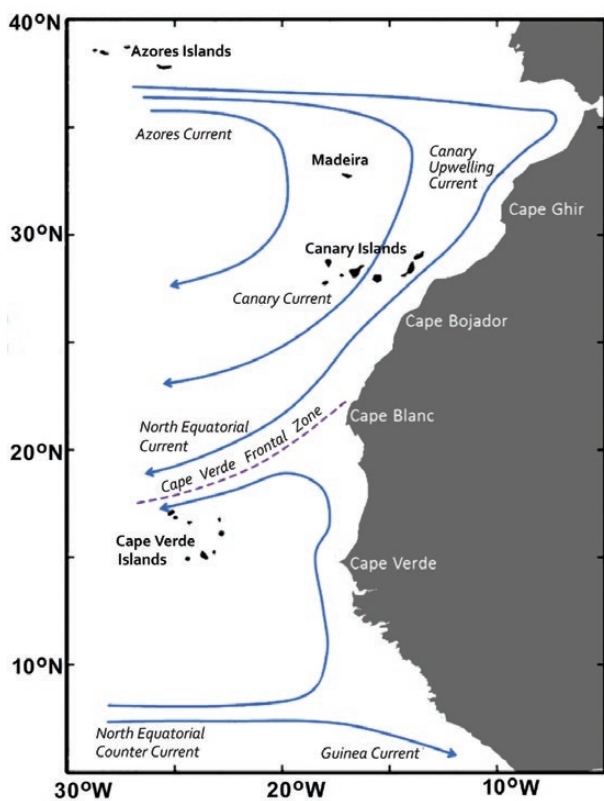
that the population of *C. stellatus* from the Cape Verde Islands is a geographically separated, evolutionarily significant unit that is a sister clade to *C. stellatus*. The genetic distance found between this evolutionarily significant unit and the other populations of *C. stellatus* is similar to, or even greater than that found between valid species of *Chthamalus*. In view of our results, we suggest that we have found another Cape Verde endemic taxonomic unit, a *Chthamalus* cf. *stellatus*, sharing a common ancestor with *C. stellatus*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Table with GenBank accession numbers used for the molecular analysis.
Appendix S2. Distance matrices.
Appendix S3. A dendrogram illustrating the hierarchical relationship between ten *Chthamalus* populations, based on the *COI* gene. The dendrogram was constructed by Ward's method (squared Euclidean distance with minimum variance amalgamation).