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Genetic and morphological assessment of *Helichrysum* Mill. from Tuscan Archipelago (Italy)

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ABSTRACT

In the Mediterranean area, the species of the genus *Helichrysum* Mill. (Asteraceae) have very similar genetic traits despite they may show different morphological characteristics. A first genetic and morphological assessment on twelve populations of *Helichrysum* from Elba, Capraia and Giglio Islands, in the Tuscan Archipelago (Italy) was carried out. To determine their taxonomic identity, additional reference specimens belonging to the three *Helichrysum* species reported from the Tuscan Islands in the literature -*H. italicum* (Roth) G. Don subsp. *italicum, H. litoreum* Guss., and *H. stoechas* L.-, were also included in the assessments. Our results show that the Tuscan Archipelago populations have low levels of morphological and genetic variation. In particular, the variability was mainly observed at the intra-population level, as revealed by the coefficient of variation and AMOVA-Gst in the morphological and genetic analyses, respectively. Basing on our results, all examined populations from the Tuscan Archipelago seem fit in *H. italicum* subsp. *italicum*. However, we detect and discuss the existence of some intermediate specimens, mainly with *H. litoreum*, reflecting the complexity of *Helichrysum*'s taxonomy and highlighting the need for additional studies to fully understand the various patterns and the underlying evolutionary events that originated them.

1. Introduction

The genus *Helichrysum* Mill. belongs to the Asteraceae family and includes approximately 600 species worldwide (Hilliard, 1983; Anderberg, 1991). It has its greatest diversity in southern Africa, where it is inferred to have originated (Galbany-Casals et al., 2014; Blanco-Gavaldà et al., 2023), but the genus is found throughout the whole of Africa, Madagascar, Macaronesia, the Mediterranean basin, and West Asia. For the Eurasian species of *Helichrysum*, three sections have been defined: section *Helichrysum*, which includes most European and Asian species; section *Virginea* (DC.) Gren. & Godr., that contains few Eastern Mediterranean species; and section *Stoechadina* (DC.) Gren. & Godr., that includes a well-defined group of suffruticose Mediterranean species. In recent years, taxonomic revisions of different species groups in section *Stoechadina* have been published (see for example, Galbany-Casals et al., 2006, 2011). In this section, species identification is challenging due to the high degree of inter- and intra-population morphological variability, and it is not uncommon to find morphological traits that overlap among species, in part due to the existence of hybridization and introgression events (Herrando-Moraira et al., 2016, 2017; Galbany-Casals et al., 2012).

In the Tuscan Archipelago, the presence of mainly three *Helichrysum* species has been reported (Paoli and Romagnoli, 1976; Baldini, 1998, 2000, 2001; Foggi et al., 2001; Rizzotto, 2011; Carta et al., 2018a, 2018b): *Helichrysum litoreum* Guss. (= *Helichrysum angustifolium* (Lam.) DC.), *Helichrysum stoechas* (L.) Moench, and *Helichrysum italicum* (Roth) G. Don subsp. *italicum*. On the islands of Capraia and Giglio, only *H. litoreum* has been recorded, while on Elba the three mentioned species have been reported: *H. litoreum* on coastal garigues, *H. italicum* subsp. *italicum* on hill and mountain garigues, and *H. stoechas* in coastal dune

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habitats. *Helichrysum litoreum* is mainly distinguished from *H. italicum* because it has longer and wider leaves, bigger synflorences with a higher number of capitula, it is not as strongly aromatic as *H. italicum,* and it has fewer glandular hairs. *Helichrysum stoechas* is distinguishable from *H. italicum* and *H. litoreum* because it has wider capitula and bigger outermost involucral bracts than these two species (Galbany-Casals et al., 2006). Additionally, *H. italicum* subsp. *microphyllum* has also been cited by Elba (Giuliani et al., 2016). However, Galbany-Casals et al. (2011), in a comprehensive work that included a molecular (AFLPs and DNA sequences) and morphological assessment of the whole *H. italicum* complex in the Mediterranean, concluded that *H. italicum* subsp. *microphyllum* is restricted to Crete, in the East Mediterranean. The gradual transition of the genetic variation that is correlated with geographic distance in the *H. italicum* complex makes it very unlikely that this subspecies, in its current circumscription, is present in the West Mediterranean, where morphologically similar species found in Corsica and Sardinia are currently recognised as subsp. *tyrrhenicum*. Moreover, a new updated inventory of the vascular flora of Elba Island only cited *H. italicum* subsp. *italicum* from Monte Capanne (Carta et al., 2018b, 2018a) and this was confirmed by a recent morphological study (Marini et al., 2022).

Among Mediterranean *Helichrysum* species, *H. italicum* subsp. *italicum* is the most studied due to its high economic value of essential oil and secondary metabolites with several biological properties, such as antimicrobial, antioxidant, anti-inflammatory, and anti-proliferative (Viegas et al., 2014; Maksimovic et al., 2017; Ninčević et al., 20199). In Mediterranean marginal land, the wild harvest of medicinal and aromatic plants stands spread and been practiced for centuries (Baldi et al., 2022). The domestication focused on the cultivation of essential oil wild plants could be an encouraging perspective to limit risks of habitat loss, incorrect taxonomic identifications, and uncontrolled concentration of bioactive compounds, which are strongly influenced by environment and genotypes (Schippmann et al., 2006; Appendino et al., 2015; Can Başer and Buchbauer, 2015). In this context, accurate identification of medicinal *Helichrysum* species based on morphological and genetic analysis becomes essential to ensure quality products from wild plants (Schippmann et al., 2002).

Investigating intra- and inter-population variation in plants is crucial for the taxonomic classification of species. The combination of morphological approach with genetic studies provides information on the magnitude of intraspecific variation, allowing for the identification of taxonomic boundaries, the detection of phenomena such as hybridization and understanding of evolutionary relationships. For example, the southern coast of Tuscany is considered a border area where the distributions of *H. italicum* and *H. litoreum* meet; this is reason that could explain the presence of *H. pseudolitoreum*, a taxon that has an intermediate morphology between *H. italicum* subsp. *italicum* and *H. litoreum*, likely product of past or current hybridization and/or introgression events (Galbany-Casals et al., 2006; Herrando-Moraira et al., 2016). Morphological characters have been widely used to study the extent of intraspecific variability and to determine the interspecific boundaries in *Helichrysum* taxa (Galbany-Casals et al., 2006, 2011; Conesa et al., 2012; Salmeri et al., 2014; Herrando-Moraira et al., 2016, 2017; Puglia et al., 2018; Azizi et al., 2019). Recently, molecular markers have contributed to a better understanding of the phylogenetic relationships, inter and intraspecific variation and hybridization phenomena in the genus *Helichrysum*, by using nrDNA ITS and ETS sequences (Galbany-Casals et al., 2004; Galbany-Casals et al., 2009, 2011, 2014; Herrando-Moraira et al., 2017), chloroplast psbA-trnH, rpl32-trnL and ndhF sequences (Galbany-Casals et al., 2011, 2012, 2014; Herrando-Moraira et al., 2017; Puglia et al., 2018), AFLP (amplified fragment length polymorphism) (Sabetta et al., 2006; Scialabba et al., 2008; Galbany-Casals et al., 2011, 2012; Melito et al., 2013) and SSRs (Arbeiter et al., 2021). ISSRs (inter-simple sequence repeats) are dominant markers highly informative and reproducible. Since repeated sequences are abundant throughout the genome, primers anneal in several regions providing a hypervariable amplification pattern in which fragments result to be greatly polymorphic between different samples. ISSR markers have been used for assessment of genetic diversity and phylogenetic studies of *Helichrysum* species occurring in Sicily (Puglia et al., 2018) and in Iran (Azizi et al., 2019).

The main purpose of this research has been to analyze the morphological and genetic variation patterns of twelve *Helichrysum* populations distributed across the three major islands of the Tuscan Archipelago (Elba, Capraia, and Giglio) referred, by the local floristic literature, to *H. italicum* subsp. *italicum, H. litoreum*, and *H. stoechas*. Adding to this, this study is a continuation of recent research on two *Helichrysum* populations on Elba Island where, based on morphological data, two taxa, *H. litoreum* and *H. italicum* subsp. *italicum,* were recognized. As well as other specimens from the Tuscan Archipelago, the present research included reference specimens from other Mediterranean geographic areas, used to contextualize the variation found and to shed light on the taxonomic identity of the Tuscan Archipelago populations.

2. Material and methods

2.1. Plant material

A total of 172 specimens of *Helichrysum* were sampled in June 2020 in twelve populations from Elba, Capraia and Giglio Islands, growing in well-distinguished locations and at different altitudes and habitats. The sampling localities were chosen based on previous reports of the presence of the different species (Table 1, Fig. 1b-e, Fig. S.5d-f). For each location, between 9 and 15 plants were sampled to comprise the intra-population variability of the species as well. A plant portion consisting of floral stems in full blooming and vegetative stems was harvested to make herbarium specimens for morphological analysis. From the same plants, a few apical sprouts were collected and transported in refrigerated containers at 4 °C until lab, then stored at −20 °C waiting for genetic analysis.

To strengthen data processing, samples of *Helichrysum* species of definite taxonomic identification were included in the morphological and genetic analysis. In detail, 49 herbarium specimens from the BC, BCN and FI herbaria, belonging to *H. stoechas, H. litoreum, H. italicum* subsp. *italicum, H. italicum* subsp. *microphyllum, H. italicum* subsp. *siculum, H. italicum* subsp. *tyrrhenicum* were included in the morphological analysis (Table 2, Fig. 1a, in detail Table S.1). In addition, since all Tuscan Archipelago DNAs were extracted from fresh leaves, to obtain a comparable both DNA extraction and ISSR analysis, fresh leaves were collected from 30 *Helichrysum* specimens in *loci classici* and border localities around the Tuscan Archipelago. The collected specimens, in this case, belong to *H. stoechas, H. litoreum*, and *H. italicum* subsp. *italicum* taxa (Table 3, Fig. 1a, in detail Table S.2).

2.2. Morphological analysis

A total of 27 morphometric characters, shown in Table S.3, were chosen according to the most recent literature, in order to better discriminate taxa within the *Helichrysum italicum* complex (Galbany--Casals et al., 2006; Herrando-Moraira et al., 2016; Puglia et al., 2018; Azizi et al., 2019; Marini et al., 2022).

The morphological measurements were carried out by analysis of digital images at different resolutions (i.e., 15x; 50x; 240x) on *exsiccata* specimens. All images were obtained using a DinoLite digital microscope and processed with the DinoCapture software (ver. 2.0). For qualitative traits, where possible, the mean of three to six measurements per specimen was used and the measurements were taken with a precision of 0.01 mm. Morphological measurements on glandular *indumentum* were performed using digital images at 225–240 × resolution, on which a color filter (red or blue) was applied to better highlight the glandular head (Fig. 2). The quantitative traits were divided into discrete groups and coded as binary or multiclass traits.

Table 1
Locations of the twelve study sites in the Tuscan Archipelago (Central Italy).

Island	Pop	Localities	Altitude range	Coordinates	References [a]	N [b]
Capraia	CI	Monte Castello's peak	350–400	43°02′57.9″N 9°48′51.6″E	(Foggi and Grigioni, 1999)	15
	CII	Punta della Bellavista	0–50	43°02′40.1″N 9°50′49.2″E	(Foggi et al., 2001)	15
	CIII	Il Frate beach	0–50	43°03′08.4″N 9°50′14.4″E	(Foggi et al., 2001)	9
Elba	EI	Cima di Monte's slopes	350–400	42°48′09.6″N 10°23′32.3″E	(Foggi et al., 2006)	15
	EII	Via dei Rosmarini, Campo	0–50	42°44′50.4″N 10°14′56.4″E	(Foggi et al., 2006)	13
	EIII	Capo di Fetovaia	0–50	42°43′52.7″N 10°08′59.5″E	(Foggi et al., 2006; Leonardi et al., 2013)	15
	EIV	Capo d'Enfola	0–50	42°49′21.8″N 10°16′18.3″E	(Leonardi et al., 2013)	15
	EV	Monte Calamita's slopes	350–400	42°43′28.4″N 10°24′15.2″E	(Foggi et al., 2006; Giuliani et al., 2016)	15
	EVI	Monte Capanne's peak	950–1000	42°46′18.6″N 10°10′05.1″E	(Giuliani et al., 2016)	15
Giglio	GI	Punta di Capo Marino	0–50	42°20′54.7″N 10°55′31.5″E	(Paolini et al., 2006; Foggi and Pancioli, 2008)	15
	GII	Punta di Capel Rosso	0–50	42°19′03.4″N 10°55′10.4″E	(Foggi and Pancioli, 2008)	15
	GIII	Poggio della Pagana's slopes	350–400	42°21′14.4″N 10°54′20.3″E	(Paolini et al., 2006; Foggi and Pancioli, 2008)	15

[a] References reporting the presence of Helichrysum specimens into the location.
[b] number of sampled specimens.

2.3. Genetic analysis

Total genomic DNA isolation was carried out from 30 to 40 mg of fresh and young leaves. The samples were placed in 2 mL tubes with three tungsten carbide beads, frozen by liquid nitrogen, and ground using a sample disruptor (Tissue Lyser, QIAGEN, Hilden, Germany). DNA was extracted following the CTAB method (Doyle and Doyle, 1987) with minor changes. The purified DNA was checked for its quality by visualization on an agarose gel, while the concentration was established using the dsDNA BR assay on a Qubit 1.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

For the genetic diversity assessment of *Helichrysum* samples collected in the Tuscan Archipelago, a collection of primers was tested. From this set, three primers (i.e., ISSR13, ISSR14, ISSR15), showing a high polymorphism level and reliability, were selected for the amplification of *Helichrysum* specimens (Table 4). The three chosen primers were previously used in the same genus by Azizi et al. (2014).

The PCR reaction total volume of 25 μL consisted of 20 ng genomic DNA, 1X GoTaq Buffer, 1 U GoTaq DNA Polymerase (Promega, Madison, WI, USA), 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.4 mM primer. Amplification was accomplished on a Primus 96 Advance thermal cycler (Peqlab Biotechnologie GmbH, Erlangen, Germany) according to the subsequent cycling conditions: 95 °C denaturation for 3 min; 35 cycles of 95 °C denaturation for 40 s, 52–58 °C annealing for 40 s (in agreement with the melting temperature of each ISSR primer), 72 °C extension for 1 min; a final 72 °C extension for 5 min.

PCR products were separated and analysed on a capillary electrophoresis analyser (QIAxcel Advanced System, QIAGEN, Hilden, Germany). OM1200 method was performed, using 5 kV and 10 s injection, 3 kV run for 1200 s. An alignment marker QX 15 bp / 5 kb was injected into the cartridge to determine the size of each scoring fragment. Peaks detected using QIAxcel ScreenGel software (QIAGEN, Hilden, Germany) were used to create a presence-absence matrix (presence = 1, absence = 0).

2.4. Statistical analysis

Correlation analyses between matrices were carried out by the Mantel test made with R Studio v. 1.4 (RStudio Team, 2021), based on *vegan* package (Oksanen et al., 2020). In detail, was analysed the correlation between the morphological and ISSR matrices and the correlation between the ISSR matrix and the geographic distances matrix. Significant differences from zero for the Mantel coefficients (rho) were assessed by comparing reference distributions obtained after 9999 interactions that randomly permuted the arrangement of the elements of the distance matrices.

An analysis of variance (ANOVA) was carried out on morphological data by using R Studio. The distribution of qualitative morphological characters was analysed by the chi-square test, whilst normality and homoscedasticity of quantitative variables were tested with Shapiro–Wilk and Levene test. Variables that did not fulfill ANOVA assumptions, even after transformation, were analysed by Kruskal–Wallis's test or Welch's test. To estimate the proportion of intrapopulation variance, we computed the average within-population coefficient of variation (CV) based on population means and standard deviations (SD) for each trait. A Principal Component Analysis (PCA) was carried out using R Studio with *FactorMineR* and *Factorextra* packages (Lê et al., 2008; Alboukadel and Mundt, 2016). The correlated morphological characters were excluded from the analyses. Substantially, the matrix subjected to multivariate analysis consisted of 221 individuals and 15 morphological traits. The cypselae characters – cypselae length and width, and cypsela duplex hair density – were not included in this analysis because data were not available for all individuals. To effort the discrimination among taxa, two Linear Discriminant Analyses (LDA) were carried out using STATISTICA software v.12. The first LDA was performed at the level of species, while the second LCA was at the subspecies level.

The genetic diversity of the 12 *Helichrysum* populations and control samples was assessed through the estimation of the number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (h), Shannon's information index (I) and the percentage of polymorphic bands (PPB) using POPGENE 1.32 software (Yeh, 1999). Furthermore, POPGENE 1.32 software was used to calculate the total genetic diversity (Ht), the mean intra-specific genetic diversity (Hs), the coefficient of gene differentiation (Gst) and gene flow (Nm= 0.5(1-Gst)/Gst) in all population. The analysis of molecular variance (AMOVA) was calculated using GenAlEx 6.5 (Peakall and Smouse, 2006) to determine the distribution of genetic diversity among and within populations in all *Helichrysum* specimens. In addition, a Principal Coordinates Analysis (PCoA) was performed according to Euclidean distances using the packages *adegenet* 2.1.3 (Jombart, 2008) and *ade4* (Chessel et al., 2004) in R-project.

3. Results

The Mantel test resulted in a significant correlation ($p < 0.01$) between the morphological and ISSR matrices, with a rho = 0.273 (Fig. S.6). Moreover, a significative correlation ($p < 0.01$) was obtained between ISSR and geographic distance matrices, with a rho = 2.279 (Fig. S.7).

3.1. Morphological analysis

The results of parametric/non-parametric statistical analysis and the basic statistical data (mean ± standard deviation; maximum and minimum) carried out on morphological traits are reported, respectively in Tables 5,6 and Table S.4.

According to the univariate analysis (Table S.4), 3 of the 27 variables did not show significant differences among taxa, i.e. measured on

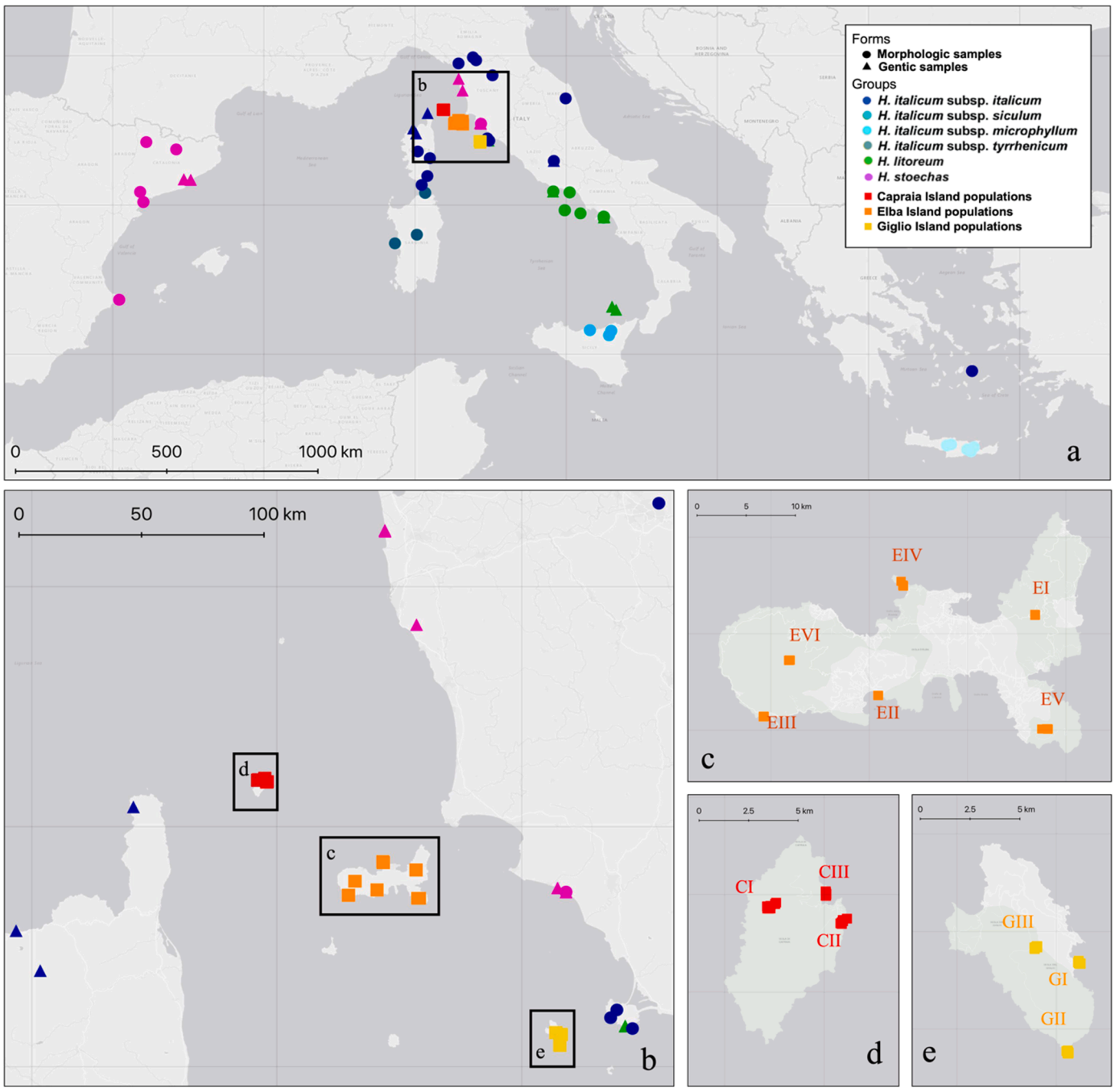

Fig. 1. Location of the analysed specimens. (A) Overall views of Mediterranean Basin were reported considered samples. (b) Particular of the Tuscan Archipelago, is interesting to note the neighbouring Helichrysum taxa. (c) Maps of sampled populations in Elba Island. (d) Maps of sampled populations in Capraia Island. (e) Maps of sampled populations in Giglio Island.

cypselae (14.Cyp.l, 15.Cyp.w, and 16.Cyp.gland). On the other hand, all the other variables exhibited significant differences among taxa ($p < 0.001$).

The highest mean values for glandular *indumentum* of leaf abaxial face (L.gland = 41.5 ± 6.9), cypsela length (Cyp.l = 0.69±0.1) and cypsela width (Cyp.w = 0.28±0.1) were found in Capraia populations. On the contrary, these populations showed the lowest mean values for caulinary leaf width (L.w = 0.65±0.1), synflorescences length (Syn.l = 24.4 ± 0.3), number of capitula (Cap.n = 23.3 ± 7.5) and pappus setae length (P.l = 2.88±0.3). Elba populations had highest mean values for pappus setae length (P.l = 2.99±0.3) and cypsela glandular density (Cyp.gland=2.87±0.6) while showed the lowest mean values for glandular *indumentum* of leaf abaxial face (L.gland=35.7 ± 6.1) and glandular *indumentum* of innermost involucral bract (B.inn.gland =37.7 ± 9.0). Giglio populations showed the highest mean values for caulinary leaf length (L.l = 18.5 ± 4.3), synflorescences length (Syn.l = 29.26± 6.2), number of capitula per synflorescence (Cap.n = 37.1 ± 14), glandular *indumentum* of innermost involucral bract (B.inn.gland =42.8 ± 13); the lowest for cypsela length (Cyp.l = 0.55±0.1), cypsela width (Cyp.w = 0.22±0.1) and the ratio between the synflorescence length and the number of cypsela (Syn.l/Cyp.n = 0.86±0.2).

The CV values indicated a relatively high variability within the populations. The most variable traits were glandular *indumentum* of leaf abaxial side on vegetative stem (03.L.gland=37.6%), leaf margin (24. LM=37.5%), glandular *indumentum* of leaf abaxial side on floral stem (17.L.gland_FL=33.5%), presence of axillary leaf fascicles on floral stem

Table 2
Number of reference specimens included as a control in morphological analysis, summarized for each taxon (N) and their location. The total number of specimens is given in bold.

Taxa	Country	Geographic region	*N*
Helichrysum italicum* subsp. *italicum			**11**
	France	Corsica	3
	Greece	Cyclades	2
	Italy	Emilia	1
		Lazio	1
		Marche	1
		Tuscany	3
Helichrysum italicum* subsp. *microphyllum			**5**
	Greece	Crete	5
Helichrysum italicum* subsp. *siculum			**3**
	Italy	Sicily	3
Helichrysum italicum* subsp. *tyrrhenicum			**6**
	France	Corsica	3
	Italy	Sardinia	3
Helichrysum litoreum			**15**
	Italy	Campania	5
		Circeo	2
		Lazio	5
		Tuscany	3
Helichrysum stoechas			**9**
	Italy	Tuscany	1
	Spain	Catalonia	8
Total			**49**

Table 3
Number of specimens included as a control in genetic analysis, summarized for each taxon (N) and their location. The total number of specimens is given in bold.

Taxa	Country	Geographic region	*N*
Helichrysum italicum* subsp. *italicum			**5**
	France	Corsica	3
	Italy	Lazio	2
Helichrysum litoreum			**10**
	Italy	Campania	3
		Eolie Islands	1
		Lazio	1
		Sicily	2
		Tuscany	3
Helichrysum stoechas			**15**
	Italy	Tuscany	5
	Spain	Catalonia	10
Total			**30**

(26.Laxfasch.FL=33.0%), number of capitula per synflorescence(05. Cap.n = 32.4%), and cypsela glandular density (16.Cyp.gland=29.1%). As opposed to that, the lowest rate of variability was found in cypsela length/cypsela width (06.Cyp.l = 7.6%) and pappus length (13.P.l = 7.6%). Populations having the highest mean CV values (mean of the CV over all the considered traits) were GIII (21.4%), CIII (20.9%), GII (20.7%) and CII (20.3%). Those having the lowest mean CV values were EIV (17.4%), EII (17.6%) and EII (17.7%).

The results of PCA for the morphological data are summarized in Fig. 3. The first two principal components explained about 49.2% of the total variation among the assessed populations (Table S.5). The graph showed the clear cluster separation between the three reference species included in the analysis: *H. italicum* (s.l.) with *H. litoreum* and *H. stoechas* clusters. In general, the Tuscan archipelago populations were clustered with the specimens referable to *H. italicum*. However, some specimens from populations EVI, EV, and GII were placed in an intermediate position between *H. italicum* and *H. litoreum*, and a single specimen from CIII was placed in an intermediate position between *H. italicum* and *H. stoechas* (Fig. S.2).

Variables describing capitula dimensions (Cap.w and Cap.l), outermost bract dimensions (B.out.w and B.out.l.), pappus length (P.l) and innermost bract dimensions (B.inn.w and B.inn.l.) were the most discriminant variables and contributed to the first principal component (Dim1; 0.87, 0.74, 0.74, 0.71, 0.56, 0.48 and 0.31 respectively), explaining 31.9% of the total variation, and that mainly separated *H. italicum* + *H. litoreum* from *H. stoechas*. The second component (Dim2) accounted for 17.3% of the total variation and differentiated individuals by leaves length (L.l), synflorescences diameter (Syn.l), capitula number per synflorescences (Cap.n) and leaves width (L.w), explaining respectively 0.67, 0.61, 0.46 and 0.42 on Dim2, and mostly separating *H. litoreum* from the other taxa.

The LCA results at the species level revealed similar results to PCA (Fig. S.8a). In particular, the Tuscan Archipelago populations were well grouped with *H. italicum* (s.l.) and separated along the first dimension from the *H. stoechas* group, while along the second dimension from the *H. litoreum* group. The morphological traits that were discriminant for the first and second dimensions are reported in Table S.6. For the first dimension, they were the outermost bract traits (B.out.l and B.out.w); capitula diameter (Cap.w), pappus length (P.l) and the presence of axillary leaves fascicles (Lax.fasch) describing respectively 0.49, 0.30, 0.36, 0.19 and −0.34 of variability. For the second dimension, leaves length (L.l), outermost bract length (B.out.l), innermost bract width (B. inn.w), innermost bract indumentum (B.inn.gland) and outermost bract width (B.out.w) were the most discriminant, with variability values equal to 0.83, 0.40, −0.34, −0.37, −0.38, respectively. The attribution percentage reported that the Tuscan Archipelago populations completely grouped with the *H. italicum* (s.l.) group (Table 7).

The LCA results at the subspecies level is shown in Fig. S.8b. In detail, the Tuscan Archipelago populations were grouped with *H. italicum* subsp. *italicum* and separated along the first dimension from the other subspecies. The morphological traits that were discriminant are reported in Table S.7. For the first dimension, leaves length (L.l), outermost bract length (B.out.l), leaf margin (L.M) and capitula width (Cap.w) were the most discriminant traits with variability values equal to 0.95, −0.31, −0.55, −0.63, respectively. For the second dimension, leaves width (L. w) glandular indumentum of leaf abaxial side of the floral stem (L. gland_FL), innermost bract length (B.inn.l) and synflorescences length (Syn.l) were the most discriminant traits with variability values equal to 0.42, 0.40, −0.54, −0.59, respectively. The attribution percentage reported that the Tuscan Archipelago population totally grouped with the *H. italicum* subsp. *italicum* group (Table 8).

3.2. Genetic analysis

3.2.1. Genetic diversity parameters

From a collection of ISSR primer pairs, three markers already used by Azizi et al. (2014, 2019) showed considerable reliability. Moreover, the protocol was based on the usage of a capillary electrophoresis system (Qiaxcel, Qiagen srl) which increased the resolution and the reliability of each detected fragment. Indeed, 88 polymorphic loci were detected using only three primers.

Table 9 shows the genetic diversity observed in the 15 *Helichrysum* populations analysed. The Na parameter varied from 1.223 (EIV) to 1.602 (EV) with a mean of 1.436. The Ne parameter ranged from 1.179 (*H. stoechas*) to 1.282 (*H. litoreum*) with an average of 1.223. The h parameter was very low with a mean of 0.137, ranging between 0.115 (*H. stoechas*) to 0.173 (EV), revealing a reduced genetic differentiation among *Helichrysum* populations. Moreover, the I index displayed a low genetic diversity with an average value of 0.213, varying between 0.185 (GIII and *H. stoechas* reference group) to 0.271 (EV). The PPB ranged from 30.68% (*H. italicum* reference group) to 60.23% (EV) with a mean of 45.07%. The population with the highest genetic diversity was EV (h = 0.173, I = 0.271, PPB= 60.23%) followed by CII and GI (h = 0.157 and 0.150, I = 0.239 and 0.233, PPB= 48.86% and 51.14% respectively). The population with the lowest genetic diversity was *H. stoechas* reference group (h = 0.115, I = 0.185, PPB= 45.45%).

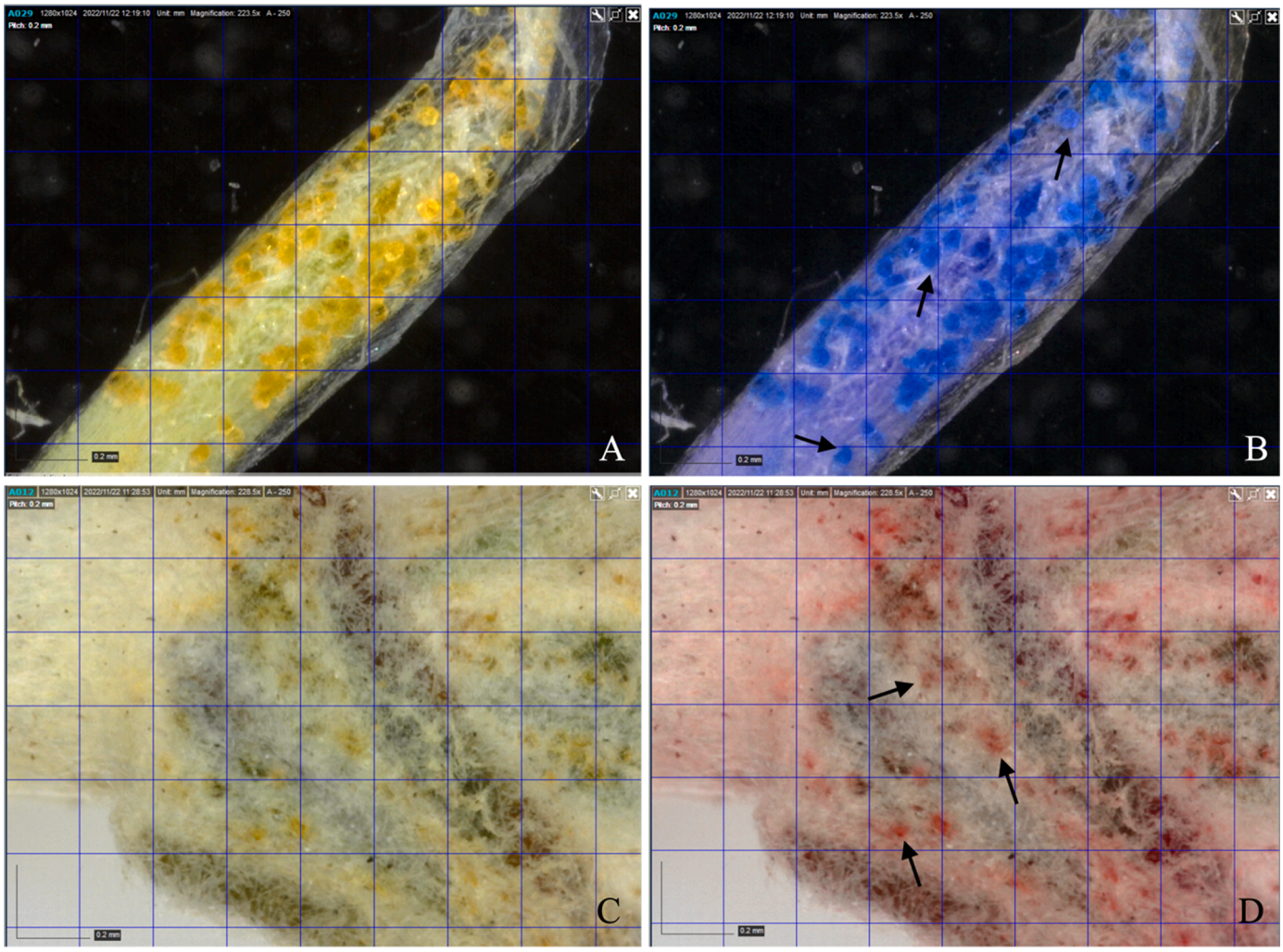

Fig. 2. Coloured filters were applied on the glandular indumentum of innermost involucral bract (A-B) and glandular indumentum of leaf abaxial side (C-D) to better highlight the glands head (arrows). The grid squares have sides of 0.2 mm.

Table 4
List of the ISSR primers used in the genetic analysis, with the indication of the UBC code, the repeat motif, the sequence, and the melting temperature (Tm).

ID	References	Repeat	Sequence (5′–3′)	Tm (°C)
ISSR13	(Azizi et al., 2014)	(CA)7GT	CACACACACACACAGT	56
ISSR14	(Azizi et al., 2014)	(CA)7AT	CACACACACACACAAT	54
ISSR15	(Azizi et al., 2014)	(CA)7AC	CACACACACACACAAC	55

3.2.2. AMOVA results

The results explained in the previous paragraphs were confirmed through the computation of the Ht, Hs, Gst and Nm parameters (Table 10). Both considering the parameters among the 12 Tuscan Archipelago populations, and the total genetic data with the reference taxa revealed the Ht and Hs with a low level of genetic variation with maximum values of 0.1711 and 0.1382 respectively. Moreover, the Gst described a minor value among the 12 Tuscan Archipelago populations then considering the 15 total groups. In detail, in the first case, the Gst value unveiled 15% of the variation, while the 19% of total genetic variation among populations considering the three references *Helichrysum* taxa. In contrast, the gene flow (Nm) showed a higher value (2.7557) within Tuscan Archipelago populations than the total value (2.0374), revealing that the reduced genetic differentiation among populations is due to a high level of gene exchange which could be justified by the outcrossing and anemochorous traits of the species.

The results of the analysis of molecular variance (AMOVA) described a similar low variation (Table 11). The genetic variation among the 12 Tuscan Archipelago populations revealed 9% variation among populations and 91% within populations. Additionally, it was tested the molecular variance considering *H. italicum* subsp. *italicum, H. stoechas* and *H. litoreum* references specimens and include the 12 Tuscan Archipelago *Helichrysum* populations as *H. italicum* subsp. *italicum.* The overall genetic variation among the three taxa considers in genetic analysis revealed 21% variation among species and 79% within species. Moreover, the pairwise differentiation among species was also determined with the PhiPT, showing low values at a significant level (p-value = 0.001).

3.2.3. PCoA results

In the Principal Coordinates Analysis (PCoA) of the genetic markers (Fig. 4), the first coordinate accounted for 10.15% of the total genetic variation, while the second one detailed 6.65%. Even though the results evidenced a reduced genetic variation explained by the two coordinates, three different clusters were observed. Particularly, it revealed a species differentiation, separating *H. italicum* from *H. stoechas* and *H. litoreum*. In general, Tuscan Archipelago populations clustered with *H. italicum* reference samples, confirming the results observed in the morphological analyses. However, as in morphological analyses, some specimens from populations GI and EV were placed in an intermediate position between *H. italicum* and *H. litoreum* (Fig. S.3).

Table 5
Morphological data on Tuscan Archipelago populations of Helichrysum. Numerical variables are reported: mean values, standard deviation, and minimum and maximum values. Categorical variables are reported in the absolute frequency for each level (Table S.3); in levels where the frequency was zero (f(x)=0) they are not reported in the table. Coefficient of variation is indicated as CV (%).

Morphological variables	Capraia			Elba						Giglio			CV (%)
	CI *N = 14*	CII *N = 15*	CIII *N = 9*	EI *N = 15*	EII *N = 13*	EIII *N = 15*	EIV *N = 16*	EV *N = 15*	EVI *N = 15*	GI *N = 15*	GII *N = 15*	GIII *N = 15*	
01.L.l	16.2 ± 3.4 (12.3–23.6)	15±3.7 (11.2–23.6)	13.6 ± 2.2 (11.5–17.9)	17.6 ± 4.2 (10.4–24.4)	15±3.9 (9.5–24.2)	13.9 ± 2.7 (9.9–19.7)	21.7 ± 3.9 (16.8–29.8)	15.2 ± 3.8 (9.8–23.9)	17±3.9 (11.2–24.1)	17.7 ± 5 (8.2–26.8)	15.7 ± 3.5 (7.7–22.9)	22.1 ± 4.4 (15–28.3)	22.2
02.L.w	0.6 ± 0.1 (0.5–0.9)	0.7 ± 0.1 (0.5–0.9)	0.7 ± 0.2 (0.4–0.9)	0.6 ± 0.1 (0.4–0.8)	0.8 ± 0.2 (0.6–1.4)	0.8 ± 0.2 (0.4–1.1)	0.9 ± 0.1 (0.7–1.2)	0.7 ± 0.1 (0.5–1)	0.6 ± 0.1 (0.5–0.8)	0.7 ± 0.2 (0.4–1)	0.8 ± 0.2 (0.5–1.1)	0.7 ± 0.1 (0.5–0.8)	20.0
03.L.gland	19.9 ± 8.8 (5–30.3)	16.1 ± 5.6 (5–27.5)	14±6.4 (4–19.5)	21.6 ± 9.3 (10.5–38)	10.5 ± 2.1 (6.5–13.5)	20.8 ± 7.8 (7–35.7)	11.4 ± 4.5 (6–21.5)	24.6 ± 7.4 (13.3–34.5)	12.2 ± 5.4 (5.3–23.5)	12.8 ± 4 (6–21)	16.5 ± 6.3 (5.5–24)	19.3 ± 8.4 (2–30.4)	37.6
04.Syn.l	23.8 ± 3.8 (17.5–31)	24.8 ± 3.1 (19.8–31.9)	24.6 ± 6.4 (14.8–35)	31±6.1 (21.5–46.2)	25.1 ± 5.2 (18–32.6)	26.1 ± 6.5 (15.4–37)	23.9 ± 3.1 (18–29.4)	34.5 ± 11 (22.5–55)	27.3 ± 5.5 (19–38.7)	30.7 ± 8 (20.9–47)	28.2 ± 5.9 (20.8–42)	29±4.7 (21.7–39)	20.7
05.Cap.n	23.4 ± 6.5 (12–34)	20.6 ± 4.1 (15.2–28.5)	25.8 ± 11.8 (9.9–53.1)	40.6 ± 9.9 (27.7–56.6)	19.2 ± 6.7 (11–30.2)	20.6 ± 6.3 (10.4–33)	18±4.1 (11.3–26)	44.3 ± 19 (21.7–88)	34.6 ± 9.6 (15.5–51.4)	34.7 ± 13.1 (18.8–62.5)	40.4 ± 16.4 (25–90.5)	36.1 ± 12.1 (21–57.8)	32.4
06.Cap.l	4.2 ± 0.3 (3.9–4.9)	4.2 ± 0.4 (2.8–4.6)	4.2 ± 0.4 (3.5–4.9)	4.1 ± 0.3 (3.6–4.6)	4.7 ± 0.2 (4.3–5)	4.5 ± 0.3 (3.9–5.1)	4.3 ± 0.4 (3.2–4.7)	4.2 ± 0.3 (3.6–4.6)	4.2 ± 0.3 (3.7–4.6)	4.2 ± 0.2 (3.7–4.5)	4.5 ± 0.4 (3.8–5)	4.4 ± 0.5 (3.7–5.6)	7.6
07.Cap.w	2.5 ± 0.3 (2.2–3.1)	2.7 ± 0.3 (2–3.1)	2.5 ± 0.4 (1.9–3.3)	2.4 ± 0.3 (1.8–2.9)	2.9 ± 0.2 (2.5–3.5)	2.9 ± 0.4 (2.4–3.9)	3.1 ± 0.3 (2.1–3.5)	2.4 ± 0.4 (1.7–3.1)	2.7 ± 0.3 (2.1–3.4)	2.5 ± 0.3 (2.2–3.1)	2.7 ± 0.3 (2.3–3.4)	2.7 ± 0.3 (2.1–3.5)	12.3
08.B.out.l	1.2 ± 0.2 (0.9–1.4)	1.3 ± 0.6 (1–3.4)	1.1 ± 0.2 (0.6–1.3)	1.1 ± 0.1 (1–1.4)	1.1 ± 0.1 (0.9–1.2)	1.1 ± 0.2 (0.9–1.5)	1.1 ± 0.2 (0.6–1.4)	1 ± 0.1 (0.9–1.2)	1 ± 0.1 (0.9–1.3)	1.2 ± 0.1 (1–1.4)	1.2 ± 0.1 (1–1.4)	1 ± 0.1 (0.8–1.3)	14.8
09.B.out.w	0.5 ± 0.1 (0.3–0.5)	0.5 ± 0.1 (0.3–0.6)	0.5 ± 0.1 (0.3–0.6)	0.5 ± 0.1 (0.4–0.7)	0.5 ± 0.1 (0.3–0.6)	0.4 ± 0.1 (0.3–0.5)	0.5 ± 0.1 (0.4–0.7)	0.5 ± 0.1 (0.3–0.7)	0.5 ± 0.1 (0.4–0.6)	0.6 ± 0.1 (0.4–0.9)	0.5 ± 0.1 (0.4–0.7)	0.4 ± 0.1 (0.3–0.6)	18.8
10.B.inn.l	3.7 ± 0.2 (3.4–4)	3.5 ± 0.7 (1.2–4.1)	3.5 ± 0.5 (3.2–4.9)	3.5 ± 0.4 (2.9–4.2)	3.8 ± 0.2 (3.3–4.1)	3.7 ± 0.3 (3.2–4.3)	3.7 ± 0.3 (3.3–4.1)	3.8 ± 0.2 (3.3–4.1)	3.7 ± 0.3 (3.3–4.3)	3.5 ± 0.2 (3–4)	3.7 ± 0.3 (3–4)	3.5 ± 0.3 (3.1–4.0)	9.3
11.B.inn.w	0.6 ± 0.1 (0.5–0.8)	0.6 ± 0.1 (0.5–0.8)	0.7 ± 0.1 (0.5–0.8)	0.6 ± 0.1 (0.4–0.9)	0.6 ± 0.1 (0.4–0.7)	0.6 ± 0.1 (0.5–0.7)	0.6 ± 0.1 (0.4–0.8)	0.6 ± 0.1 (0.5–0.8)	0.6 ± 0.1 (0.5–0.7)	0.6 ± 0.1 (0.4–0.8)	0.7 ± 0.1 (0.5–1)	0.6 ± 0.1 (0.5–0.7)	13.5
12.B.inn.gland	36.4 ± 6.2 (25–47)	42.3 ± 10.1 (26–57)	42±7 (30–49.5)	37.4 ± 8.9 (28.5–63)	35.5 ± 7.6 (22–46)	43.7 ± 10.2 (23.5–61)	42.9 ± 6.8 (37–58)	37.7 ± 9.3 (22–47.5)	28.9 ± 11.5 (9.8–49)	45.1 ± 9.6 (33–62.3)	40.5 ± 9.4 (26–51.5)	42.9 ± 19.1 (23.5–88.6)	24.6
13.P.l	2.9 ± 0.2 (2.7–3.3)	2.9 ± 0.2 (2.6–3.4)	2.8 ± 0.4 (2.6–3.8)	3 ± 0.2 (2.7–3.5)	3 ± 0.2 (2.7–3.3)	3 ± 0.2 (2.7–3.4)	2.9 ± 0.3 (2.4–3.4)	3 ± 0.2 (2.6–3.4)	3.1 ± 0.2 (2.8–3.4)	2.9 ± 0.2 (2.6–3.3)	3 ± 0.3 (2.5–3.4)	2.9 ± 0.2 (2.5–3.4)	7.6
14.Cyp.l	0.6 ± 0.1 (0.4–0.8)	0.7 ± 0.1 (0.5–0.9)	0.7 ± 0.1 (0.6–0.8)	0.5 ± 0.1 (0.4–0.6)	0.7 ± 0.1 (0.5–0.8)	0.7 ± 0.1 (0.5–0.9)	0.7 ± 0.1 (0.5–0.8)	0.5 ± 0.1 (0.3–0.7)	0.6 ± 0.1 (0.4–0.7)	0.5 ± 0.1 (0.4–0.6)	0.5 ± 0.1 (0.4–0.7)	0.6 ± 0.1 (0.5–0.8)	14.3
15.Cyp.w	0.2 ± 0.1 (0.1–0.3)	0.3 ± 0.1 (0.2–0.4)	0.3 ± 0.1 (0.2–0.4)	0.1 ± 0 (0.1–0.2)	0.3 ± 0.1 (0.2–0.4)	0.3 ± 0 (0.2–0.3)	0.4 ± 0.1 (0.3–0.4)	0.2 ± 0.1 (0.1–0.3)	0.2 ± 0.1 (0.1–0.3)	0.2 ± 0 (0.1–0.2)	0.2 ± 0.1 (0.1–0.3)	0.3 ± 0 (0.2–0.4)	22.5
16.Cyp.gland	2.6 ± 0.6 (1.9–3.5)	1.9 ± 0.5 (1.4–3.1)	2.3 ± 0.7 (1.4–3.5)	4.1 ± 0.7 (3.5–5)	1.9 ± 0.4 (1–2.5)	2.2 ± 0.5 (1.6–3.4)	1.6 ± 0.5 (0.5–2.6)	3.2 ± 0.9 (1.8–4.7)	4.2 ± 0.9 (2.8–5.5)	2.6 ± 1 (1.8–4.4)	2.1 ± 0.7 (1.5–3.3)	2.5 ± 1.2 (1.1–5.1)	29.1
17.L.gland_FL	22.1 ± 6.1 (11–33)	25.5 ± 6.6 (14.5–35)	20.8 ± 8.3 (10.5–32.3)	26.9 ± 5.5 (17.2–35)	12.4 ± 3.8 (5.8–18.7)	27.5 ± 7.4 (19.5–47.8)	14.3 ± 4.4 (6.7–20.7)	37.4 ± 11.9 (18.3–60.8)	26.9 ± 9.9 (13.5–50.7)	18±7.7 (4–34.7)	20±7.9 (10.3–37.7)	26.4 ± 13.2 (10.3–61.3)	33.5
18.L.l/L.w	27.9 ± 5.5 (20.4–38.5)	23.4 ± 5.9 (15–34.5)	20.8 ± 6.1 (14.3–32.5)	31.2 ± 6.3 (22.3–43.2)	19.2 ± 3.7 (12.7–26.1)	19.5 ± 3.7 (12.4–25.2)	24.1 ± 3.7 (19–30.7)	23±4.2 (18.1–33.3)	27.5 ± 5 (20.3–36.7)	25±5.9 (13.2–34.6)	19.5 ± 3.7 (14.9–26.5)	33.1 ± 6 (22.8–46.8)	20.5
19.Cap.l/Cap.w	1.7 ± 0.2 (1.3–1.9)	1.6 ± 0.2 (1.4–1.9)	1.7 ± 0.1 (1.5–1.9)	1.7 ± 0.2 (1.4–2.1)	1.7 ± 0.1 (1.4–1.9)	1.6 ± 0.2 (1.1–1.8)	1.4 ± 0.1 (1.3–1.7)	1.8 ± 0.2 (1.5–2.3)	1.6 ± 0.2 (1.3–1.9)	1.7 ± 0.2 (1.2–2)	1.7 ± 0.2 (1.2–2.1)	1.7 ± 0.2 (1.3–2.2)	10.3
20.B.out.l/B.out.w	2.5 ± 0.5 (1.7–3.4)	2.9 ± 1.1 (2–6.2)	2.2 ± 0.3 (1.7–2.8)	2.3 ± 0.5 (1.5–3.3)	2.3 ± 0.6 (1.7–3.4)	2.6 ± 0.6 (2.1–4)	2 ± 0.5 (1.3–2.8)	2.2 ± 0.4 (1.5–3.1)	2.2 ± 0.4 (1.7–3.2)	2.2 ± 0.4 (1.2–2.8)	2.7 ± 0.5 (1.8–3.3)	2.5 ± 0.5 (1.6–3.3)	21.4
21.B.inn.l/B.inn.w	6 ± 0.7 (4.4–6.9)	5.7 ± 1.3 (2–7.8)	5.4 ± 0.5 (4.5–6)	5.8 ± 1.4 (4.2–9.3)	6.5 ± 0.7 (5.4–8)	6.3 ± 0.7 (4.9–7.1)	5.8 ± 0.9 (4.4–8.4)	6.6 ± 0.9 (4.7–8.2)	6.4 ± 0.6 (5.2–7.6)	5.6 ± 1 (4.1–8.5)	5.3 ± 0.9 (3.8–7.5)	6 ± 0.9 (5.0–7.8)	14.8
22.Cyp.l/Cyp.w	2.8 ± 0.7 (1.8–4.2)	2.4 ± 0.3 (2–3.3)	2.4 ± 0.4 (2–3.2)	3.8 ± 0.9 (2.9–5.5)	2.7 ± 0.4 (2.1–3.4)	2.5 ± 0.3 (2–3.2)	2 ± 0.3 (1.5–2.5)	2.5 ± 0.4 (1.8–3)	2.8 ± 0.7 (1.9–4.3)	2.8 ± 0.4 (2.5–3.5)	2.6 ± 0.3 (2.1–3.1)	2.5 ± 0.4 (1.9–3.5)	16.8
23.Syn.l/Cyp.n	1.1 ± 0.2 (0.7–1.6)	1.3 ± 0.2 (0.9–1.8)	1.1 ± 0.3 (0.7–1.6)	0.8 ± 0.2 (0.5–1)	1.4 ± 0.3 (0.9–2)	1.4 ± 0.2 (1–1.6)	1.4 ± 0.3 (1.1–1.9)	0.8 ± 0.2 (0.6–1.3)	0.9 ± 0.2 (0.5–1.3)	1 ± 0.2 (0.7–1.3)	0.8 ± 0.2 (0.5–1.0)	0.9 ± 0.2 (0.6–1.4)	21.6
24.L.M	f(1)=4 – f(2)=6 – f(3)=4	f(1)=5 – f(2)=10	f(1)=4 – f(2)=3 – f(3)=2	f(1)=5 – f(2)=4 – f(3)=6	f(1)=8 – f(2)=5	f(1)=12 – f(2)=3	f(1)=15 – f(2)=1	f(1)=10 – f(2)=5	f(1)=7 – f(2)=6 – f(3)=2	f(1)=9 – f(2)=6	f(1)=8 – f(2)=5 – f(3)=2	f(1)=3 – f(2)=7 – f(3)=5	37.5
25.Lax.fasch	f(1)=2 – f(2)=12	f(1)=7 – f(2)=8	f(1)=5 – f(2)=4	f(1)=8 – f(2)=7	f(1)=7 – f(2)=6	f(1)=5 – f(2)=10	f(1)=3 – f(2)=13	f(1)=11 – f(2)=4	f(1)=1 – f(2)=14	f(1)=3 – f(2)=12	f(1)=3 – f(2)=12	f(1)=7 – f(2)=8	28.4
26.Lax.fasch.FL	f(1)=10 – f(2)=4	f(1)=12 – f(2)=3	f(1)=7 – f(2)=2	f(1)=12 – f(2)=3	f(1)=11 – f(2)=2	f(1)=13 – f(2)=2	f(1)=10 – f(2)=6	f(1)=14 – f(2)=1	f(1)=11 – f(2)=4	f(1)=12 – f(2)=3	f(1)=8 – f(2)=7	f(1)=14 – f(2)=1	33.0
27.P. apical cell	f(1)=13 – f(2)=1	f(1)=13 – f(2)=2	f(1)=8 – f(2)=1	f(1)=15	f(1)=13	f(1)=15	f(1)=15 – f(2)=1	f(1)=14 – f(2)=1	f(1)=14 – f(2)=1	f(1)=13 – f(2)=2	f(1)=14 – f(2)=1	f(1)=12 – f(2)=3	20.6
CV (%)	18.5	20.3	20.9	19.0	17.6	17.7	17.4	19.9	19.2	20.1	20.7	21.4	

Table 6
Morphological data on Helichrysum species from herbarium specimens. Numerical variables are reported: mean values, standard deviation, and minimum and maximum values. Categorical variables are reported in the absolute frequency for each level (Table S.3); in levels where the frequency was zero (f(x)=0) they are not reported in the table.

Morphological variables	*Helichrysum italicum* subsp. *italicum* *N = 14*	*Helichrysum italicum* subsp. *microphyllum* *N = 5*	*Helichrysum italicum* subsp. *siculum* *N = 3*	*Helichrysum italicum* subsp. *tyrrhenicum* *N = 6*	*Helichrysum litoreum* *N = 12*	*Helichrysum stoechas* *N = 9*	CV (%)
01.L.l	21.7 ± 7.6 (12.6–37.4)	5.1 ± 1.7 (3.2–7.9)	9.8 ± 0.3 (9.4–10)	7.9 ± 2.4 (5.1–11.1)	38.7 ± 7.1 (29.1–53.9)	17.3 ± 7.9 (11.1–30.7)	27.7
02.L.w	0.8 ± 0.2 (0.6–1.1)	0.6 ± 0.1 (0.5–0.8)	0.5 ± 0 (0.5–0.5)	0.7 ± 0.1 (0.6–0.8)	1.1 ± 0.2 (0.8–1.6)	0.8 ± 0.2 (0.6–1)	16.8
03.L.gland	15.2 ± 8.3 (7–38.5)	25.1 ± 14.1 (11–37.5)	16.9 ± 4.1 (14–19.8)	13.3 ± 6 (6.5–21.8)	4 ± 3.6 (0.7–9.7)	11.1 ± 6.8 (4–20.7)	55.3
04.Syn.l	28.8 ± 7.2 (11–40.8)	16.8 ± 3.9 (11.8–20.8)	31.4 ± 23.1 (17.8–58)	23.5 ± 4.7 (15.6–29.5)	45.4 ± 19.2 (25.7–80.8)	29.1 ± 6.9 (19.8–42.2)	34.6
05.Cap.n	26.1 ± 10.1 (14.3–46)	11.7 ± 5.8 (4.6–18)	26±32.1 (5–63)	18.9 ± 6.6 (6–24.4)	52.4 ± 37.1 (16–141.5)	13.8 ± 3.4 (7.7–18.2)	57.0
06.Cap.l	4.5 ± 0.3 (4.1–5.1)	4.7 ± 0.4 (4.3–5.2)	5.1 ± 0.3 (4.9–5.4)	4.9 ± 0.4 (4.5–5.5)	4.8 ± 0.4 (4.2–5.3)	6.5 ± 1.1 (5.1–8.4)	9.1
07.Cap.w	3 ± 0.3 (2.6–3.5)	3.2 ± 0.2 (3–3.4)	3.5 ± 0.2 (3.3–3.7)	3.1 ± 0.4 (2.6–3.5)	3.4 ± 0.3 (3.1–4)	7.1 ± 1.3 (4.6–9.1)	10.3
08.B.out.l	1.2 ± 0.2 (1–1.7)	1.3 ± 0.2 (1.2–1.6)	1.2 ± 0.1 (1.1–1.3)	1.5 ± 0.3 (1.2–1.9)	1.5 ± 0.3 (1.1–2)	3.4 ± 0.4 (2.8–4)	14.4
09.B.out.w	0.5 ± 0.1 (0.4–0.7)	0.5 ± 0.1 (0.4–0.5)	0.4 ± 0 (0.4–0.5)	0.6 ± 0.1 (0.5–0.7)	0.6 ± 0.1 (0.4–0.8)	1.5 ± 0.3 (1.1–2)	14.0
10.B.inn.l	3.9 ± 0.3 (3.4–4.4)	3.8 ± 0.4 (3.4–4.2)	4.5 ± 0.1 (4.4–4.6)	3.9 ± 0.2 (3.7–4.1)	3.6 ± 0.4 (3.1–4.2)	4.8 ± 0.5 (4.3–5.7)	7.7
11.B.inn.w	0.7 ± 0.1 (0.4–0.8)	0.6 ± 0.1 (0.5–0.7)	0.7 ± 0 (0.7–0.8)	0.7 ± 0.2 (0.5–0.9)	0.6 ± 0.1 (0.4–0.7)	0.9 ± 0.1 (0.8–1)	14.8
12.B.inn.gland	38.3 ± 8.6 (25.3–60)	22.6 ± 4.8 (15–27)	22±1.4 (21–23)	31.7 ± 8.9 (23–42.7)	21.8 ± 6.1 (15.8–33)	23.1 ± 6.8 (15.3–37.5)	22.6
13.P.l	3.1 ± 0.3 (2.6–3.5)	3.1 ± 0.2 (2.8–3.4)	3.5 ± 0.1 (3.4–3.6)	3.2 ± 0.2 (2.9–3.4)	3 ± 0.2 (2.6–3.5)	3.8 ± 0.2 (3.5–4.1)	6.2
14.Cyp.l	0.7 ± 0.1 (0.5–0.8)	0.9 ± 0.1 (0.7–0.9)	0.9 ± 0 (0.9–0.9)	0.7 ± 0.1 (0.6–0.8)	0.7 ± 0.1 (0.6–0.9)	0.7 ± 0.1 (0.6–0.8)	11.2
15.Cyp.w	0.3 ± 0.1 (0.1–0.4)	0.4 ± 0.1 (0.3–0.5)	0.4 ± 0 (0.4–0.4)	0.3 ± 0.1 (0.2–0.4)	0.3 ± 0.1 (0.2–0.4)	0.3 ± 0.1 (0.2–0.4)	28.6
16.Cyp.gland	2.4 ± 0.6 (1.8–3.5)	1.2 ± 0.2 (1–1.3)	1.6 ± 0.2 (1.5–1.8)	1.2 ± 1 (0–2)	2.1 ± 0.8 (1.3–3.1)	1.8 ± 0.9 (1–2.7)	38.7
17.L.gland_FL	17±8.7 (5.1–36)	29.9 ± 15.3 (10.6–44.8)	17.7 ± 3.6 (13.8–21)	15.5 ± 7.7 (5–25.5)	3.4 ± 2.7 (0.6–8.3)	16.8 ± 12.4 (6.3–43.2)	54.5
18.L.l/L.w	29.9 ± 9.8 (18.1–49.2)	8.4 ± 1.7 (6.4–10.6)	20.5 ± 2.5 (18.2–23.1)	11.6 ± 2.7 (8.5–15.3)	35.8 ± 3.9 (29.2–42.9)	22.6 ± 6.6 (17.7–35.4)	21.6
19.Cap.l/Cap.w	1.5 ± 0.1 (1.3–1.7)	1.5 ± 0.1 (1.3–1.6)	1.5 ± 0 (1.4–1.5)	1.6 ± 0.2 (1.3–1.7)	1.4 ± 0.1 (1.2–1.5)	0.9 ± 0.1 (0.8–1.1)	8.0
20.B.out.l/B.out.w	2.5 ± 0.4 (1.9–3.6)	2.7 ± 0.3 (2.2–3.1)	2.6 ± 0.2 (2.4–2.7)	2.5 ± 0.3 (2.1–3)	2.6 ± 0.3 (2.3–3.2)	2.3 ± 0.5 (1.7–3.2)	14.0
21.B.inn.l/B.inn.w	6.2 ± 1.4 (4.5–8.9)	6.6 ± 0.6 (5.7–7.3)	6.5 ± 0.4 (6–6.9)	6.3 ± 1.6 (4.4–8)	6.9 ± 1.3 (4.7–8.6)	5.4 ± 0.5 (4.6–6.1)	15.4
22.Cyp.l/Cyp.w	2.7 ± 1.1 (1.8–5.3)	2.4 ± 0.6 (1.8–2.9)	2.5 ± 0.2 (2.3–2.6)	2.5 ± 0.6 (1.9–3.4)	3 ± 0.8 (2–4.1)	2.5 ± 0.6 (1.8–2.9)	24.4
23.Syn.l/Cyp.n	1.2 ± 0.4 (0.8–1.9)	1.8 ± 0.6 (1.1–2.6)	2.4 ± 1.5 (0.9–3.9)	1.5 ± 0.6 (1–2.7)	1.1 ± 0.4 (0.5–1.7)	2.2 ± 0.3 (1.8–2.7)	36.7
24.L.M	f(1)=5 - f(2)=6 - f(3)=3	f(1)=1 - f(2)=2 - f(3)=2	f(1)=1 - f(2)=1 - f(3)=1	f(1)=1 - f(2)=4 - f(3)=1	f(1)=12	f(1)=5 - f(2)=1 - f(3)=3	36.0
25.Lax.fasch	f(1)=5 - f(2)=9	f(2)=5	f(1)=1 - f(2)=2	f(2)=6	f(1)=12	f(1)=9	10.8
26.Lax.fasch.FL	f(1)=12 - f(2)=2	f(1)=3 - f(2)=2	f(1)=1 - f(2)=2	f(1)=3 - f(2)=3	f(1)=11 - f(2)=1	f(1)=8 - f(2)=1	33.1
27.P. apical cell	f(1)=11 - f(2)=3	f(1)=5	f(1)=3	f(1)=6	f(1)=7 - f(2)=5	f(1)=8 - f(2)=1	16.9
CV (%)	30.0	21.2	18.1	25.0	24.9.	25.0	

4. Discussion

This study investigated for the first time the morphological and genetic diversity of *Helichrysum* of the Tuscan Archipelago (Italy).

In general, the 12 studied populations exhibited relatively low values of diversity both at morphological and molecular levels. Within the population, morphological traits exhibited a mean CV ranging from 17.4 to 21.4 (Table 5). These values are significantly lower than the CV observed for other Asteraceae species, such as *Artemisia absinthium* L. (10.6–45.3%) (Nguyen et al., 2017), *Santolina rosmarinifolia* L. aggregate (CV: 10.0 −68.0%) (Rivero-Guerra, 2011), *Crepis sancta* (L.) Bomm. (6.1–106.1) (Imbert, 2001), *Solidago gigantea* Ait. (8.54–63.0%) (Weber, 1997). On the other hand, studies conducted on populations of Asteraceae species reported a CV value around 20% as a threshold to be indicative of low-medium morphological variation (Nooryazdan et al., 2010; Rivero-Guerra, 2011). Using ISSR markers to measure the genetic polymorphism, we found a strongly reduced genetic diversity within the analysed populations as it has also been detected by other authors investigating *H. italicum* (Arbeiter et al., 2021; Ninčević et al., 2021). In addition, regarding the Shannon information index, our result (I = 0.213) was comparable with the values observed by Ninčević et al. (2021) in the *H. italicum* populations on the Eastern coast of the Adriatic Sea (I = 0.355) and by Scialabba et al. (2008) in Sicilian *Helichrysum* taxa (I = 0.255).

The AMOVA genetic divergence among Tuscan Archipelago populations is rather low (9%), and this was confirmed by the Gst value (0.1526) indicating that most of the genetic variation was mainly related to the within-population component. Our results were in accordance with the ones obtained by Ninčević et al. (2021), which revealed only 6.92% of genetic variation among several *H. italicum* populations in

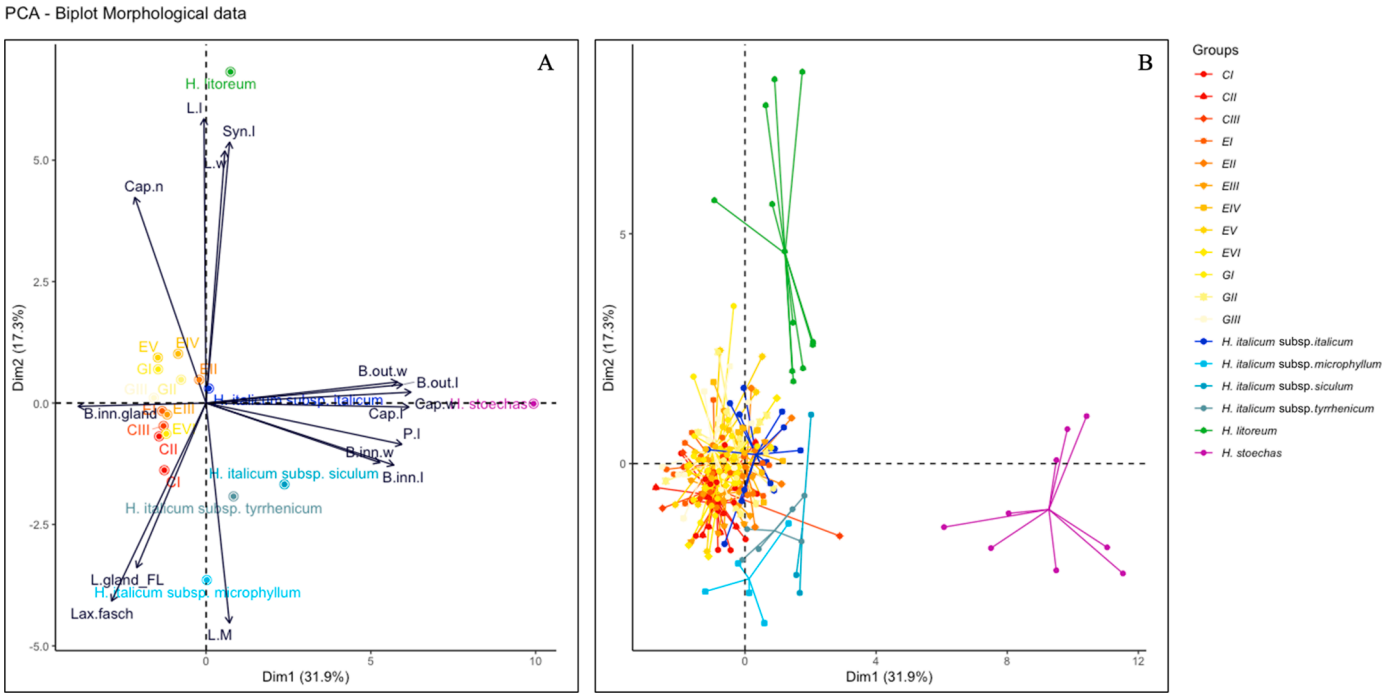

Fig. 3. Principal component graph of morphological characters based on the first two components. (A) Centroid PCA with eigenvalues that represent morphological significant variables. (B) Star-plot PCA that shows the distribution of singular specimens.

Table 7
LDA results at the species level. Percentage of attribution to each group based on the discriminant morphological traits is highlighted.

	Attribution (%)	*H. litoreum*	*H. italicum* (s.l.)	*H. stoechas*
H. litoreum	100	8	0	0
H. italicum (s.l.)	100	0	137	0
H. stoechas	100	0	0	9
Total	100	8	137	9

Balkan Adriatic coast through the analysis of the molecular variance, as well as a low average F_{ST} value of 0.036. This low differentiation found in the Archipelago populations is presumably caused by a relatively high gene flow among individuals, as confirmed by the detected Nm value of 2.7557 and suggested by the correlation of genetic data with geographic distance. To this aim, Hutchison and Templeton (1999) observed that the variation among populations is predominantly caused by genetic drift, leading to a gene flow value lower than 1. Consequently, in our study, the discrimination among populations is prevented by a great gene flow (Nm > 1), dwindling the effect of genetic drift. The degree of gene flow among populations might be the result of several factors such as geographical distance among the populations, population size, mating system and life history traits. *Helichrysum* species have been reported as outcrossing, entomophilous and anemochorous (Galbany-Casals et al., 2011; Ninčević et al., 2021). As reported by Herrando Moraria et al. (2017), the putative excellent dispersal ability of the *Helichrysum* tiny achenes (~1 mm) combined with long-distance dispersal events could favor gene flow events between far populations. In our case, gene flow could occur with some frequency on *Helichrysum* populations in the Tuscan Archipelago, as the islands appear to be well exposed to winds from both Corsica and Italy (Vittorini, 1976). These reproductive characteristics of the mating system have been proven to facilitate the transfer of genetic material among populations and the consequent admixture (Govindaraju, 1988).

The multivariate analyses (PCA, LDA and PCoA) carried out on the Tuscan Archipelago populations and reference taxa both at the morphological and genetic level, clearly distinguish three clusters, and all the 12 Tuscan Archipelago populations were mostly grouped together and clustered with the reference group of *H. italicum* subsp. *italicum*, with a significant correlation of morphological and genetic data. A medium rate of variation among groups was found at the morphological level: for *H. litoreum* and *H. stoechas* the CV was 25%, while for *H. italicum* subsp. *italicum* was 30%, the highest value recorded in our study. This agreed with what has been reported by Galbany et al. (2006) where *H. italicum* was described as morphologically highly variable. However, here we have included a low number of samples of *H. stoechas*, which is also a widely distributed species in the Mediterranean basin and highly variable in terms of morphology, so the comparison of the CV of this species with *H. italicum* obtained here may not be representative of the total variation found in its whole distribution area. Moreover, based on the AMOVA results considering the Tuscan Archipelago populations and the three reference species, our study reported a 21% of variability among species (Table 11). Although Azizi et al. (2019) analysed a larger number of *Helichrysum* species (i.e., 19) by using ISSR molecular markers, they detected that only 24% of the total genetic variation was among the species.

Table 8
LDA results at the subspecies level. Percentage of attribution to each group based on the discriminant morphological traits is highlighted.

	Attribution (%)	*H. italicum* subsp.*italicum*	*H. italicum* subsp. *tyrrhenicum*	*H. italicum* subsp. *siculum*	*H. italicum* subsp. *microphyllum*
H. italicum subsp. *italicum*	100	126	0	0	0
H. italicum subsp. *tyrrhenicum*	80	0	4	0	1
H. italicum subsp. *siculum*	50	0	1	2	0
H. italicum subsp. *microphyllum*	100	0	0	0	6
Total	97,8	126	5	3	7

Table 9
Genetic parameters of 12 Tuscan Archipelago Helichrysum populations and the three reference taxa. The parameters are number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (h), Shannon's information index (I), and the percentage of polymorphic loci (PPB) are described.

Population	Na	Ne	h	I	PPB
GI	1.511	1.243	0.150	0.233	51.140
GII	1.466	1.226	0.141	0.218	46.590
GIII	1.421	1.189	0.118	0.185	42.050
EI	1.500	1.236	0.149	0.232	50.000
EII	1.375	1.203	0.124	0.189	37.500
EIII	1.432	1.192	0.121	0.190	43.180
EIV	1.223	1.186	0.120	0.191	44.320
EV	1.602	1.271	0.173	0.271	60.230
EVI	1.511	1.238	0.147	0.230	51.140
CI	1.386	1.233	0.137	0.206	38.640
CII	1.489	1.260	0.157	0.239	48.860
CIII	1.409	1.194	0.124	0.193	40.910
H. italicum subsp. *italicum*	1.307	1.206	0.120	0.177	30.680
H. litoreum	1.455	1.282	0.167	0.250	45.450
H. stoechas	1.455	1.179	0.115	0.185	45.450
Mean	**1.436**	**1.223**	**0.137**	**0.213**	**45.076**

Table 10
Genetic differentiation parameters calculated within the 12 Tuscan Archipelago Helichrysum populations and considering H. italicum subsp. italicum, H. stoechas and H. litoreum references specimens (Total). The parameters were: the total genetic diversity (Ht), the mean intra-specific genetic diversity (Hs), the coefficient of genetic differentiation (Gst) and the gene flow (Nm) are reported.

Population	Ht	Hs	Gst	Nm
Tuscan Archipelago populations	0.1631	0.1382	0.1526	2.7557
Total	0.1711	0.1374	0.1971	2.0374

Table 11
AMOVA results: the first test was carried out considering only the 12 Tuscan Archipelago Helichrysum populations; the second test was carried out considering H. italicum subsp. italicum, H. stoechas and H. litoreum references specimens and including the 12 Tuscan Archipelago Helichrysum populations as H. italicum subsp. italicum.

Source	df	SS	MS	Est. Var.	%	PhiPT
Among Tuscan Archipelago populations	11	167.020	15.184	0.668	9%	
Within Tuscan Archipelago populations	142	942.668	6.639	6.639	91%	
Total	153	1109.688		7.306	100%	0.091***
Among Species	2	93.160	46.580	1.905	21%	
Within Species	179	1276.653	7.132	7.132	79%	
Total	181	1369.813		9.037	100%	0.210***

*** p-value <0.001.

Helichrysum italicum (Roth) G. Don subsp. *italicum* occurs throughout the Italic peninsula (Pignatti, 1977, 1982; Galbany-Casals et al., 2006; Pignatti et al., 2017), and the occurrence of individuals with intermediate morphological characteristics has been reported in sympatric areas where more than one *Helichrysum* taxa belonging to sect. *Stoechadina* occur (Galbany-Casals et al., 2011; Herrando-Moraira et al., 2017). In the case of *H. litoreum*, the *locus classicus* is reported on the garrigues of the Amalfi Coast (Pignatti et al., 2017), but it is also widespread in the Aeolian Islands, in the Pontine Islands and Capri Island, even in the Circeo promontory in the Lazio Region (Aghababyan et al., 2009).

In our research, the 12 populations of the Tuscan Archipelago differ from *H. litoreum* because this species has wider and longer leaves, larger synflorescence diameter and a higher number of capitula. According to taxonomic evidence (Galbany-Casals et al., 2006; Pignatti et al., 2017), these morphological characters are considered to discriminate between *H. litoreum* and *H. italicum*. However, in the PCA and PCoA graphs, some specimens from Tuscan Archipelago populations and *H. litoreum* were relatively close. In detail, some individuals from Elba Island populations (especially EIV, but also EV and GI) appeared to be more dispersed in the multivariate space, approaching *H. litoreum* specimens. This finding can partially account for the different results presented in the previous study (Marini et al., 2022) where a rather clear morphological differentiation between EVI (Monte Capanne) and EIV (Capo d'Enfola) suggested the identification of two different species: *H. italicum* (EVI) and *H. litoreum* (EIV). This was in accordance with some taxonomic studies on Elba Island (Foggi et al., 2006; Giuliani et al., 2016; Carta et al., 2018b), where the occurrence of *H. litoreum* was reported from Capo d'Enfola. However, in our experiment, the presence of the reference clusters of *H. italicum* and *H. litoreum* reduced the morphological differences between EIV and EVI; in fact, it is evident that most specimens from both populations belong to the *H. italicum* cluster. This evidence makes Capo d'Enfola a controversial *Helichrysum* population, for which more in-depth studies are needed. Nevertheless, it can be hypothesized that some individuals in the population are the result of past or present hybridization and/or introgression events caused by gene flow between *H. litoreum* and *H. italicum* subsp. *italicum*, discussed above (Galbany--Casals et al., 2006; Herrando-Moraira et al., 2016).

Floristic literature reported the occurrence of *H. italicum* subsp. *microphyllum* in Monte Capanne (Foggi et al., 2006; Giuliani et al., 2016; Carta et al., 2018b). However, Galbany-Casals et al. (2011), based on morphological and genetic data, showed that in the *H. italicum* complex there is high gene flow between subspecies and a high correlation between genetic distance and geographic distance. Based on these results, subsp. *microphyllum* was considered to be restricted to Crete Island in the eastern Mediterranean, while morphologically similar populations found in the western Mediterranean, in Corsica and Sardinia, are currently recognized to belong to subsp. *tyrrhenicum*. In our present work, we have several specimens belonging to both subsp. *microphyllum* and subsp. *tyrrhenicum* have been included in the morphological analyses, and the results clearly show them separated from Tuscan specimens collected in Monte Capanne. Thus, our current results are in accordance with the recently updated inventory of the vascular flora of Elba Island, in which only *H. italicum* subsp. *italicum* was reported on Monte Capanne (Carta et al., 2018b, 2018a).

Furthermore, the species of *Helichrysum stoechas* (L.) Moech. was reported to occur naturally on the beaches of the northern coast of Tuscany (Galbany-Casals et al., 2012; Ciccarelli, 2014; Herrando-Moraira et al., 2016). As mentioned above, in the Tuscan Archipelago, *H. stoechas* was reported only from Elba Island, on the stable dunes of Lacona beach (Carta et al., 2018b, 2018a). In our multivariate analysis, we include three sampled from Lacona beach (Dune 1, Dune 2, Dune 3; in detail Table S.2) and was clearly identified as *H. stoechas*, clustering together with the other reference specimens belong to *H. stoechas*. On the other hand, all the 12 populations of the Tuscan Archipelago, clearly differ from *H. stoechas* cluster both for morphological and genetic parameters. In detail, based on morphological analysis, the differences were mainly driven by the capitula and florets traits between *H. stoechas* and *H. italicum: H. stoechas* has larger and rounded shape capitula, wider and bigger outermost bracts and longer pappus than *H. italicum*. Accordingly, Galbany-Casals et al. (2006) monograph defined that outermost capitula bract dimensions was one of the most discriminating morphological traits between *H. stoechas* and *H. italicum.* Interestingly, one specimen from Capraia is placed in an intermediate position between *H. italicum* and *H. stoechas* in the morphological analysis. However, this specimen is placed within *H. italicum* variation in the genetic analysis. This, and the apparent absence of *H. stoechas* in Capraia island, may suggest that this morphologically intermediate appearance could be caused by ancient hybridization/hybridization with geographically distant populations, for example, those from continental Italy, or caused by an environmental adaptation. The examination of additional

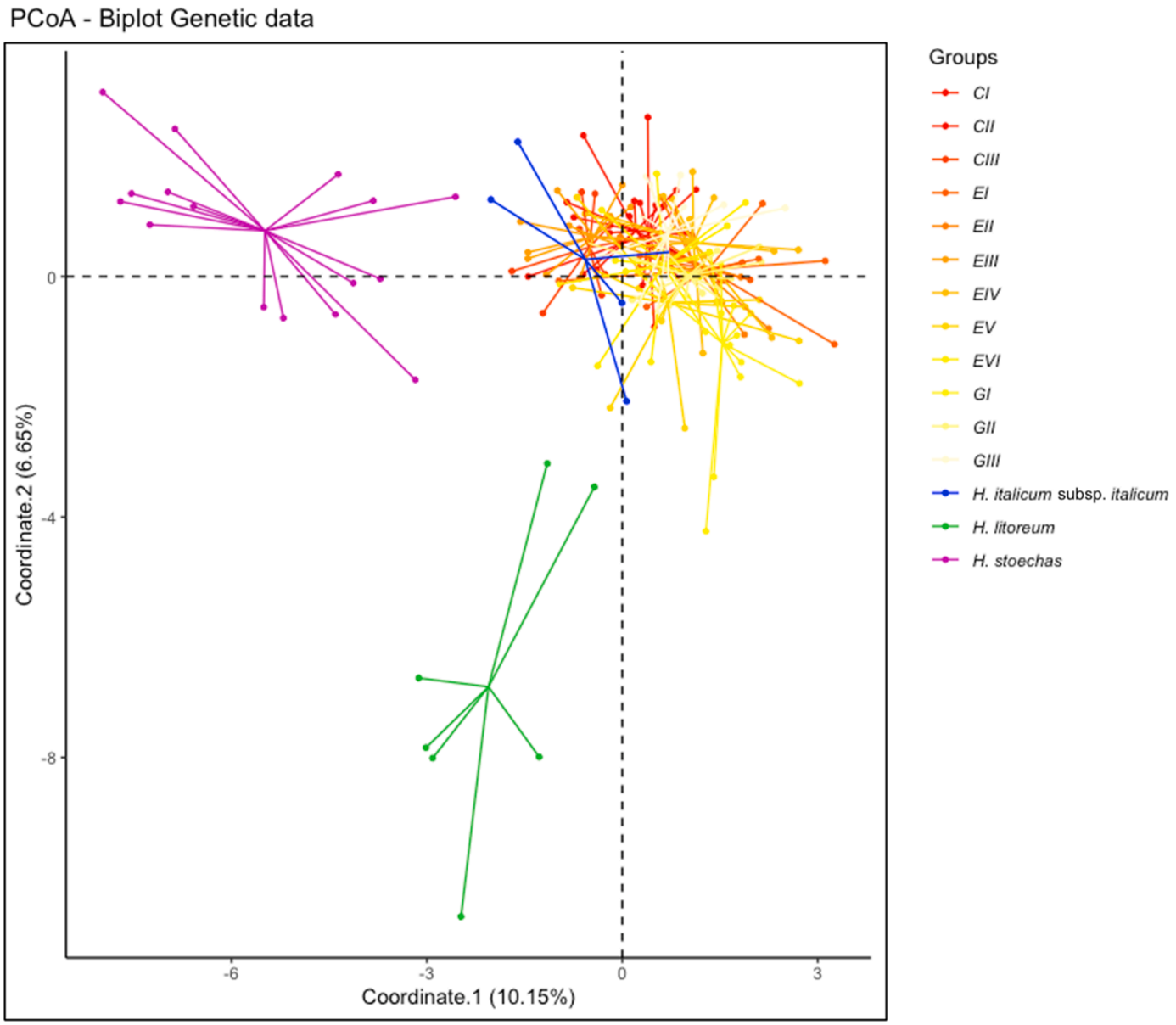

Fig. 4. Principal Coordinates Analysis (PCoA) based on the first two coordinates.

markers, such as chloroplast sequences, and additional specimens, could provide helpful information (Galbany-Casals et al., 2012). Intermediate forms between *H. stoechas* and other species of *Helichrysum*, including *H. italicum,* have previously been reported from Spain and Greece (Galbany-Casals et al., 2012; Herrando-Moraira et al., 2016). Moreover, further analyses could be necessary to completely verify the presence of *H. stoechas* in other populations of the Tuscan Archipelago.

5. Conclusions

The investigation of morphological and molecular variation in twelve wild populations of *Helichrysum* provides valuable information on the complex taxonomy of this genus. The high degree of morphological and genetic similarity among the Tuscan Archipelago populations, and the lack of clear gaps in the pattern of variation, suggest that all the specimens examined could belong to *H. italicum* subsp. *italicum*. However, Future studies are needed that analyze in detail the presence and distribution of *H. italicum* subspecies in the Tuscan Archipelago. The study confirmed the discriminant morphological traits between the main taxa: *Helichrysum litoreum* has longer and wider leaves, bigger synflorescences with a higher number of capitula and fewer glandular hairs than *H. italicum; H. stoechas* is distinguishable from *H. italicum* and *H. litoreum* because it has wider capitula and bigger outermost involucral bracts than these two species. Nevertheless, few individuals in the Elba and Giglio populations seem to have intermediate traits between *H. litoreum* and *H. italicum* subsp. *italicum*, and one specimen from Capraia has intermediate traits between *H. stoechas* and *H. italicum* complex. More in-depth studies are needed to verify the hypothesis that these cases are the result of past or present hybridization and/or introgression events and to deeply verify and investigate the correct distribution of *Helichrysum* species in the Tuscan Archipelago.

The low genetic diversity found within *Helichrysum* populations in the Tuscan Archipelago may reduce the fitness of the individuals and may also pose a risk to the species' adaptability from the perspective of the climate changes occurring in the Mediterranean area. *Helichrysum* species, and particularly *H. italicum* subsp. *italicum*, are usually harvested in the wild because of their bioactive essential oil constituents, which make them attractive to the cosmetic and pharmaceutical industries. Human activities, such as habitat fragmentation and degradation, can affect the genetic variation of the populations. Therefore,

protecting wild populations becomes essential and the wild harvest of these populations should be allowed only with official permission.

CRediT authorship contribution statement

Lorenzo Marini: Formal analysis, Investigation, Writing – original draft, Visualization, Methodology. **Lorenzo Bini:** Formal analysis, Investigation, Writing – original draft, Visualization. **Massimo Gori:** Investigation, Methodology. **Stefano Biricolti:** Resources, Writing – review & editing, Methodology. **Mercè Galbany-Casals:** Resources, Writing – review & editing, Methodology. **Bruno Foggi:** Conceptualization, Validation, Writing – review & editing, Methodology. **Enrico Palchetti:** Conceptualization, Project administration, Funding acquisition. **Piero Bruschi:** Conceptualization, Funding acquisition, Validation, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112360.

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