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# Species identification of the psammophilous tenebrionid beetles *Phaleria acuminata* Küster, 1852 and *Phaleria bimaculata* (Linnaeus, 1767) from central Mediterranean beaches: geometric morphometrics and molecular insights from species to population level

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**Abstract** Dominating global arid environments, from desert to coastal dunes, most Tenebrionidae are highly specific in their habitat preferences and display limited dispersal potential, thus exhibiting a remarkable degree of regional genetic and morphological differentiation. The tenebrionid genus *Phaleria* is speciose and widely distributed, with *P. acuminata* and *P. bimaculata* having a wide Mediterranean distribution, with numerous morphological differentiations at population level, often described as different taxa of doubtful taxonomical significance. In order to investigate the variability of the central Mediterranean populations of *P. bimaculata* and *P. acuminata* and to compare the results obtained with different identification techniques, these species were sampled on sandy beaches in Sicily (southern Italy) and on circum-Sicilian and Maltese islands. Collected samples were studied through the application of geometric morphometrics and the sequencing of a fragment of the mitochondrial COII gene. Geometric morphometrics and molecular analyses gave congruent results, allowing a sound separation of the two species. At the population level, the two species showed different patterns. *P. acuminata* showed a remarkable morphological and molecular homogeneity throughout the sampled area. Conversely, two well-characterized subclades were detected within *P. bimaculata*, and within the

two lineages, a low-to-absent inter-populations differentiation was observed, in spite of the physical isolation of the sampled sandy beaches and of their geographical distance. These two *P. bimaculata* lineages, hereby named "Tyrrhenian sub-clade" and "Southern sub-clade," might be compatible with the hypothesis of subspecific status already proposed for the populations from the Aeolian archipelago (as *P. bimaculata marcuzzii* Aliquò).

**Keywords** Sandy beaches · *Phaleria* spp. · Central Mediterranean · Species delimitation · Molecular systematics · Geometric morphometrics

## Introduction

The Mediterranean Sea is a basin that, with its noteworthy environmental diversity along coastlines, can be considered "a great natural laboratory" where to measure and assess macroscopic and cryptic variability. The biocenoses inhabiting the coastal habitats are subject to various forms of environmental pressures, and their study is assuming greater importance. In the face of an increasing pressure due to anthropogenic activities and climate changes, there is an unrelenting search for reliable target species for assessing and monitoring biodiversity (e.g., Lo Brutto et al. 2011; Sarà et al. 2012); accordingly, a sound taxonomic identification of the target taxa, as well as information on their morphological and genetic diversity, is certainly necessary.

The coleopteran family Tenebrionidae dominates the arid habitats of the world, from deserts to coastal dunes (Fallaci et al. 1997). Members of this family play an important role in the food chain of sandy littoral systems since they contribute substantially to the consumption of

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beach debris (Colombini et al. 1994). In view of their limited dispersal capacity and their high habitat specificity, darkling beetles also exhibit a noteworthy degree of endemicity, as reported in several studies, including the ones by Contreras-Diaz et al. (2003) for tenebrionid species from the Canary Islands and by Fattorini and Leo (2000a) for the Aegean Islands.

The tenebrionid beetles *Phaleria bimaculata* and *P. acuminata* belong to a speciose and a widely distributed psammophilous genus; *P. bimaculata* (Linnaeus, 1767) has a wide Mediterranean distribution (Löbl and Smetana 2008) with morphologically distinct populations that have been often described as new taxa of doubtful taxonomical meaning (e.g., Canzoneri 1968; Marcuzzi 1996; de Jong 2010). The congener *P. acuminata* Küster, 1852 is distributed along the sandy coastlines of the Mediterranean (Löbl and Smetana 2008), Bulgaria, and the Black Sea (Dajoz 1984). Though the two species show overlapping ranges, they have not been compared using genetic and morphometric methods to date. Their genetic diversity is nearly unknown, and studies to identify molecular diagnostic characters have never been performed. Unfortunately, no phylogeny of the genus *Phaleria* is currently available, and there is thus no evidence supporting the sister-species relationship between *P. acuminata* and *P. bimaculata*.

The two studied species (*P. acuminata* and *P. bimaculata*) have been recorded in large numbers from Maltese (Deidun et al. 2007, 2009) and Sicilian beaches (Canzoneri 1968; Aliquò and Leo 1997–1998; Fattorini and Leo 2000b; Deidun et al. 2011), while a third species, *P. reveilleri* Mulsant and Rey 1858, which is sometimes reported to occur in Sicily (e.g., Canzoneri 1968; but see also Aliquò and Soldati 2010), was not recorded in the frame of our surveys. *P. bimaculata* and *P. acuminata* have been rarely recorded in syntopy (e.g., Mifsud and Scupola 1998; Deidun et al. 2010), and they demonstrate slightly different eco-ethological characteristics. They seem to occupy different beach zones (Deidun et al. 2010) and have different grain-size preferences: *P. acuminata* shows a predilection for fine-sand beaches, and *P. bimaculata* prefers coarser sand (in some extreme cases, *P. bimaculata* is also found on cobble beaches, e.g., Canzoneri 1968; Gardini 1975; Minelli et al. 2002).

From a taxonomic perspective, the *P. acuminata* populations from the central Mediterranean area show a scarce differentiation, being somehow consistent and homogenous in terms of morphology of exoskeleton characters (Canzoneri 1968; Aliquò and Soldati 2010).

Conversely, the *P. bimaculata* populations occurring within the same geographical area demonstrate a high degree of polymorphism, with several populations already being recognized as distinct taxa of intra-specific rank

(subspecies, "races," "nationes," cf. Canzoneri 1968), or at least described as such (Canzoneri 1968). One such example is the subspecies *P. bimaculata marcuzzii* Aliquò 1993, which was described for the Aeolian Islands (Fig. 1), with its *locus typicus* located on the island of Vulcano (Aliquò 1993). The *P. bimaculata* populations from the Aegadian Islands are also considered different from the nominal subspecies by Canzoneri (1970), but they have not been formally described as such.

The results of a geometric preliminary morphometric analysis of different *P. bimaculata* populations from the Maltese and Sicilian archipelagos, reported in Deidun et al. (2011), confirmed the high degree of inter-populations morphological variability within *P. bimaculata* from such a geographical region. The results proved to be compatible with the possible subspecific taxonomic status of the Aeolian *P. bimaculata* populations (i.e., *P. bimaculata marcuzzii*). In light of these results, Deidun et al. (2011) recommended the inclusion of a higher number of *Phaleria* populations in the geometric morphometric analyses, and the implementation of molecular techniques to test and corroborate the results of both traditional morphology and geometric morphometry analyses. Following these guidelines, the primary aims of this paper are (1) to describe the morphological and molecular diversity of the central Mediterranean populations of two widespread *Phaleria* species and (2) to compare the accuracy and consistency of different identification techniques, i.e., traditional morphology, molecular identification, and geometric morphometry. Finally, the study also aims (3) to gauge the putative degree of compartmentalization along close beaches through the characterization of the within-species and between-species variation of *P. bimaculata* and *P. acuminata* sampled over different spatial scales.

## Materials and methods

### Sampling

A total of 25 *Phaleria* spp. populations from sandy beaches on different central Mediterranean archipelagos (Maltese, Pelagian, Aegadian, and Aeolian ones) and on the Sicilian mainland were sampled once, over the March–October 2010 period (Fig. 1). The full list of sampled sandy beaches is given in Table 1, while Fig. 1 gives the geographical location of the sampled sites. The supralittoral zone of these beaches was sampled by means of individual pitfall traps or by means of constellations of pitfall traps, each of which consisted of five plastic cups (diameter = 7.5 cm) buried with their mouth flush with the surface of the sand and connected by means of thin wooden walkways. Such walkways
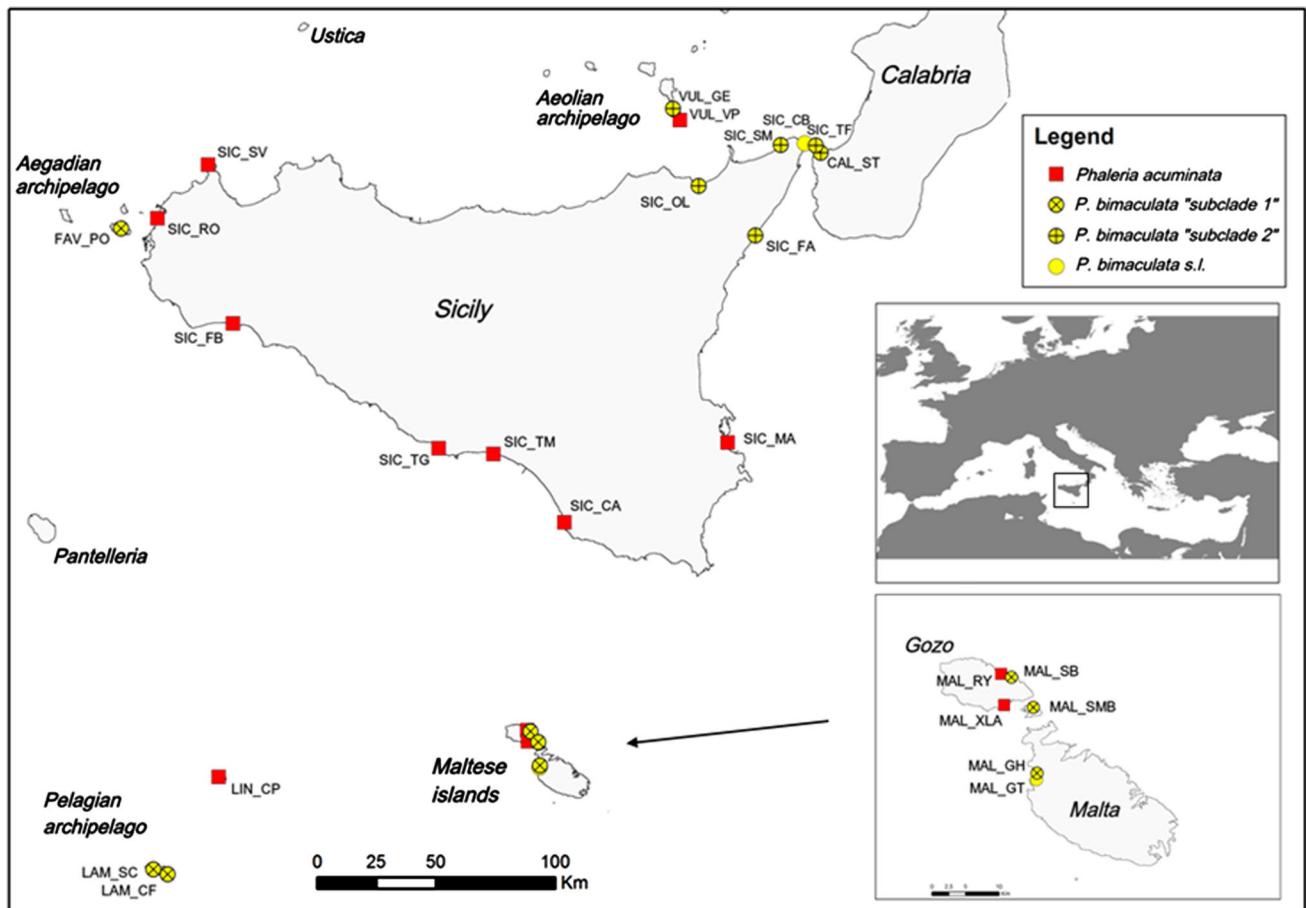
**Fig. 1** Map of the studied *Phaleria* spp. populations. The locality codes are those reported in Table 1. The two *P. bimaculata* sub-clades are those highlighted by the molecular analyses

increase the efficiency of the traps as they divert any wandering animal that makes contact with the walkways into the traps (L. Chelazzi, personal communication, see Gauci et al. 2005). Where substrate type (e.g., too coarse a sediment) or beach attributes (e.g., too narrow a beach) precluded the deployment of pitfall trap constellations, individual, non-connected traps (plastic cups) were used instead. A mixture of freshwater and vinegar (3:1) was placed in each trap as an attractant, with the traps being deployed at dusk and emptied at dawn. Collected *Phaleria* spp. specimens were sorted out and fixed in situ in 95 % ethanol.

Identification of the collected specimens was carried out under a stereomicroscope according to the morphological characteristics described by Canzoneri (1968). When necessary, selected specimens were dissected in order to study the form of the aedeagus.

DNA extraction, amplification, and sequence analyses

Prior to DNA extraction, heads and legs of selected specimens (Table 1) were soaked in double-distilled water for

2–3 h. DNA was then extracted using whole specimens and following the "DNEasy—Animal Tissue Kit" (QIAGEN) protocol.

A fragment of the cytochrome c oxidase subunit II mitochondrial gene (COII) was amplified using the primer pairs described by Contreras-Diaz et al. (2003) and the following thermal cycle: 5′ at 95 °C followed by 35 cycles with 1′ at 95 °C, 1′ at 50 °C, and 1′ at 72 °C; a final extension of 8′ at 72 °C concluded the PCR. The PCR mix consisted of 17.63 µl of double-distilled water, 2.5 µl Buffer 10×, 2 µl MgCl$_2$ solution (25 mM), 0.12 µl dNTPs (20 mM), 0.25 µl of each primer (25 µM), 0.25 µl *Taq* polymerase 5u/µl, and 2 µl of DNA template, for a total reaction volume of 25 µl.

After PCR, 5 µl of each PCR product was separated by electrophoresis on a 2 % agarose gel at 70 V for 1 h and visualized with a UV Transilluminator. When PCR products showed a clear and single band of the correct length, they were purified using the Exo-SAP-IT kit and sequenced with an ABI 3130xL (Applied Biosystems) sequencer. The forward primer was used for direct sequencing of the PCR product, and if the sequences were not of sufficient quality,

**Table 1** List of the studied populations and of the specimens included in the molecular and morphometrical analyses

| Site code | Locality | Coordinates (WGS84) | Taxon | # of specimens studied for the elytra | # of specimens studied for the pronotum | # of specimens included in the molecular analyses |
|---|---|---|---|---|---|---|
| CAL_ST | Calabria–Santa Trada | 38.242210 N; 15.673196 E | PB2 | 3 | 3 | 1 |
| FAV_PO | Favignana–Port | 37.928888 N; 12.325555 E | PB1 | 10 | 10 | 2 |
| LAM_CF | Lampedusa–Cala Francese | 35.495555 N; 12.624722 E | PB1 | 0 | 0 | 1 |
| LAM_SC | Lampedusa–Spiaggia dei Conigli | 35.513055 N; 12.557222 E | PB1 | 15 | 14 | 1 |
| LIN_CP | Linosa–Cala di Ponente | 35.867222 N; 12.851111 E | PA | 0 | 0 | 1 |
| MAL_GH | Malta–Ghajn Tuffeha | 35.928888 N; 14.343888 E | PB1 | 0 | 0 | 2 |
| MAL_GT | Malta–Gnejna Bay | 35.920278 N; 14.343055 E | PB | 15 | 13 | 0 |
| MAL_RY | Gozo–Ramla beach | 36.061388 N; 14.284166 E | PA | 11 | 10 | 1 |
| MAL_SB | Gozo–San Blas | 36.056944 N; 14.300833 E | PB1 | 0 | 0 | 1 |
| MAL_SMB | Comino–S. Marija Bay | 36.016666 N; 14.337222 E | PB1 | 15 | 8 | 1 |
| MAL_XLA | Gozo–Xatt l-Ahmar | 36.019795 N; 14.289272 E | PA | 16 | 17 | 0 |
| SIC_CA | Sicily–Camarina | 36.847222 N; 14.452500 E | PA | 15 | 15 | 1 |
| SIC_CB | Sicily–Casa Bianca | 38.279253 N; 15.595860 E | PB | 15 | 14 | 0 |
| SIC_FA | Sicily–Foce dell'Agrò | 37.931388 N; 15.357222 E | PB2 | 4 | 4 | 1 |
| SIC_FB | Sicily–Foce del Belice | 37.581388 N; 12.870277 E | PA | 3 | 3 | 2 |
| SIC_MA | Sicily–Magnisi | 37.150138 N; 15.223411 E | PA | 0 | 0 | 1 |
| SIC_OL | Sicily –Oliveri | 38.120000 N; 15.086111 E | PB2 | 15 | 15 | 2 |
| SIC_RO | Sicily–Ronciglio | 37.971008 N; 12.497649 E | PA | 0 | 0 | 1 |
| SIC_SM | Sicily–Sindaro Marina | 38.272562 N; 15.481321 E | PB2 | 12 | 13 | 1 |
| SIC_SV | Sicily–San Vito | 38.178396 N; 12.733155 E | PA | 0 | 0 | 1 |
| SIC_TF | Sicily–Torre Faro | 38.270733 N; 15.648744 E | PB2 | 9 | 9 | 1 |
| SIC_TG | Sicily–Torre Gaffe | 37.122500 N; 13.855000 E | PA | 13 | 13 | 1 |
| SIC_TM | Sicily–Torre Manfria | 37.103055 N; 14.115277 E | PA | 15 | 15 | 1 |
| VUL_GE | Vulcano–Gelso beach | 38.368888 N; 14.995833 E | PA | 15 | 15 | 2 |
| VUL_VP | Vulcano–Ponente beach | 38.411666 N; 14.963611 E | PB2 | 15 | 15 | 1 |

PA, *Phaleria acuminata*; PB, *P. bimaculata* s.l. (no molecular data available); PB1, *P. bimaculata* "Southern sub-clade"; PB2, *P. bimaculata* "Tyrrhenian sub-clade"

the complement/reverse sequences were obtained additionally.

Chromatograms were imported and edited with Chromas Lite 2.01 (Technelysium Pty Ltd) and exported to be aligned with ClustalX (Thompson et al. 1997). The sequences were deposited in GenBank with the accession numbers (A.N.) JX982338–JX982365 (Table 2). A COII sequence of the tenebrionid beetle *Nesotes helleri* (Reitter 1922) was downloaded from GenBank (A.N.: AJ299304.1) to be used as out-group in the molecular analyses.

Bayesian (BA) and maximum likelihood (ML) analyses were performed as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) and PhyMl v.3 (Guindon et al. 2010). For both analyses, the best evolutionary model for each dataset was selected using mrModeltest (Nylander 2004). According to the Akaike Information Criterion (AIC), the best-fit model for ML analysis proved to be a general time-reversible plus invariant sites model (GTR + I); as a measure of branch support, bootstrap

values were calculated with 1,000 replicates in the ML trees. BA analysis was carried out with the following settings: ngen = 500,000 nchains = 4 nrun = 2 sample = 500 temp = 0.1; default priors; the best BA parameters selected by AIC were the following: Prset statefreqpr = dirichlet (1,1,1,1); Lset nst = 6 rates = propinv. The node stability of BA trees was evaluated from their posterior probabilities.

Geometric morphometrics

The study involved the use of the geometric morphometrics technique, which is nowadays a standard protocol in morphological research. The geometric morphometrics techniques (Bookstein 1991; Rohlf and Marcus 1993; Adams et al. 2004; Zelditch et al. 2004; Viscosi and Cardini 2011) were used to quantify and analyze the inter- and intraspecific differentiation in the shape of two external anatomical structures in the collected *Phaleria* spp. specimens,

**Table 2** GenBank accession numbers of the studied specimens

| Site code | Specimen code | Taxon | GenBank accession numbers |
|---|---|---|---|
| CAL_ST | CAL_ST_281 | PB2 | JX982340 |
| FAV_PO | FAV_PO_1 | PB1 | JX982350 |
| FAV_PO | FAV_PO_2 | PB1 | JX982351 |
| LAM_CF | LAM_CF_284 | PB1 | JX982361 |
| LAM_SC | LAM_SC_256 | PB1 | JX982364 |
| LIN_CP | LIN_CP_285 | PA | JX982354 |
| MAL_GH | MAL_GH_11 | PB1 | JX982360 |
| MAL_GH | MAL_GH_6 | PB1 | JX982344 |
| MAL_RY | MAL_RY_283 | PA | JX982341 |
| MAL_SB | MAL_SB_188 | PB1 | JX982342 |
| MAL_SMB | MAL_SMB_308 | PB1 | JX982343 |
| SIC_CA | SIC_CA_8 | PA | JX982345 |
| SIC_CB | SIC_CB_307 | PB2 | JX982362 |
| SIC_FA | SIC_FA_1 | PB2 | JX982363 |
| SIC_FB | SIC_FB_1 | PA | JX982348 |
| SIC_FB | SIC_FB_5 | PA | JX982349 |
| SIC_MA | SIC_MA_305 | PA | JX982356 |
| SIC_OL | SIC_OL_1 | PB2 | JX982358 |
| SIC_OL | SIC_OL_253 | PB2 | JX982359 |
| SIC_RO | SIC_RO_304 | PA | JX982355 |
| SIC_SM | SIC_SM_306 | PB2 | JX982365 |
| SIC_SV | SIC_SV_303 | PA | JX982353 |
| SIC_TF | SIC_TF_282 | PB2 | JX982347 |
| SIC_TG | SIC_TG_2 | PA | JX982352 |
| SIC_TM | SIC_TM_5 | PA | JX982346 |
| VUL_GE | VUL_GE_1 | PA | JX982357 |
| VUL_GE | VUL_GE_309 | PA | JX982339 |
| VUL_VP | VUL_VP_2 | PB2 | JX982338 |

PA, *Phaleria acuminata*; PB1, *P. bimaculata* "Southern sub-clade"; PB2, *P. bimaculata* "Tyrrhenian sub-clade"

i.e., the pronotum and the elytra. Statistical variations in the shape of the pronotum and of the right elytra in different *P. bimaculata* and *P. acuminata* individuals were analyzed using multivariate statistics.

The pattern of morphological body-shape variation was analyzed in eighteen *Phaleria* spp. populations. Analyses were performed on 206 pronota and 216 elytra (Table 1). Head and limbs from sampled specimens were dissected to be used for the DNA extraction and subsequent molecular analyses.

After dissection, the remaining body parts were mounted on an entomological card. The specimens were positioned along a horizontal plane; for each individual, only the right portion of the body was examined, with the aim of removing any possible bias caused by bilateral asymmetry. Dorsal images were digitized using a Leica D-LUX 3 ≪LMS≫ camera mounted on the optical stereomicroscope Wild M3.

The digital images of the pronotum and the elytra were processed separately with MAKEfAN6 (Sheets 2003). For each body structure, the *landmarks* and *semi-landmarks* configuration was identified following criteria of homology (Bookstein 1991). Within the pronotum, the Cartesian x, y coordinates of four *landmarks* and six *semi-landmarks* were recorded. In the elytra, the Cartesian x, y coordinates of four *landmarks* and twelve *semi-landmarks* were recorded.

The position of the pronotum *landmarks* and *semi-landmarks* adopted in this study is shown in Fig. 2a, while the position of elytra *landmarks* and *semi-landmarks* is shown in Fig. 2b.

The bidimensional coordinates of the anatomical *landmarks* and *semi-landmarks* on the outline of the dorsal view of the pronotum and the right elytra were collected and digitized by means of TPSDIG2 (Rohlf 2004). In order to better perform statistical analysis, the *landmarks* and *semi-landmarks* were successively recognized with TPSUTIL 1.45 (Rohlf 2008).

**Fig. 2** *Landmarks* and *semi-landmarks* positioning on *Phaleria* specimens. **a** Four *landmarks* (1, 3, 9, and 10) and six *semi-landmarks* for pronotum (2, 4, 5, 6, 7, and 8); **b** four *landmarks* (1, 2, 3, and 16) and twelve *semi-landmarks* (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15) on the right elytra. The *landmarks* were digitized on half of each structure to remove the variability introduced by the possible asymmetry
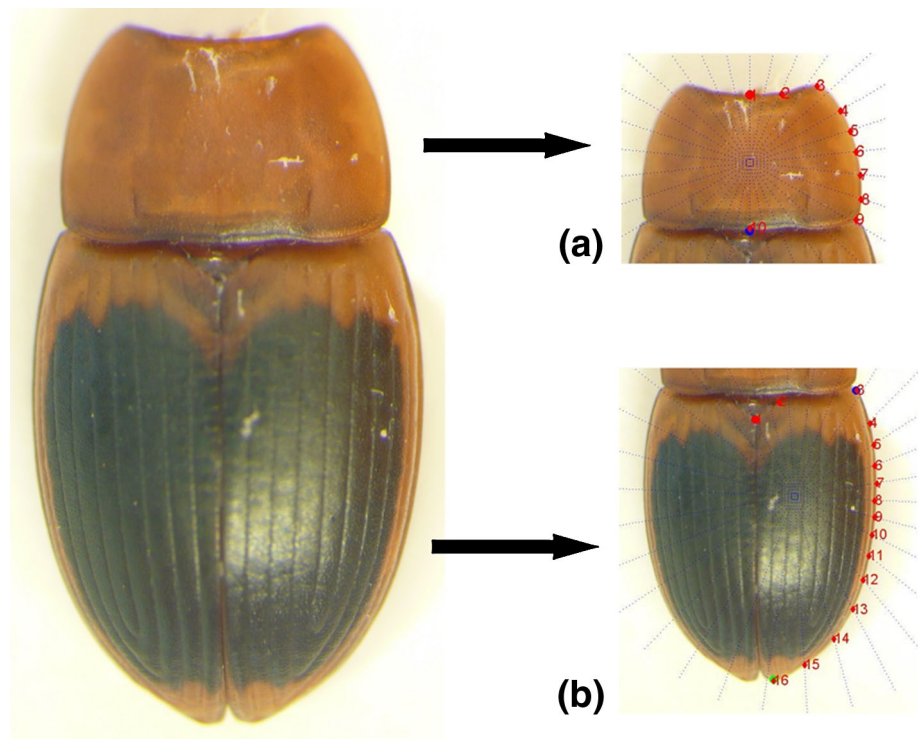


The resulting coordinates were subjected to a generalized procrustes analysis (GPA), which removes all the information that is unrelated to shape (Rohlf and Slice 1990). The perpendicular projection or minimum procrustes distance criterion, in this study, was used to align the semi-landmarks along their respective curves. In this case, the coordinates of the outlines were slid along a tangential direction, in order to minimize the procrustes distance between the specimen and a reference.

For the comparison of the configurations of *landmarks* and *semi-landmarks,* the relative warps method (RWM) was used (Bookstein 1991; Rohlf 1993). The relative warps are principal component vectors of the partial warps, variables generated for thin-plate spline transformations (Bookstein 1989), and were used to describe the major trends in shape variation among specimens within the sample (Rohlf 1993, 1996). Thin-plate spline deformation grids were generated to facilitate description of shape variation. The analyses were performed by means of RELATIVE WARPS 1.39 (Rohlf 2004).

The Discriminant function

Discriminant function analysis (DFA) of the morphometric data was performed using MORPHOJ 1.01 (Klingenberg 2011) and used in order to test the separation of the morphotypes attributable to the two species (Zelditch et al. 2004). The DFA examines the separation between two groups of observations, known a priori. The procedure carries out a leave-one-out cross-validation to assess the reliability of classification. The analysis automatically includes a parametric *T*-square test for the difference between group means.

## Results

The ML and BA molecular analyses gave congruent results, which are summarized in Fig. 3. The BA tree, rooted on the tenebrionid species *Nesotes helleri*, singles out two well-supported clades, corresponding to the populations ascribed to *P. acuminata* and *P. bimaculata* based on morphological analyses. The only exception is the sample from Vulcano-Gelso (VUL_GE), which was identified as *P. bimaculata marcuzzii* by Deidun et al. (2011), but which clustered with the *P. acuminata* clade in the present study.

The *P. acuminata* clade is poorly structured, as the populations are rather homogeneous, both on the Sicilian mainland and on the circum-Sicilian islands. Conversely, it is possible to distinguish two supported sub-clades (hereby reported as the "Tyrrhenian" and the "Southern" sub-clades, respectively) within the *P. bimaculata* clade (Fig. 3), although the molecular distance between them is rather low (Table 3).

As stressed in the RWM plot (Fig. 4), there are noteworthy inter-specific differences in the shape of the pronotum and the elytra. When compared with those of

**Fig. 3** **a** Phylogeographic reconstruction of *Phaleria* spp. from central Mediterranean area based on a 660-bp-long fragment of the mitochondrial gene COII. The tree topologies based on BA and ML analyses are congruent at the higher nodes. Support at nodes is represented as "BA posterior probability/ML bootstrap." The node support for ML tree is based on 1,000 bootstrap replicates. ML analysis is based on the GTR + I evolutionary model. See Tables 1 and 3 for the locality codes and GenBank accession numbers. **b** An iconographic representation of the genetic diversity outlined in *Phaleria* spp. The direction of the two branches into which the MAW bifurcates and of the AIS through the Sicily Strait are evidenced, according to Malanotte-Rizzoli et al. 1997; Millot 1999; Pinardi et al. 2005
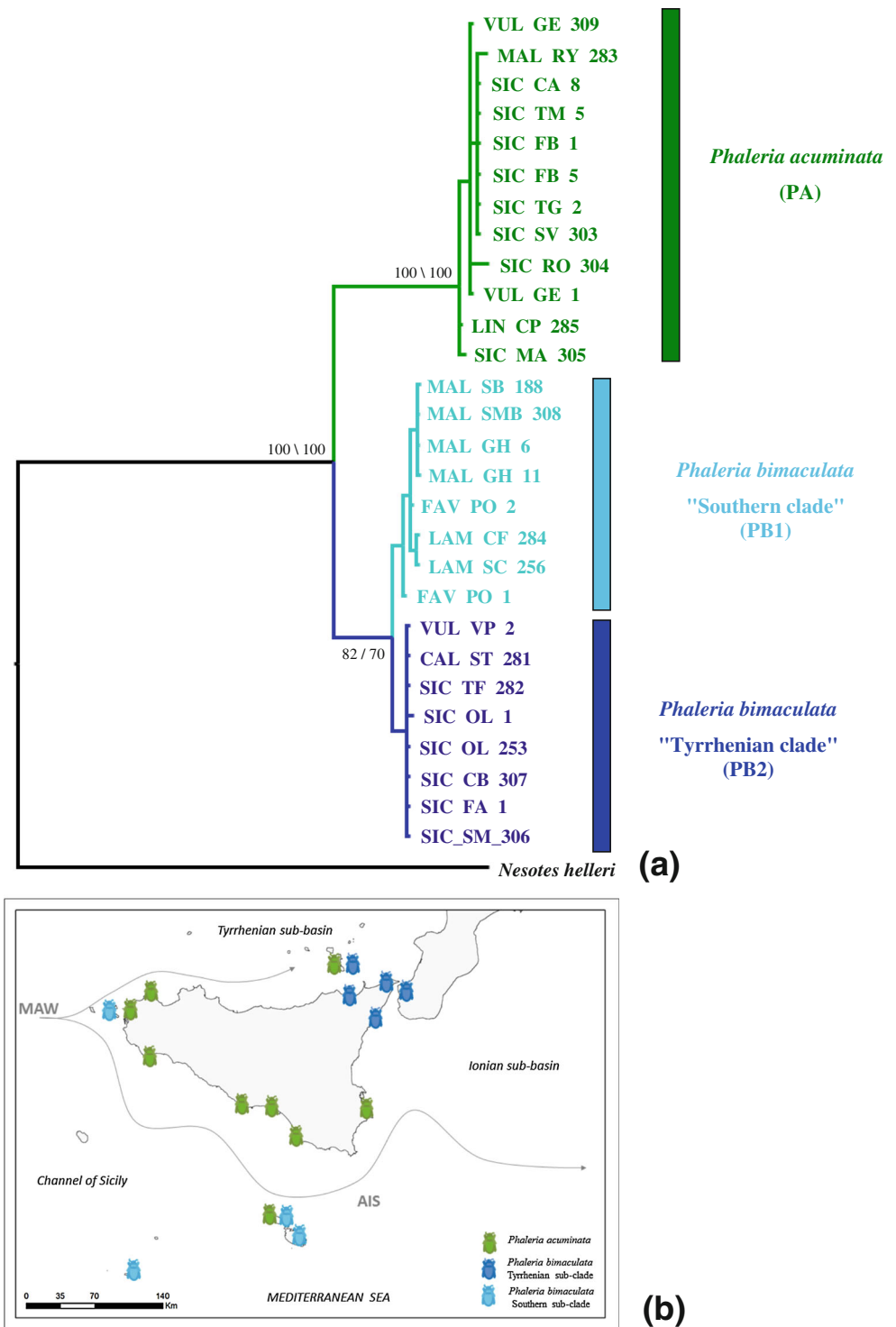
*P. bimaculata*, *P. acuminata* elytra are more elongated and elliptical, and the pronotum of the latter is narrower, with a less curved margin.

DFA was carried out separately for elytra and pronotum data, imposing the grouping of the specimens according to their species and sub-clade genetic affiliation. DFA allowed to clearly distinguish *P. acuminata* from *P.*

*bimaculata* for both elytra and pronotum (Fig. 5a, b); furthermore, based on pronotum-only data, a significant morphometric difference was detected among the populations belonging to the two molecular clades singled out for *P. bimaculata* (see Fig. 5c); conversely, no significant differences were observed when these populations were compared solely on the elytra data. The two *Phaleria*

**Table 3** Molecular distances among the clades and sub-clades singled out in Fig. 5a

|  | PA | PB1 | PB2 | Out. |
|---|---|---|---|---|
| *P. acuminata* (PA) |  | 0.06 | 0.06 | 0.18 |
| *P. bimaculata* "Southern sub-clade" (PB1) | 0.07 |  | 0.01 | 0.17 |
| *P. bimaculata* "Tyrrhenian sub-clade" (PB2) | 0.07 | 0.01 |  | 0.17 |
| Out-group (*Nesotes helleri*) | 0.27 | 0.26 | 0.26 |  |

Uncorrected p distances (above the diagonal) and distances based on the best evolutionary model selected by AIC (GTR + I, below the diagonal)

populations from the island of Vulcano, i.e., VUL_GE and VUL_PV (within the Aeolian archipelago, the *locus typicus* of *P. bimaculata marcuzzii*), did not cluster together in the DFA: One of them clustered within the *P. acuminata* populations, and the second one clustered within the *P. bimaculata* s.l. populations belonging to the "Tyrrhenian sub-clade."

All the pairwise comparisons performed by the DFA were highly significant ($p < 0.0001$) and the relative classifications proved to be reliable. The classification table (Table 4) shows the percentage correct attributions to the a priori molecularly identified groups (i.e., PA: *P. acuminata* vs. PB: *P. bimaculata;* PB1: *P. bimaculata* "Southern sub-clade" vs. PB2: *P. bimaculata* "Tyrrhenian sub-clade").

## Discussion

There is a perfect agreement between geometric morphometry and molecular analyses in assigning the studied *Phaleria* populations to the species *P. acuminata* or *P. bimaculata* s.l. Previously, a good agreement was observed also with the identification results from the conventional morphological investigation, with the only exception being the population from Vulcano-Gelso (VUL_GE), which was assigned to *P. bimaculata marcuzzii* by Deidun et al. (2011), while it is ascribed to *P. acuminate* in the present study on the bases of both morphometric and molecular evidence. Upon a careful morphological reanalysis, in fact, the Vulcano-Gelso population proved to belong to *P. bimaculata* s.l., thus providing evidence that the original identification of the same samples as reported by Deidun et al. (2011) was erroneous. The taxonomical value and the species-level discriminatory power of the geometric morphometrics analyses in Tenebrionidae, already demonstrated for different genera (e.g., Taravati et al. 2009; Peric-Mataruga et al. 2008), are thus also confirmed for the genus *Phaleria*, where the elytra and the pronotum proved to be

**Fig. 4** Scatterplots of two first relative warps scores obtained from ▶ the RWM of the shape of two external morphological structures. Plots show deformation grids relative to each axis; **a** pronotum: RW1 + RW2 accounted for a total of 79.11 %; **b** elytra: for a total of 71.01 %

consistent discriminant character among the studied species (Fig. 5a, b; Table 4).

The morphogenetic approach allowed us to further discriminate two well-characterized sub-clades within *P. bimaculata*; these are geographically segregated, and they are separated by modest molecular distances (Table 3) and by the pronotum morphometry (Fig. 4c). One of these groups includes all the *P. bimaculata* populations sampled along the Strait of Sicily, from the Maltese archipelago to the Pelagian and the Aegadian archipelago (the "Southern sub-clade"), and the other one includes all the populations from the northeastern Sicilian coast, Vulcano island (Aeolian archipelago), and southernmost Italian mainland (the "Tyrrhenian sub-clade") (Fig. 3). This second group thus includes also the *P. bimaculata* populations currently ascribed to the subspecies *P. bimaculata marcuzzii*.

At the current state of knowledge, both the mutually exclusive hypotheses that (1) *P. bimaculata marcuzzii* is a valid subspecies with a wider distribution than previously thought, i.e., extending over the entire Aeolian archipelago and the southeastern Tyrrhenian coasts, and that (2) *P. bimaculata marcuzzii* is in fact a junior synonym of another *P. bimaculata* subspecies, are equally plausible and deserve further investigation. A future broader sampling protocol, which includes the topotypical populations of the presumptive subspecies and different "forms" of *P. bimaculata*, is needed in order to check whether these alleged taxa of infra-specific rank coincide with well-defined molecular lineages.

Tenebrionid beetles lack a planktonic larval stage; however, the processes of water transport and rafting (passive dispersal through floating debris) between islands, described already for species like the oniscid isopod genus *Idotea* (ClarkinE et al. 2012), cannot be a priori dismissed (Fattorini 2002). Though other species inhabiting sandy beaches and exhibiting passive dispersal patterns, such as talitrid amphipods, show a population genetic structure shaped by the surface circulation of water masses (Pavesi et al. 2012), the geographical segregation of the two *P. bimaculata* sub-clades is not consistent with the prevailing pattern of surface currents within the central Mediterranean. The modified Atlantic water (MAW) stream bifurcates into two major streams just off the westernmost tip of Sicily (Fig. 3b), with one stream meandering along the northern coast of Sicily, in the southern Tyrrhenian, and one stream proceeding in a southeastern fashion, just south of Adventure Bank, in the Strait of Sicily (Malanotte-Rizzoli et al.
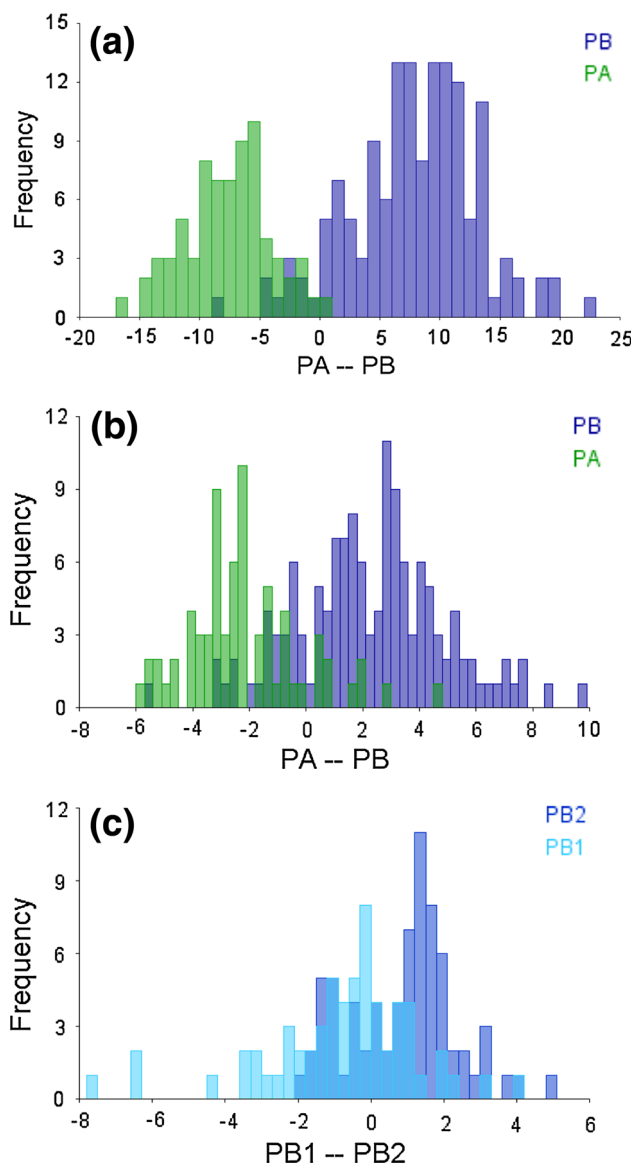
**(a)**
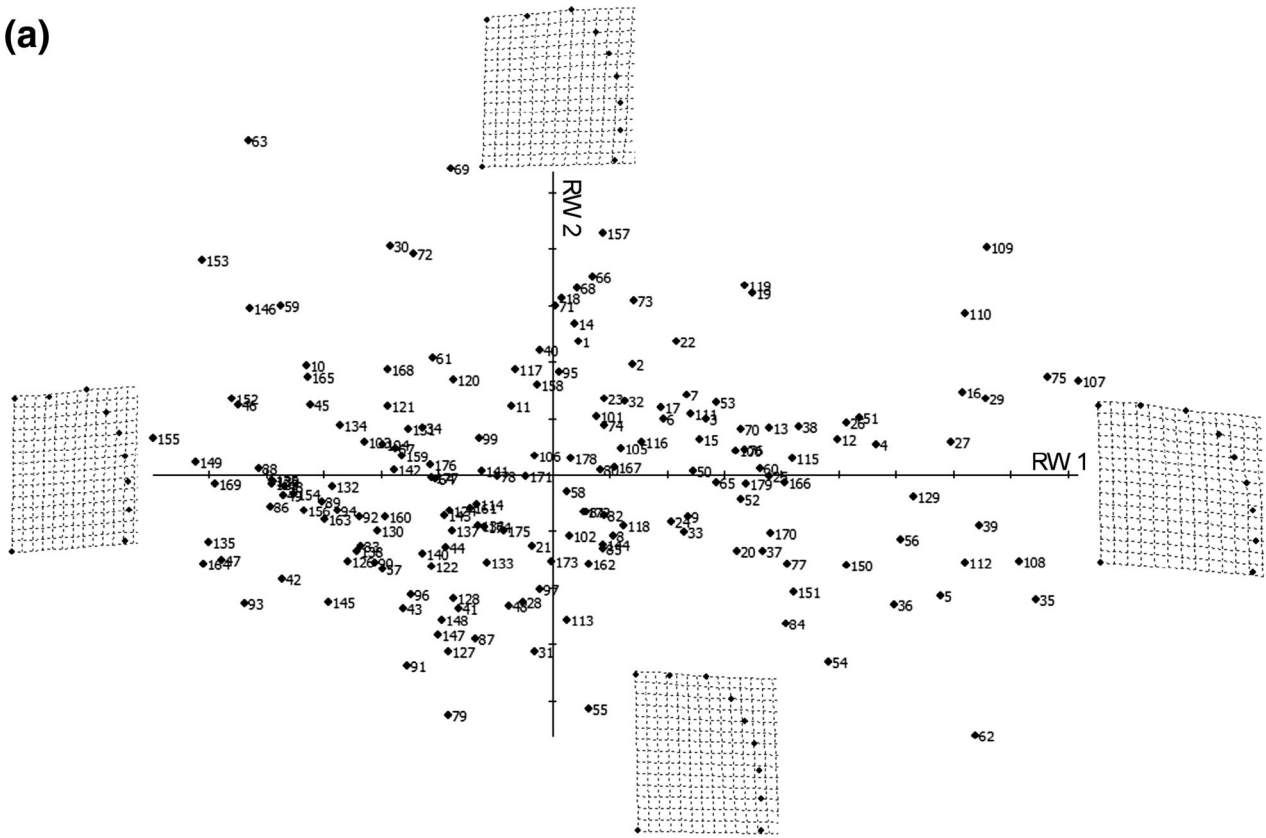


**(b)**
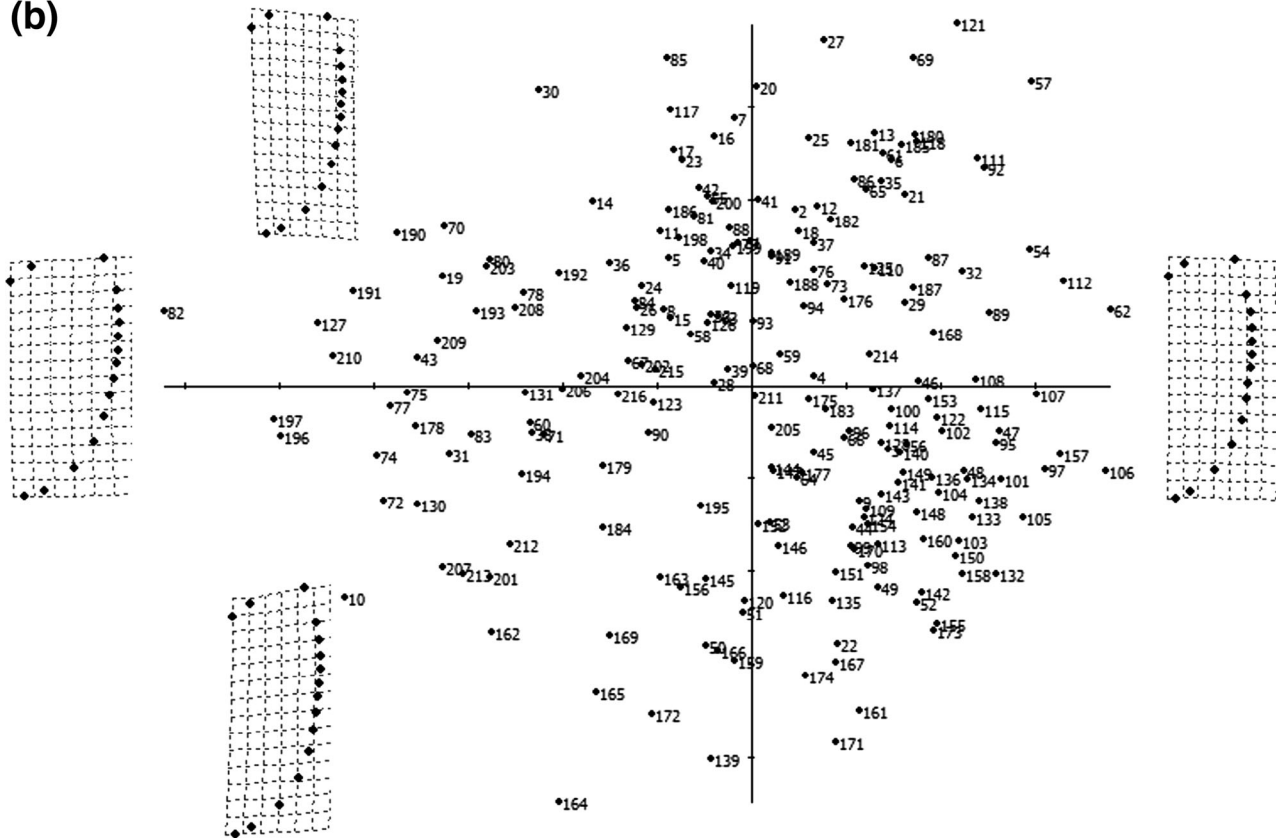
**Fig. 5** Discriminant function analysis between *P. acuminata* (PA) and *P. bimaculata* (PB): **a** elytra, **b** pronotum; and discriminant function analysis between the "Southern sub-clade" (PB1) and the "Tyrrhenian sub-clade" (PB2) within *P. bimaculata*: **c** pronotum

**Table 4** Percentages of correct classification using all shape or only "size-corrected" shape in DFAs, based on the leave-one-out cross-validation procedure

| All shape | | |
|---|---|---|
| Pronotum | | |
| | PA (%) | PB (%) |
| PA | 88.7 | 11.3 |
| PB | 16.3 | 83.7 |
| | PB1 | PB2 |
| PB1 | 80.6 | 19.4 |
| PB2 | 23.3 | 73.7 |
| Elytra | | |
| | PA | PB |
| PA | 100 | 0 |
| PB | 4.9 | 95.1 |

PA, *Phaleria acuminate*; PB, *P. bimaculata;* PB1, *P. bimaculata* "Southern sub-clade"; PB2, *P. bimaculata* "Tyrrhenian sub-clade"

1997; Millot 1999; Pinardi et al. 2005). The Maltese populations and the Aegadian ones are thus hydrodynamically linked by virtue of the MAW and of the Atlantic Ionic Stream (AIS) meandering their way through the Sicily Strait in a southeasterly direction. However, against such a hydrodynamic regime, gene flow should also be maintained between the Aegadian and the Tyrrhenian localities thanks to the northern stream of the MAW; however, this is incongruent with results emerging from the current study.

At a finer geographical scale, previous ecological studies on Maltese sandy beaches (e.g., Deidun and Schembri 2008; Gauci et al. 2005) have commented on the pocket nature of the same beaches surveyed, i.e., beaches that are headland delineated. Such a geomorphology thwarts long-distance longshore transport of sediment and propagules between adjacent or close beaches, translating in semi-isolated macrobenthic assemblages (Deidun et al. 2003; Deidun and Schembri 2008). However, results from the present study do not support this hypothesis, since *Phaleria* spp. specimens collected from adjacent Maltese pocket beaches belonged either to different species or to the same *P. bimaculata* sub-clade. In order to further test the actual level of compartmentalization of the studied coastal environments, the realization of a broader comparative study involving diverse molecular markers and taxa with different dispersal capacities is thus advisable.

The exploration and definition of character diagnostics assume a fundamental role in biological conservation; it is noteworthy to point out that in Australia, the Mediterranean *P. bimaculata* has been accidentally introduced in the vicinity of Melbourne (Doyen et al. 1989); in such cases, the availability of sound identification methods, as the ones described in this paper, is of pivotal importance for an early recognition of the biological invasions, which otherwise might be overlooked and thus inadequately managed. Molecular techniques are known to be extremely effective in order to unveil these "cryptic invasions" (e.g., Marrone et al. 2011, and references therein), and the geometric morphometry proves to be an extremely promising cost-effective alternative.

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