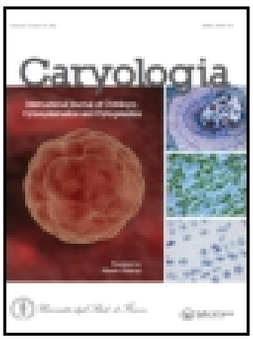

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A probable anthropic origin of *Nerium oleander* L. (Apocynaceae) population in Montecristo island (Italy, Tuscany): evidence from loci polymorphism and ISSR analysis

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ABSTRACT

Nerium oleander (hereafter oleander) is a Mediterranean evergreen shrub representing the only species in the genus *Nerium* (Apocynaceae). Oleander is widespread across the entire Mediterranean basin both as spontaneous native plant and as ornamental cultivated plant. Concerns regarding the native origin of oleander populations may arise particularly in case of ecosystem restoration interventions, when the introduction of alien species or populations should be avoided. Within this work we evaluate the origin of oleander population on the Island of Montecristo (Central Italy), aiming to provide useful information for the implementation of conservation actions on the Island. We used ISSR markers to assess its native or feralized origin by comparison with two reference populations. Our results indicate a probable anthropic origin for the population of Montecristo, which share several characters close to the anthropic reference population, such as a relatively high polymorphism detected and a close ISSR profile.

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Anthropic; domestication; Mediterranean basin; native population; naturalization

Introduction

Nerium oleander L. (hereafter oleander) is a Mediterranean evergreen shrub representing the only species in the genus *Nerium* (Apocynaceae). It usually inhabits temporary streams, primarily ravines, and other highly seasonal streams with extreme variation in the flooding condition, typically with dry conditions for several months during summer (Herrera 1991). It establishes low ligneous galleries and thickets, usually associated with other typical riparian species of the thermo-Mediterranean zone such as some tamarisk species (*Tamarix africana* Poir. and *T. gallica* L.) and chaste tree (*Vitex agnus-castus* L.). These formations represent a habitat worthy of conservation according to the Council Directive 92/43/EEC of 21 May 1992 (Habitat code 92D0: Southern riparian galleries and thickets (*Nerio-Tamaricetea* and *Securinegion tinctoriae*)). Furthermore, oleander is cultivated worldwide as an ornamental plant and naturalizes very easily, so much so that in many regions it is considered subspontaneous (Bella et al. 2006).

Oleander is a species with hermaphrodite flowers, theoretically fully self-compatible, although the spatial separation of pollen and stigma prevents self-fertilization (Herrera 1991), leading to high heterozygosity when the plants are reproduced by seeds. Seed propagation is mainly barochorous and hydrochorous. Indeed, while oleander can be propagated by seed (Pagen 1988), given its allogamy and being highly heterozygous, it shows great variability in seedling populations and growers generally use cuttings and vegetative propagation (Portis et al. 2004). These methods generally focus on the maintenance of selected traits typical of cultivated varieties, mainly related to flower's color and shape (often reflected in a double-corolla), and other discriminating characters such as the presence of foliage variegation and growth habit (Portis et al. 2004).

The native range of the species is usually considered to embrace the entire Mediterranean basin, spanning from Spain and Morocco to the Arabian Peninsula, including also Ethiopia and Niger in Africa and Afghanistan, Iran, Iraq, India and central China in Asia (IUCN – www.iucn.org/). The presence of oleander around the Mediterranean Basin has been documented since the Miocene (Palamarev 1989), indicating that this species belong to the tropical element of the Mediterranean shrub flora (Herrera 1991). However, oleander is so widely cultivated that no precise region of origin has been identified, although southwest Asia has been suggested (Mateu-Andrés et al. 2015), and it is hard to verify the natural origin of the Mediterranean populations. Nowadays, oleander is widely distributed worldwide. It has been introduced in several other parts of Africa and in Azores, Japan, Indonesia, Australia, New Zealand, North, Central and South America (IUCN).

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Recently, the EU Life project RESTO CON LIFE (LIFE13 NAT/IT/000471 – "Island conservation in Tuscany, restoring habitats not only for birds") drew attention to the oleander populations of the Tuscan Archipelago, particularly concerning their natural or allochthonous origin. The project has among its aims the restoration of the habitats on the island of Montecristo, which hosts a population of oleander whose natural origin is under assessment. Although this population occupies the typical habitat of the species, based on the current knowledge it is difficult to assess whether it is native or derives from the cultivated plants still present on the island. Two main factors may help to separate the potentially natural populations from the naturalized ones: (i) plants inhabiting the reference habitat of the species (Habitat 92D0); and (ii) plants not showing the typical traits of domesticated varieties (i.e. "double flowers"). Nevertheless, in a re-naturalization process of the typical natural habitat by individuals deriving from cultivated ones, a shuffle of the traits usually occurs with the loss of the typical characters of the cultivated plants.

In recent decades, molecular approaches have become useful tools in the determination of relationships among natural and anthropogenic populations. Previous studies on oleander using molecular markers showed conflicting features regarding genetic variability of this species. Portis et al. (2004) successfully distinguished commercial varieties via amplified fragment length polymorphisms (AFLP), highlighting a high variability related to the fact that oleander is substantially allogamous and highly heterozygous. On the other hand, Mateu-Andrés et al. (2015), investigating several natural populations from the Mediterranean region, found a low genetic variability among populations based on cpSSR and noncoding cpDNA regions. This lack of differentiation between the natural oleander populations of the Mediterranean area can be explained by the hypothesis of a recent recolonization occurring during the post-glacial period (Cottrell et al. 2005; Fussi et al. 2010, 2012). Considering the low resolution of the methods discussed above we focused on the application of inter simple sequence repeat markers (ISSRs). This genome scanning technique is widely used in plant population genetics due to the reliability and reproducibility of the results (Rakoczy-Trojanowska and Bolibok 2004). In particular, it has been applied to assess genetic variation and population differentiation in a number of species from different areas (Hatcher et al. 2004; Ge, Yu, et al. 2005; Ge, Zhang, et al. 2005; Luo et al. 2007; Coppi et al. 2010).

This work focuses on the evaluation of the origin of the Montecristo oleander population, aiming to provide useful information for the implementation of conservation actions on the island. Into this context, we aimed (1) to assess the genetic structure of the oleander population of Montecristo; (2) to evaluate the use of ISSR molecular markers in the resolution of the natural/anthropogenic origin of an oleander population; and subsequently (3) to investigate the supposed native condition of this species within the island.

We thus studied the genetic profiles of the population of Montecristo and some other populations. The results obtained may provide a necessary guideline for a possible habitat reconstruction on the island. This evaluation is of primary importance toward the restoration of the habitat if the species are considered native, or eventually avoid the spread of new individuals if the population is considered of anthropic origin.

Methods

Area of study and sampling

The present work focus on the oleander populations in the Tuscan Archipelago, particularly in the islands of Montecristo and Capraia. The island of Montecristo (Tuscany, Italy) is situated in the Tyrrhenian Sea (42° 19′ N, 10° 19′ E), west of the Tuscan coast. Over the past 30 years, the island has been subjected to a specific regime of protection, being an integral reserve of the State Forestry Corp., and it is now included in the Tuscan Archipelago National Park. The island, formed by a magmatic intrusive body, has a surface extension of 10.4 km^2 and is mainly mountainous. Oleander is present on the island of Montecristo in only one main established population whose natural origin is here under assessment (hereafter referred as Mont_NAT), distributed in the creek *Vado di Cala Maestra*. In addition, several sparse individuals of anthropic origin (hereafter referred as Mont_ANT) can be retrieved within the settlement, at the cave Grotta del Santo and along the trail which connects the settlement to the cave.

The island of Capraia, which is located 44.8 km north of Montecristo (42° 02′ N, 9° 49′ E) and is also mainly granitic and mountainous, hosts the only other established oleander formations described for the Tuscan Archipelago (Foggi et al. 2001). According to the Authors, this population could be considered of natural origin, and is distributed in two different areas, respectively located in the creek Vado dell'Aghiale and in the creek Vado del Porto.

Sampling design

We sampled oleander individuals from the populations of Montecristo and Capraia, being the two established populations of the Tuscan Archipelago. The samples were collected in order to represent the spatial distribution and genetic variability of the oleander populations of the Tuscan Archipelago. The Montecristo population represented the focus of the study, and included both individuals of a certain anthropogenic origin (coming from the three main sub-sites *Sentiero Del Santo*, *Grotta del Santo* and *Cala Maestra* settlement) and of

potential natural origin (coming from the creek *Vado di Cala Maestra*) (Figure 1(b)). Capraia individuals belonged to the only other established population in the Tuscan Archipelago, and were sampled in two sites *Vado dell'Aghiale* and *Vado del Porto* (Figure 1(a)), aiming to provide the comparison to a close population, whose native origin is uncertain. To provide a further outgroup of certain anthropic origin a further sampling of oleander individuals was carried out in Pistoia (Italy), selecting randomly 12 cultivated individuals along the roadsides.

The plant material used for the molecular analysis was collected from 96 individuals from the different sampling sites in Montecristo (50), Capraia (34) and Pistoia (12) (see Table 1). The sampling was carried out in the autumn of the year 2014. Fresh leaflets were collected from individuals at least 5 m apart, and rapidly dried in silica-gel.

DNA extraction and ISSR profiling

Genomic DNA was extracted from 30–100 mg of leaf tissue, using a CTAB 2X protocol with minor modifications (Mengoni et al. 2006). Quality of extracted DNA was checked by agarose gel electrophoresis (0.6% w/v) in TBE 19 buffer (Tris 0.89 M, EDTA 2 mM, boric acid 0.89 M, pH 8.3) after ethidium bromide (1 μg ml^{-1}) staining. The quantity of DNA was estimated by means

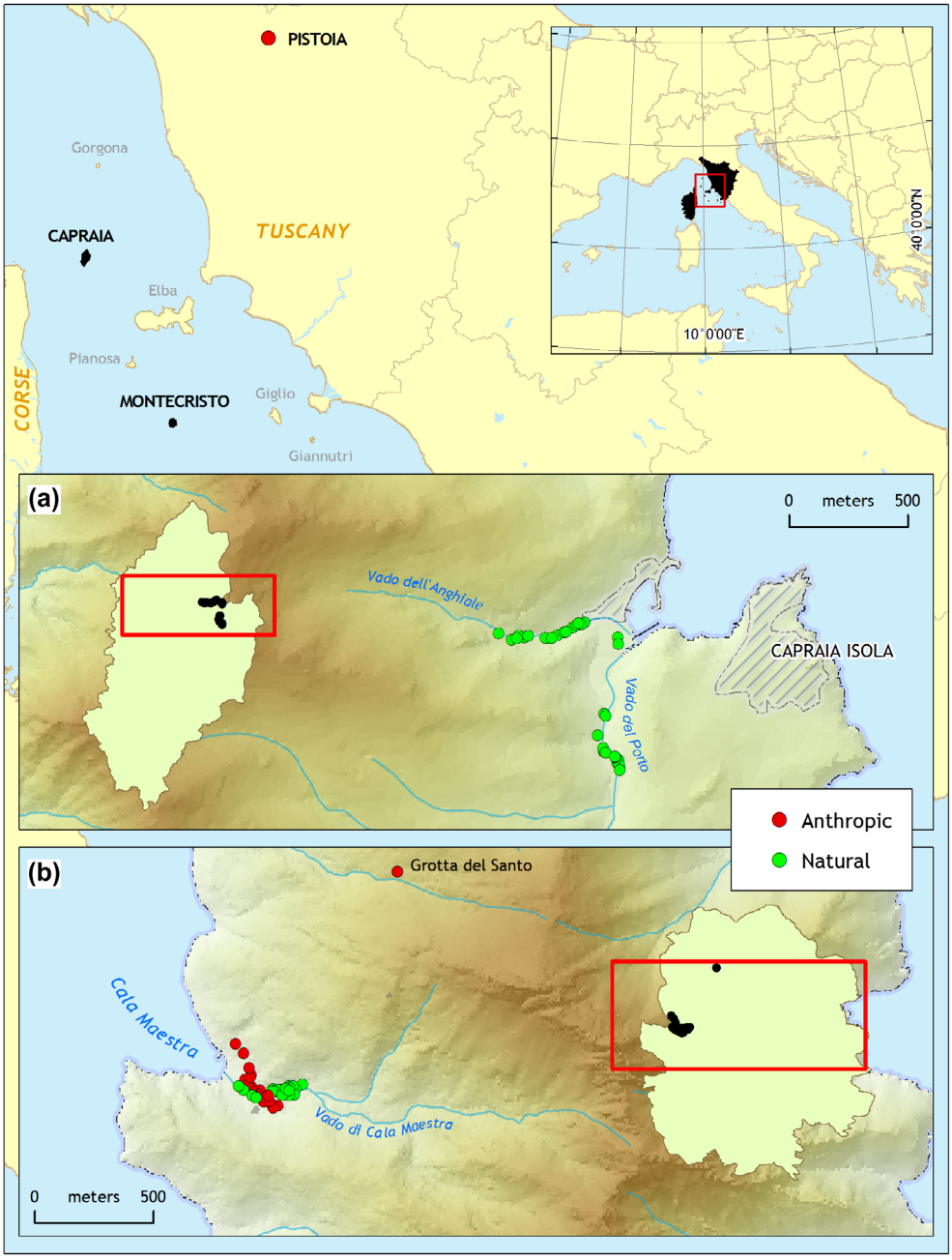

Figure 1. Area of study and sampling sites. (a) detail of the Island of Capraia and relative sampling sites; (b) detail of the Island of Montecristo and relative sampling sites.

Table 1. Number of accessions per sampling site (SS) and island. For each site the natural (NAT) or anthropic (ANT) origin of the individuals is indicated.

Sampling Site	Origin	Number of accessions
Isola di Capraia		**34**
Vado dell'Aghiale	NAT	19
Vado del Porto	NAT	15
Isola di Montecristo		**50**
Vado di Cala Maestra	NAT*	25
Centro Abitato	ANT	9
Sentiero	ANT	12
Grotta	ANT	4
Pistoia	ANT	**12**
TOT		**96**

Note: Figures in bold denote the total amount of sampled individuals.
*Natural origin for the individuals coming for de Creek Vado di Cala Maestra for Montecristo is supposed a priori and is one of the main subjects of study in the paper.

Table 2. Nucleotide sequences and fluorescent staining of each primer exploited in the analyses.

Primers	Label
ISSR4 *(CAAGAGAGAGAGA)*	**HEX:** *(5'-Hexachloro-fluorescein-CE-phosphoramidite)*
ISSR8 *(CACACACACACARG)*	**TET:** *(5'-Tetrachloro-fluorescein-CE phosphoramidite)*

of spectrophotometric readings using a BioPhotometer (Eppendorf, Hamburg, Germany).

The ISSR protocol had provided a preliminarily screening of six combinations of primer pairs on four individuals of each population. Two out of the six primers yielded clear and reproducible banding patterns (Table 2) and were adopted in the further analyses. Polymerase chain reactions were performed in a total volume of 25 µl containing 10 ng of DNA, 2 µl of 109 reaction buffer (Dynazyme II, Finnzyme, Espoo, Finland), 1.5 mM MgCl2, 200 µM deoxynucleoside triphosphates, 2 µl of 10 mM primer and 1.4 U of Taq DNA polymerase (Dynazyme II, Finnzyme). Thermocycling was carried out after an initial denaturing phase of 5 min. at 94 °C followed by 35 cycles each of 40 s at 94 °C, 45 s at 43 °C, and 90 s at 72 °C. A final cycle was set for 45 s at 94 °C, 45 s at 42 °C, and a final extension step of 5 min at 72 °C. Separation of amplification products was performed by capillary electrophoresis on an ABI310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). To ensure reproducibility of the results, replicate ISSR profiles were generated for a total of six individuals from Montecristo and Capraia. All the amplified bands were treated as dominant genetic markers and all ISSR profiles obtained were translated in a rectangular binary matrix.

Data analysis

The analyses were conducted on two levels to assess both the genetic structures of the oleander populations and the natural/anthropogenic origin of this species within the island of Montecristo through the use of ISSR. Firstly, we pooled the individuals according to the population origin, thus distinguishing Montecristo, Capraia and Pistoia. As a secondary step, we separated the samples from Montecristo in two groups according to their (supposed) natural/anthropogenic origin (Mont_NAT vs. Mont_ANT, as previously described in the sampling design section).

Genetic diversity within groups was analyzed by studying the percentage of polymorphic sites in the dataset. Within-population genetic variation was computed as "average gene diversity over loci" (Nei 1987) using the program Arlequin 2.000 (Schneider et al. 2000). The distribution of mismatches, i.e. the observed number of differences between pairs of individuals, was plotted to evaluate whether this distribution tended to a multimodal (demographic equilibrium) or to a unimodal/bimodal pattern, as postulated for populations passed through recent demographic expansions (Slatkin and Hudson 1991; Rogers and Harpending 1992).

The inter-group genetic distances were estimated computing a matrix of linearized pairwise F_{ST} values (Slatkin 1995) which was then used to generate a neighbor-joining dendrogram (Saitou and Nei 1987) using the software MEGA 3.0 (Kumar et al. 2004).

The hierarchical structure of genetic variation within and across groups was assessed by an analysis of molecular variance (AMOVA, Excoffier et al. 1992), as implemented in Arlequin 2.000 (Schneider et al. 2000). The AMOVA was used to analyze the partition of total genetic variation at two different hierarchical levels: within groups and between groups. The statistical support to different hypothetical groupings of individuals was tested in terms of variance components and percentage of explained variation.

Results

Only 80 out of 96 samples provided clear and repeatable profile analyses and were subsequently taken into account during the interpretation of the data. The combination of primers used for the amplification lead to the identification of 127 loci, 44 for ISSR4 and 83 for ISSR8.

Internal genetic diversity within population was higher for Montecristo regarding both the percentage of polymorphic loci and the average gene diversity over loci, while Capraia and Pistoia showed variable outputs (Table 3). Particularly Capraia showed the lowest value regarding the average gene diversity.

Table 3. Estimates of genetic variation of three populations of *N. oleander*.

Pop ID	N.	% pl	avgd	% rf	N.sf
Capraia	23	59	0.171	31	19
Montecristo (all individuals)	47	83	0.206	49	28
Anthropic	23	69	0.199	38	10
Natural	24	72	0.212	39	12
Pistoia	10	47	0.191	20	1

Notes: The following parameters are shown: N. numbers of analyzed individuals; %pl, percentage of polymorphic loci in each sampling site; avgd, average gene diversity over loci; %rf percentage of rare fragments (occurring in up to 10 individuals within the whole dataset) per individual. N.sf, number of private fragments (fragments confined to one population only).

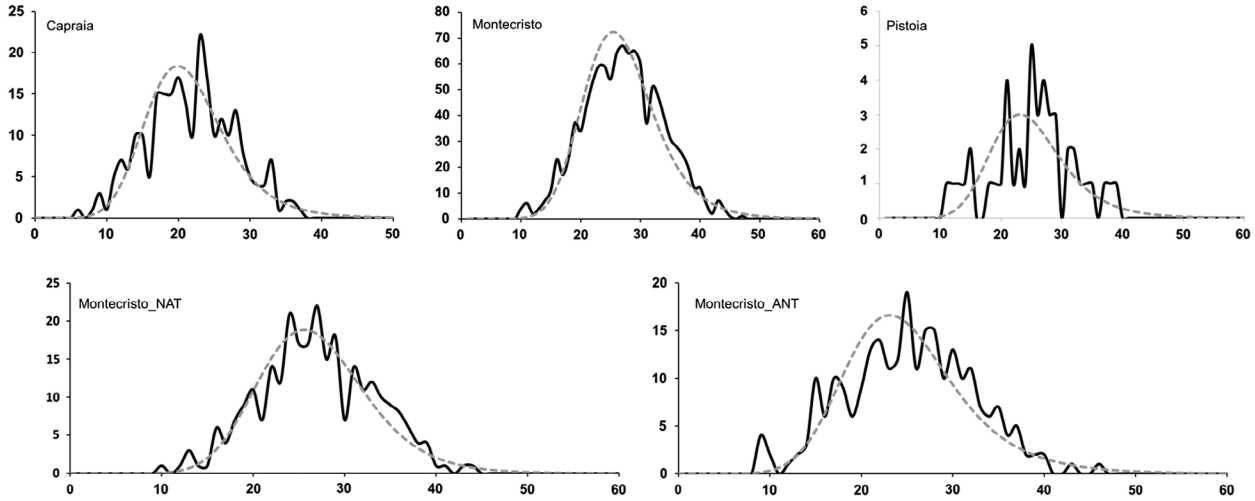

Figure 2. Distribution of number of pairwise differences among samples. The abscissa shows the number of pairwise differences, the ordinate the number of loci showing the mismatch.

Table 4. AMOVA results. Both AMOVA tests resulted significant at $p < 0.001$ after 1023 permutations.

Sampling sites	Variance partition	Df	SS	Variance	% Variance
(1) Across sites	Inter-pop	2	119.89	2.14	14.78
	Intra-pop	77	949.60	12.33	85.22
	Total	79	1069.49	14.47	–
(2) Across sites + Nat vs. Ant origin	Inter-pop	3	133.68	1.66	11.87
	Intra-pop	76	935.81	12.31	88.13
	Total	79	1069.49	13.97	–

Notes: Test (1) took into account the Capraia, Montecristo and Pistoia populations. Test (2) took into account also the supposed natural or anthropic origin of the populations, i.e. Capraia, Mont_ANT, Mont_NAT, Pistoia. Df = degree of freedom; SS= sum of squares.

Considering the population of Montecristo, divided according to the supposed natural or anthropogenic origin of individuals, we found minor differences in the percentage of polymorphic loci: 69% in natural and 72% in the anthropized ones (Table 3). Mont_NAT presented a higher average gene diversity in respect to Mont_ANT, but also when compared to Pistoia and Capraia. Particularly, Pistoia and Mont_ANT presented close values.

The analysis of mismatches revealed slight differences between the three populations. Capraia and Montecristo displayed comparable profiles, close to a unimodal pattern, while, for the population of Pistoia, a multi-modal distribution could be observed (Figure 2). Looking at the profile of mismatches obtained according to the natural/anthropic origin of Montecristo individuals, the profile of mismatches is attributable to a unimodal pattern, similarly to that of Capraia.

The AMOVA analysis results (Table 4), obtained with the geographic subdivision, indicate that even if exists a differentiation between the sampled populations (14.78%), the main source of variation was retrieved within each site (85.22%). Consistently, the subdivision into two groups of the samples from Montecristo showed a small portion of genetic differentiation between the samples (11.87%) linked to the separation among the supposed natural or anthropogenic origin of the population (Table 4), again with a great contribution of variance within each group.

The neighbor-joining tree resulted in two distinct clades: one containing the populations of Pistoia and Montecristo and the other, consisting of the population of Capraia (Figure 3(a)). The topology of the dendrogram confirms the clear separation between Capraia and the group of Pistoia and Montecristo. A similar topology was reached considering also the subdivision into two groups of the samples from Montecristo, with a clear separation of Mont_NAT, which segregate from a first group constituted by Mont_ANT individuals and Pistoia ones (Figure 3(b)).

Discussion and conclusion

Studies based on genetic diversity metrics, obtained by dominant or co-dominant molecular markers (e.g. AFLP, RAPD or ISSR), require a continuous increase of expertise. Parameters such as genetic diversity at an intra-populational level, associated with plants' life-history traits, may be useful to establish comparisons also between phylogenetically well-differentiated species (Hamrick and Godt 1996). In addition, the utility as reference data, of such data frames, become evident for local study of plant species characterized by overdispersion (Coppi et al. 2015).

This study, despite being conducted in a small portion of the distribution area of oleander, highlights medium levels of genetic diversity, slightly higher than those reported by Hamrick and Godt (1996) for long-lived outcrossing taxa (0.180) with a widespread distribution (0.183). Low levels of genetic diversity were confirmed also for taxa distributed in the Mediterranean basin, either on shrub species, such as *Cistus monspeliensis* L. and *Cistus ladanifer* L. (Guzmán and Vargas 2009; Fernández-Mazuecos and Vargas, 2010, 2011), and

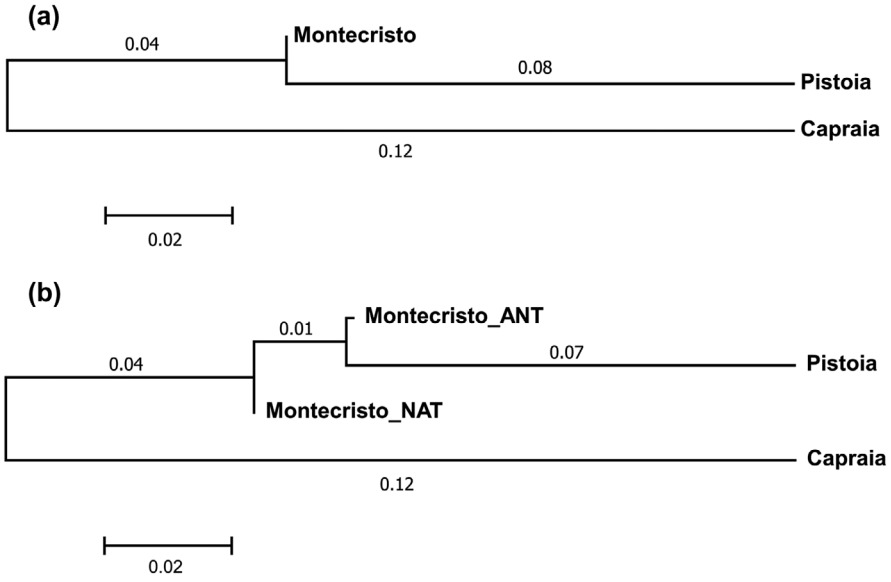

Figure 3. Neighbor-joining dendrogram based on Slatkin's linearized Fst matrix showing the genetic relationships among grouping of sampling sites. (a) Neighbor-joining of the three sampled population. In (b) Montecristo individuals are divided according to their supposed natural (Mont_NAT) or anthropic (Mont_ANT) origin. Scale bar indicates value of genetic distance.

trees, such as *Pinus pinea* L. (Vendramin et al. 2008) and *Fraxinus ornus* L., the latter widely distributed in the islands and in the north of the Mediterranean basin (Heuertz et al. 2006). The causes of this condition are linked to the action of different factors including, the time of persistence of a species in a given region (Hewitt 1996), the different reproduction features of the species (Vendramin et al. 2008) and finally the occurrence of genetic drift (Nora et al. 2014).

A recent study, performed on Mediterranean natural populations of oleander, showed that also for this species there is a high homogeneity at both intra- and inter-population level (Mateu-Andrés et al. 2015). This low variability may be linked to the hypothesis that sees oleander as the protagonist of a recent and rapid recolonization occurred during the last post-glacial period (Cottrell et al. 2005; Fussi et al. 2010, 2012). During the glacial phase, in fact, the low temperatures would have caused the reduction of the distribution of the species, allowing only to the individuals survived in the areas of shelter a subsequent phase of recolonization (Hewitt 1996). Despite our data being essentially consistent with those reported above, only the Capraia population showed low levels of genetic diversity (0.17), while Montecristo and Pistoia appeared more differentiated at the population level. The relatively high polymorphism, and genetic diversity, detected in Montecristo (both "ANT" and "NAT") is a pivotal result, which seems to support an anthropic origin of such population. This hypothesis is also suggested by the general relativeness among Montecristo and Pistoia, which may arise from a possible common anthropic origin of these two populations. A long history of repeated introduction and arrival of new germplasm in both populations may have resulted in higher levels of diversity compared to those characterizing natural populations.

As to the general low genetic diversity of the Capraia population, this seems to be in accordance with its supposed natural origin (following the concepts in Foggi et al. 2001). Nevertheless, the resemblances between Montecristo and Capraia displayed by the analyses of mismatches suggest that both populations are affected by dynamics typical of small isolated populations. Our interpretation here is that the Montecristo population is involved in a "feralization" process, originated from the first introductions for ornamental purposes, as with most of the introductions to this island (Lazzaro et al. 2014; Celesti-Grapow et al. 2016). On the other hand, these results may point to an anthropic origin also for Capraia individuals, whose process of naturalization may have had a longer time to shape the population genetic traits.

The data concerning the hierarchical organization of genetic differentiation, together with those coming from the analyses of genetic diversity, reinforce the suggestion that no natural origin for the Montecristo population should be supposed. The analyses of the values of genetic distance confirm a certain degree of similarity among the populations of Montecristo and Pistoia, separating these two populations from Capraia. The further subdivision of Montecristo individuals according to natural criteria shows a well-defined genetic structure, which highlights a separation of the individuals located in the creek from those of other sampling sites. Individuals of the cave, the path and the town, recognized a priori as non-natural, are, in fact, of greater affinity with those from Pistoia, of certain artificial origin. The data obtained allowed the existence of a genetic structure to be highlighted

for the oleander population of Montecristo. At intra-population level the Montecristo samples have values of genetic diversity similar to those observed for Pistoia, but rather higher compared to those of Capraia. Based on genetic distance values among the populations we could hypothesize an artificial origin for the oleander populations of Montecristo. Particularly we can assume a series of introductions, repeated over time, of germplasm of different origin. Subsequently the population of Vado di Cala Maestra originated from the establishment of feral individuals descending from these introduced plants. The genetic separation of the different sampling sites is confirmed, moreover, by the analysis of molecular variance, which shows that the highest percentage of variance is explained by the spatial separation of individuals, within the individual sampling sites of Montecristo.

In conclusion, our study confirms the suitability of the ISSR technique as easy and cheap tool aiming to retrace the origin of populations for which a history of introduction needs to be tracked down. Specifically for oleander in Montecristo, even if our study took into consideration only the "feral" populations of the Tuscan archipelago and a single certain anthropic sample population, the data suggest that no natural origin of this population should be reasonably supposed.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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