Contribution to the molecular taxonomy of cultivated populations of mastic trees on Chios Island

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Research

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Abstract

Background: The mastic tree (formal name: *Pistacia lentiscus* var. Chia) is extensively cultivated on the southern part of the Greek island of Chios. Its extensive cultivation is due to the production of a special resin, known as mastic gum, or mastiha, which is widely used in several dietary, pharmaceutical and other products since ancient times. Mastic tree is an evergreen, dioecious (consisting of male and female individuals), woody plant. Furthermore, a number of morphological characteristics suggest the existence of several different plant groups, called here “morpho-varieties”, but it is probable that there exist an unknown number of different, deviating genotypes on the island. Nevertheless, five major morphologically determined morpho-varieties of mastic tree are mainly cultivated, namely “Votomos”, “Maroulitis”, “Mavroschinos”, “Stenophyllos” and “Fardyphyllos”. The morpho-varieties exhibit mostly, but not always, distinct morphology, on which their taxonomy was initially based. Since it is observed that different plant populations differ in the quantity and quality of the produced resin, it is important to explore whether these differences have a genetic basis, or are due to other factors, such as agricultural practices, or soil composition. To this end, the genetic diversity among these five morpho-varieties and within each one of them was investigated in the present study, using in a first approximation their Random Amplified Polymorphic DNA profiles and subsequently the sequence divergence of their Internal Transcribed Spacer regions 1 and 2.

Results: The results confirm the existence of a considerable genotypic diversity among the five morpho-varieties studied and, furthermore, reveal the existence of genotypic diversity within each one of them, as well as the existence of a number of further deviating genotypes. The possibility, this diversity to be partly a result of reticulate evolution events is also discussed.

Conclusions: This study provides evidence that the population of cultivated mastic trees on the island of Chios consists mainly of two species, i.e. *Pistacia lentiscus* and *Pistacia* x *saportae*, although it is clear that there also exist genetically deviating mastic trees, as well as individual plants, probably produced through hybridization between the two afore mentioned species.

Background

The mastic tree is an evergreen dioecious shrub or a small tree [1 and ref. therein]. It is cultivated exclusively on the southern part of the Greek island of Chios [2]. Fossils of mastic tree leaves found on the island of Chios, indicating its presence already 6.000 years ago, imply that it is a native plant of the island [3]. The male and female trees are morphologically and genetically different and they also differ considerably in a quantitative and qualitative manner, in respect to the produced resin.

The mastic tree produces a special resin from its wounded trunk and thick branches, known as mastic gum and carrying the commercial name “mastiha” (mastic), which was used since ancient times, among others, also as a medicine (Herodotus, 5th century B.C.). The resin and the ethanol-soluble and acidic fractions of the essential oil of *P. lentiscus* var. Chia have been analyzed by Gas chromatography - Mass spectroscopy (GC-MS) and the majority of their components were identified [4-6].

Recent studies have shown that both mastiha and its essential oil exhibit antibacterial activity against several pathogens, such as *Streptococcus* sp. mutants [7], *Helicobacter pylori* [8], and other Gram positive and Gram negative bacteria [9]. *P. lentiscus*’s resin has been traditionally regarded also as an anti-cancer agent [10], especially on tumors of breast, liver, stomach, spleen and uterus [11]. Furthermore, recent studies demonstrated that Chios’s mastiha induces apoptosis [12] and possesses antiproliferative activity [13] in colon cancer cells, while mastiha oil inhibits growth and survival of human K562 leukemia cells and attenuates angiogenesis [14]. Furthermore, mastiha gum has also been

associated with cardiovascular and hepatic protective activity, inhibiting human LDL oxidation *in vitro* [15] and, thanks to the contained triterpenes, it also acts on peripheral blood mononuclear cells to elicit an antioxidant/ antiatherogenic effect [16].

Regarding the taxonomy of mastic tree, it belongs to the Anacardiaceae family and it is generally considered as a variety of the species *Pistacia lentiscus* L. (*P. lentiscus*). Its exact taxonomic position is though subject to controversy, since it has been considered by some authors as a variety of *P. lentiscus* L., i.e. *P. lentiscus* var. *chia*, or as a cultivar named *P. lentiscus* cv. Chia by Browicz [2 and references therein]. The name "*chia*" was first mentioned by Desfontaine [17], while later, in 1809 Poiret described the mastic tree as a variation of the species *P. lentiscus* [2]. Browicz [2] was the first to notice that the male trees on plantations were morphologically different, depending on the area, in which they were cultivated. These distinct morphological characteristics imply the existence of different male genotypes, which also differ in respect to the quantity and quality of the produced resin. There are five major morphologically determined groups (called here morpho-varieties) of the cultivated mastic trees on the island of Chios: "Votomos", "Maroulitis", "Mavroschinos", "Fardyphyllos" and "Stenophyllos" (or "Siderakikos", or "Psilophyllos"). These different morpho-varieties may sometimes exhibit distinct phenotypes, but these morphological characters (e.g. shape and number of leaflets of the composite leaves) can sometimes be observed also on individuals considered to belong to a different morpho-variety. There are only few reports concerned specifically with the molecular taxonomic analysis of *P. lentiscus*. Zografou et al. [3] studied the genetic diversity mainly among male plants of *P. lentiscus* var. Chia, using Randomly Amplified Polymorphic DNA (RAPD) analysis and Inter-Simple Sequence Repeat (ISSR) markers. They analyzed a limited number of plants belonging to four morpho-varieties, i.e. "Votomos", "Maroulitis", "Mavroschinos" and "Stenophyllos", as well as, a female tree and concluded that some morpho-varieties are genetically closer related with each other ("Maroulitis" and "Mavroschinos"), than with others ("Siderakikos"). The female tree exhibited an even more distant genetic relationship compared with the male trees. A second study, by Abuduli et al. [18], investigated the genetic diversity and phylogenetic relationships of 12 male and 12 female *P. lentiscus* L. trees, originating from various locations of Turkey and one plant from one location on Chios. They used four different techniques, i.e., Simple Sequence Repeat (SSR), RAPD, ISSR and Internal Transcribed Sequence 1 (ITS1). The authors concluded that there exists a great variability among the examined mastic tree genotypes. To a similar conclusion came also the authors Turhan-Serttaş and Özcan [19] using ISSR and Inter-retrotransposon Amplified Polymorphism (IRAP) markers, after examination of specimens originating mainly from Anatolia (Turkey).

The objectives of the present study were: 1) to investigate the phylogenetic relationships among the different cultivated morpho-varieties of male mastic trees, 2) to investigate the genetic diversity among different individuals within each morphologically determined variety and 3) to compare our results with those of known related *Pistacia* species. To this end and in a first approximation, RAPD analysis was employed. The initial results prompted us to use cloning and sequencing of the Internal Transcribed Spacers (ITS) 1 and 2, located between the ribosomal genes (rDNA) [20-26]. The latter method was expected to provide more detailed results, since these regions are not subjected to any natural selection process and therefore are expected to carry a higher degree of variation. In this study, we present data, which verify the existence of a considerable genetic variation, both among different morpho-varieties, and also within some of the morpho-varieties. We further show that most plants examined in this study belong to two different species, i.e. *P. lentiscus* L. and *Pistacia* x *saportae* (*P.* x *saportae*), although, there also exist some genotypes deviating from both species. Notably, the ITS composition of at least one plant seems to be a hybrid between the two prevailing species and this could be explained as a result of a reticulate evolution event.

Results

RAPD-based taxonomic analysis

Total DNA was extracted from leaves of 25 different individual plants (5 plants from each of the morpho-varieties “Votomos”, “Maroulitis”, “Mavroschinos”, “Stenophyllos” and “Fardyphyllos”) and was used as a template in RAPD reactions. Only primer OPG2 yielded the most reliable results, in contrast to primers OPA2 and OPH13 (Table 1), which yielded variable or very poor results (data not shown). Four representative figures of RAPD gels loaded with the reaction products using primer OPG2 are presented in Figures 1, 2, 3 and 4. Figure 1 clearly indicates the existence of at least four distinct kinds of genotypes within “Votomos”. This finding prompted us to examine more “Votomos” individuals (s. below, “ITS-based taxonomic analysis”). In Figure 2 are presented the results obtained from the RAPD reactions using primer OPG2 and DNA from five individuals belonging to “Maroulitis”. In this case, it is obvious that this morpho-variety exhibits a genotypic uniformity. Figure 2 depicts also the results of a comparison of RAPD profiles of “Maroulitis” and “Mavroschinos” plants, where it is obvious that the profiles of the two morpho-varieties differ significantly. Figure 3 presents the results of the amplification of “Mavroschinos” DNA using primer OPG2. The results indicate that individuals belonging to this morpho-variety exhibit a genotypic homogeneity in this assay. Furthermore, the RAPD reactions with DNA from “Stenophyllos” and “Fardyphyllos”, using again primer OPG2, indicate that these morpho-varieties differ from each other, but they also exhibit a limited genotypic variability within themselves (Fig. 4). In specific, among the “Stenophyllos” plants, S1 and S3 exhibit similar profiles, while plants S2 and S5 seem more closely related to each other and different from all other plants of the same morphological variety (Fig. 4). Plant S4 gives rise to a RAPD profile, which differs from the profiles of all other “Stenophyllos” plants. Concerning the plants considered to belong to the morpho-variety “Fardyphyllos”, plants F1 and F2 give rise to identical RAPD profiles, while plants F3, F4 and F5 have RAPD profiles dissimilar from each other and from the ones of plants F1 and F2.

Table 1 RAPD- and ITS- primers used in this study.

Primer	Sequence	Source
OPA-2	(5′- TGC CGA GCT G_- 3′)	[27]
OPG-2	(5′-_GGC ACT GAG G_-_3′)	[27]
OPH-13	(5′-_GAC GCC ACA C_- 3′)	[27]
ITS 18S	5′- GTG AAC CTG CGG AAG GAT C - 3′	This study
ITS 26S	5′- GTA ATC CCG CCT GAC CTG -_3′	This study

ITS-based taxonomic analysis

To verify or disprove the results of the RAPD analysis, a second approach was chosen, i.e. the determination of the sequence of ITS regions 1 and 2. In addition, the apparent genetic heterogeneity of the “Votomos”-group observed in the RAPD analysis, prompted us to examine four more “Votomos” individuals. To this end, total DNA from the same individual plants of the five morpho-varieties (enriched with four additional individuals belonging to “Votomos”) was extracted from young leaves and the ITS regions 1 and 2 of their rDNA, along with the *5.8S rRNA* gene, were amplified by PCR, using specific primers (Table 1). The PCR products of the different samples, when separated in 1% (w/v) agarose gels, all showed an approximately 650-bp band (data not shown). All purified PCR products (except for the “Stenophyllos” individual 5, which for unknown reasons could not be cloned) were cloned and sequenced. Alignment of the sequences of both strands of each plasmid insert revealed full complementarity between the two strands for each individual plasmid clone. Sequences were further processed and separated in their component regions ITS1-*5.8S rRNA*-ITS2. Using the algorithm BLAST, all sequences were compared with sequences deposited in the data bank. Table 2 lists the accession numbers of the deposited sequences along with the accession numbers of the reference/out-group sequences used in the bioinformatic analyses. The results of this search are presented in Tables 3, 4, 5, 6 and 7, which present the combined results for both ITS sequences (1 and 2) for the individuals studied for each morpho-variety.

Table 2 List of accession numbers of deposited sequences and the sequences of reference plants used in this study.

Deposited ITS1 sequences	Deposited ITS2 sequences
SUB7801037 S1MT790494	SUB7813560 S1MT793048
SUB7801037 S2MT790495	SUB7813560 S2MT793049
SUB7801037 S3MT790496	SUB7813560 S3MT793050
SUB7801037 S4MT790497	SUB7813560 S4MT793051
SUB7801037 Mx1MT790498	SUB7813560 Mx1MT793052
SUB7801037 Mx2MT790499	SUB7813560 Mx2MT793053
SUB7801037 Mx3MT790500	SUB7813560 Mx3MT793054
SUB7801037 Mx4MT790501	SUB7813560 Mx4MT793055
SUB7801037 Mx5MT790502	SUB7813560 Mx5MT793056
SUB7801037 F1MT790503	SUB7813560 F1MT793057
SUB7801037 F2MT790504	SUB7813560 F2MT793058
SUB7801037 F3MT790505	SUB7813560 F3MT793059
SUB7801037 F4MT790506	SUB7813560 F4MT793060
SUB7801037 F5MT790507	SUB7813560 F5MT793061
SUB7801037 Mr1MT790508	SUB7813560 Mr1MT793062
SUB7801037 Mr2MT790509	SUB7813560 Mr2MT793063
SUB7801037 Mr3MT790510	SUB7813560 Mr3MT793064
SUB7801037 Mr4MT790511	SUB7813560 Mr4MT793065
SUB7801037 Mr5MT790512	SUB7813560 Mr5MT793066
SUB7801037 Bo1MT790513	SUB7813560 Bo1MT793067
SUB7801037 Bo2MT790514	SUB7813560 Bo2MT793068
SUB7801037 Bo3MT790515	SUB7813560 Bo3MT793069
SUB7801037 Bo4MT790516	SUB7813560 Bo4MT793070
SUB7801037 Bo5MT790517	SUB7813560 Bo5MT793071
SUB7801037 Bo6MT790518	SUB7813560 Bo6MT793072
SUB7801037 Bo7MT790519	SUB7813560 Bo7MT793073
SUB7801037 Bo8MT790520	SUB7813560 Bo8MT793074
SUB7801037 Bo9MT790521	SUB7813560 Bo9MT793075
P. lentiscus	KY549574.1
P. x *saportae*	EF193103.1
P. terebinthus	EF193097.1
P. vera	MH444780.1

Table 3 Composition of the ITS1 and ITS2 sequences of plants belonging to “Votomos” (Bo).

Plant	ITS1 (%) identity	Relative species	ITS2 (%) identity	Relative species
Bo1	100	*P.* x *saportae*	99	*P.* x *saportae*
Bo2	100	*P.* x *saportae*	100	*P.* x *saportae*
Bo3	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P.* x *saportae*
Bo4	100	*P.* x *saportae*	99	*P.* x *saportae*
Bo5	100	*P.* x *saportae*	100	*P.* x *saportae*
Bo6	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan
Bo7	100	*P.* x *saportae*	100	*P.* x *saportae*
Bo8	100	*P.* x *saportae*	100	*P.* x *saportae*
Bo9	99	*P. lentiscus* voucher Golan, *P. lentiscus*	99	*P.* x *saportae*, *P. lentiscus*

Table 4 Composition of the ITS1 and ITS2 sequences of plants belonging to “Maroulitis” (Mr).

Plant	ITS1 (%) identity	Relative species	ITS2 (%) identity	Relative species
Mr1	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan
Mr2	99	*P. lentiscus* voucher Golan, *P. lentiscus*	98	*P. lentiscus* voucher Golan
Mr3	99	*P. lentiscus* voucher Golan, *P. lentiscus*	98	*P. lentiscus* voucher Golan
Mr4	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan
Mr5	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan

Table 5 Composition of the ITS1 and ITS2 sequences of plants belonging to "Mavroschinos" (Mx).

Plant	ITS1 (%) identity	Relative species	ITS2 (%) identity	Relative species
Mx1	100	*P.* x *saportae*	100	*P.* x *saportae*
Mx2	99	*P. lentiscus* voucher Golan, *P. lentiscus*	99	*P. lentiscus* voucher Golan, *P. lentiscus*
Mx3	99	*P.* x *saportae*	100	*P.* x *saportae*
Mx4	100	*P. lentiscus* voucher Golan	99	*P. lentiscus* voucher Golan, *P. lentiscus*
Mx5	100	*P.* x *saportae*	99	*P.* x *saportae*, *P. lentiscus*

Table 6 Composition of the ITS1 and ITS2 sequences of plants belonging to "Stenophyllos" (S).

Plant	ITS1 (%) identity	Relative species	ITS2 (%) identity	Relative species
S1	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan
S2	100	*P. lentiscus* voucher Golan, *P. lentiscus*	99	*P. lentiscus* voucher Golan, *P. lentiscus*
S3	100	*P. lentiscus* voucher Golan, *P. lentiscus*	99	*P. lentiscus* voucher Golan, *P. lentiscus*
S4	100	*P. lentiscus* voucher Golan, *P. lentiscus*	100	*P. lentiscus* voucher Golan

Table 7 Composition of the ITS1 and ITS2 sequences of plants belonging to "Fardyphyllos" (F).

Plant	ITS1 (%) identity	Relative species	ITS2 (%) identity	Relative species
F1	99	*P. lentiscus* voucher Golan, *P. lentiscus*	98	*P. lentiscus* voucher Golan, *P. lentiscus*
F2	99	*P. lentiscus* voucher Golan, *P. lentiscus*	98	*P. lentiscus* voucher Golan, *P. lentiscus*
F3	100	*P. lentiscus* voucher Golan, *P. lentiscus*	99	*P. lentiscus* voucher Golan, *P. lentiscus*
F4	98	*P.* x *saportae*	98	*P.* x *saportae*, *P. lentiscus*
F5	99	*P. lentiscus* voucher Golan, *P. lentiscus*	98	*P. lentiscus* voucher Golan, *P. lentiscus*

Regarding the morpho-variety “Votomos”, the results presented in Table 3 show clearly that: i) the majority of the plants (Bo1, Bo2, Bo4, Bo5, Bo7 and Bo8) belonging to the “Votomos” group, contain ITS1 sequences, which are 100% identical with the corresponding one of *P.* x *saportae* [a hybrid between *P. lentiscus* and *Pistacia terebinthus* (*P. terebinthus*); 25], while their ITS2 sequences are 99-100% identical with the ITS2 sequence of *P.* x *saportae*. Therefore, they can be considered as members of a single group of genotypes, belonging to the hybrid species *P.* x *saportae*. ii) One plant (Bo6) contains ITS1 and 2 sequences 100% identical with the corresponding ones of *P. lentiscus* voucher Golan 1.1009 clone 1 and therefore is considered to belong to the species *P. lentiscus*. iii) Plants Bo3 and Bo9 both contain ITS1 sequences, which are 99-100% identical with the corresponding ones of *P. lentiscus* voucher Golan 1.1009 clone 1 and *P. lentiscus*, while their ITS2 sequences are 99-100% identical with the corresponding one of *P.* x *saportae*. These plants constitute a separate group. In the dendrogram of ITS sequences (Fig. 5), plant Bo3 clusters next to *P. lentiscus*, while plants Bo1, Bo2, Bo4, Bo5, Bo7 and Bo8 are closely related to *P.* x *saportae*. Plants Bo3 and Bo9 form a separate clade located between *P. lentiscus* and *P.* x *saportae*. These results are further confirmed by the statistical analysis presented in the PCoA diagram (Fig. 6), where the “Votomos” plants appear separated in three groups. Similarly the STRUCTURE analysis (Fig. 7) supports the existence of intervarietal genetic heterogeneity.

Concerning the group “Maroulitis”, plants Mr1, Mr4 and Mr5 contain ITS1 and ITS2 sequences, which are 100% identical with the corresponding ones of *P. lentiscus* voucher Golan 1.1009 clone 1 (Table 4), while plants Mr2 and Mr3 contain ITS1 sequences, which are 99% identical with the corresponding region of *P. lentiscus* voucher Golan 1.1009 clone 1 and *P. lentiscus*, but their ITS2 sequences are only 98% identical with the corresponding region of *P. lentiscus* voucher Golan 1.1009 clone 1. The dendrogram of ITS sequences (Fig. 5) reflects the above-mentioned split of the “Maroulitis” genotypes into two groups: plants Mr1, Mr4 and Mr5 constitute one group closely related to *P. lentiscus*, while the second group (Mr2 and Mr3) are genetically closely related to each other (and to some “Fardyphyllos” plants) but remoter related to *P. lentiscus* compared with the first “Maroulitis” group. In the PCoA diagram (Fig. 6), the degree of genetic relatedness among “Maroulitis” plants is not presented as accurately as in the dendrogram, but still all “Maroulitis” plants group together with *P. lentiscus*. STRUCTURE analysis (Fig. 7) lends further support to the grouping of the PCo analysis, presenting all “Maroulitis” plants as a homogeneous population, closely related with *P. lentiscus*.

The morpho-variety “Mavroschinos”, in contrast to the RAPD analysis results, is a genetically heterogeneous population, when the ITS analysis is taken into account (Table 5). According to the latter analysis, the “Mavroschinos” plants constitute a genetically heterogeneous population, showing a division in two sub-groups: one of them includes three plants, which are identical, or nearly identical, with *P.* x *saportae*, while two other plants contain ITS sequences, which are identical, or nearly identical with *P. lentiscus* voucher Golan 1.1009 clone 1 and *P. lentiscus*. In detail, plants Mx1, Mx3 and Mx5 contain ITS1 sequences, which are 100% identical with the corresponding one of *P.* x *saportae*, while their ITS2 sequences are 99-100% identical with the corresponding one of *P.* x *saportae* as well. Two other plants (Mx2 and Mx4) contain ITS1 and 2 sequences, which are 99-100% identical to the corresponding sequences of *P. lentiscus* voucher Golan 1.1009 clone 1 and *P. lentiscus*. The same separation of the “Mavroschinos” plants is also observed in the dendrogram of the ITS sequences (Fig. 5), in which Mx1, Mx3 and Mx5 are found in the same cluster with *P.* x *saportae*, while Mx2 and Mx4 cluster along with *P. lentiscus*. The PCoA diagram (Fig. 6) though differs from the above clustering, showing a division in three groups, a division also present in the diagram of the STRUCTURE analysis (Fig. 7).

All four plants (S1 to S4) belonging to “Stenophyllos” group contain ITS1 and ITS2 sequences 99-100% identical with the corresponding ones of *P. lentiscus* voucher Golan 1.1009 clone 1 (Table 6). Similarly, in the dendrogram of the ITS sequences (Fig. 5), all four “Stenophyllos” plants cluster together and along with the *P. lentiscus* reference plant. In the PCoA diagram (Fig. 6) all four plants are included in the same group again together with the *P. lentiscus* reference plant. The same degree of genetic homogeneity is also reflected in the diagram of the STRUCTURE analysis (Fig. 7).

Finally, three plants (F1, F2 and F5) of the morpho-variety “Fardyphyllos” contain ITS1 sequences 99-100% identical with the ones of *P. lentiscus* voucher Golan 1.1009 clone 1 and *P. lentiscus*, while their ITS2 sequences are 98-99% identical with the corresponding ones of the latter species (Table 7). Plant F3 is related to the plants F1, F2 and F5, differing from them in respect to ITS1 in only one nucleotide (out of 262 nucleotides aligned, data not shown) and in three positions (two nucleotides and one gap, out of 240 bps aligned, data not shown) in respect to the ITS2 sequences (Table 7). All those four plants are genetically related to the *P. lentiscus* reference plant as it is apparent in: i) the dendrogram of ITS sequences (Fig. 5), ii) the PCo analysis (Fig. 6) and iii) the STRUCTURE analysis (Fig. 7). To the contrary, plant F4 contains an ITS1 sequence 98% identical with its counterpart of *P.* x *saportae* voucher T10 (clones 3 and 4), but its ITS2 is 98% identical with the corresponding sequences of various vouchers/clones of *P.* x *saportae*, and also with the ITS2 of *P. lentiscus* (Table 7). This curious composition of its ITS sequences is also apparent in the corresponding dendrogram (Fig. 5), in which F4 appears as a discrete genotype, clustering alone, apart from any reference plant used in this study. This is not in agreement with the PCo analysis (Fig. 6), according to which, plant F4 groups together with the *P.* x *saportae* group of plants. This is not supported by the STRUCTURE analysis (Fig. 7), which is in agreement with the dendrogram (Fig. 5).

Discussion

The plant *P. lentiscus* grows among other places also in the Mediterranean basin, where it is widely-spread. On the island of Chios (Greece), *P. lentiscus* (also known as *P. lentiscus* var. *Chia*) is intensively cultivated on the southern parts of the island and constitutes an important component of the island’s terrestrial environment. Furthermore, the tree populations cultivated in the southern part of the island produce a resin called mastiha, which is processed to several different products and makes a significant contribution to the island’s economy. Thus, studying this plant is important for both environmental and economic reasons.

Taxonomic analysis of the cultivated genotypes of the mastic tree is therefore an important aspect, of an accurate certification of this species and its products. Previous works have suggested that the cultivated genotypes are members of different varieties [2] and others [3] have already detected genotypic diversity among the different cultivated genotypes. To further investigate this diversity and since morphological analysis of the plants is sometimes confusing, a dual approach was employed, involving RAPD-based and ITS-based molecular taxonomy.

RAPD-based analysis included a total of 25 plants (five plants of each of the morphologically determined varieties “Votomos”, “Maroulitis”, “Mavroschinos”, “Stenophyllos” and “Fardyphyllos”; Figures 1, 2, 3 and 4). ITS-based analysis included 28 plants (i.e. 24 of the above-mentioned plus four additional plants belonging to the group “Votomos”).

RAPD analysis of the “Votomos” group of plants revealed the existence of a strong genotypic variability (Fig. 1), according to which each plant exhibits a different, unique profile. This finding is not supported by the more detailed ITS marker analysis, according to which, most “Votomos” plants (Bo1, Bo2, Bo4, Bo5, Bo7 and Bo8) are 99-100% identical the corresponding regions of the hybrid species *P.* x *saportae*. Plants Bo6 and Bo9 are 99-100% identical with the reference plants *P. lentiscus* voucher Golan and *P. lentiscus*. Plant Bo3, we consider as very interesting case, because its ITS1 sequence is 100% identical with the ITS1 of *P. lentiscus* voucher Golan and *P. lentiscus*, while its ITS2 sequence is 100% identical with the ITS2 of *P.* x *saportae* (Table 3). This could be considered as a case, where an event of reticulate evolution has taken place. This possibility is further discussed below.

The RAPD profiles of the plants belonging to “Maroulitis” group are genotypically homogeneous at the intra-varietal level (Fig. 2). This is in full agreement with the results of the ITS marker analysis according to which all five plants are 98-100% identical with the corresponding regions of the reference plants *P. lentiscus* voucher Golan and *P. lentiscus* (Table 4). Therefore, it seems that the morpho-variety “Maroulitis” is relatively genetically homogeneous, although two

plants (Mr2 and Mr3) are not 100% identical with the other three “Maroulitis” plants and with the reference plants. This genetic homogeneity of the “Maroulitis” plants is also reflected in the dendrogram of the ITS sequences, where all plants appear genetically related to the reference plant *P. lentiscus* (Fig. 5). The PCoA diagram and the STRUCTURE analysis (Fig. 6 and Fig. 7, respectively) are in agreement with the dendrogram.

Similarly, all five “Mavroschinos” plants exhibit identical RAPD profiles but their banding patterns clearly differ from the ones of the “Maroulitis” plants (Fig. 2 and 3). Again, this finding of genetic homogeneity within the “Mavroschinos” group of plants is not supported by the ITS analysis (Table 5, Fig. 5). According to the latter analysis, three “Mavroschinos” plants are genetically related to *P.* x *saportae*, while two other plants are related to *P. lentiscus*. Therefore, we consider the members of the “Mavroschinos” group constitute a mix of *P.* x *saportae* and *P. lentiscus* plants.

Among the plants belonging to the morphological variety “Stenophyllos”, RAPD analysis (Fig. 4) indicates that these plants constitute a heterogeneous population, but this conclusion is not supported by the ITS analysis, according to which all examined individuals belong to the species *P. lentiscus* (Table 6, Fig. 5, 6 and 7). Since the ITS sequence analysis is a reliable taxonomic tool [20, 21], we consider the members of the “Stenophyllos” group as a homogeneous *P. lentiscus* population.

Based on the RAPD analysis, the plants belonging to “Fardyphyllos” group exhibit also a genotypic variability (Fig. 4), even though not as strong as the one of “Votomos” plants. This is in agreement with the ITS sequence analysis, according to which, most “Fardyphyllos” plants (F1, F2, F3 and F5) belong to two different clades (Fig. 5) closely related (98-100%), but not identical, with the corresponding regions of *P. lentiscus*, while plant F4 has a deviating, *P.* x *saportae* - like genotype (Table 7), which is though not close enough to the hybrid species, in order to cluster along with it in the dendrogram (Fig. 5).

All these observations support the notion that on the island of Chios exist many more genotypes, in addition to the ones of *P. lentiscus* and *P.* x *saportae*, than the five morphologically different “*P. lentiscus* var. *Chia*” cultivated populations known today. This plethora of genotypes may be due to regular mutation accumulation processes, but also to reticular evolution processes, as it exemplified by plant Bo3. There are several other examples of plants for which one could suspect the occurrence of reticulate evolution events. For example, plant Bo9 could also contain hybrid ITS sequences originating from the ITS1 of *P. lentiscus* voucher Golan and an ITS2 from another, so far, unknown genotype. Certainly, the case of plant Bo3 constitutes a strong indication for such an event. Furthermore, Yi et al. [26] have already presented evidence for reticulate evolution within the genus *Pistacia*. In a different study, Chiang et al. [28] detected, among others, genetic diversity of nuclear ITS sequences in experimental hybrids of Begonia x taipeiensis. The authors suggested intermolecular genetic recombination as the molecular mechanism, which could result in such a phenomenon. One possible way to explain the higher rate of reticulation of ITS sequences, in comparison to other genomic areas may reside in the process of nucleolus formation and rDNA transcription. It is known that during these processes many different chromosomes contribute their rDNA containing regions to form nucleolus. Therefore, it is expected that highly homologous genes/sequences originating from different chromosomes find themselves in a close spatial proximity within the nucleolus, a fact that leads to a high concentration of homologous sequences and therefore, could favour intermolecular genetic recombination. Finally, our findings could lead to a reconsideration of the generally accepted practice to determine the sequence of only one of the two ITS regions and use for taxonomical purposes, since in cases of recombination between different species, this would lead to erroneous conclusions.

Conclusions

In conclusion, to our knowledge, this is the first report revealing the presence of the hybrid *P.* x *saportae* in the cultivated population of mastic trees on the island of Chios. It is also the first report of such discordance in the composition of ITS1/ITS2 sequences, i.e. the existence of reticulation events between species *P. lentiscus* and the hybrid species *P.* x *saportae*. Furthermore, it is the first study, which establishes that the members of the morphologically determined, cultivated varieties are not genetically homogeneous populations.

Methods

Plant material

Initially, young leaves were collected from five different male plants of each morphologically determined variety from different randomly selected places on the island of Chios. At a later time were collected more leaf samples from four individual plants, belonging to the morpho-variety “Votomos”, in order to verify the initial results. Collected young leaves were washed, dried and frozen at -80 ºC until used.

Reagents, growth media and enzymes

Inorganic and organic chemicals, growth media, molecular biology reagents and various enzymes were purchased from several different suppliers. For RAPD amplification reactions *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) was used, while for ITS amplification reactions was used Phusion (FINNZYMES, Espoo, Finland). As DNA molecular mass markers, DNA of *λ*/*Hind*III [Marker 1 (M1); HT Biotechnology Limited, Cambridge, UK] and ladder DNA 1.0 kb- [Marker 2 (M2); Invitrogen, Carlsbad, CA, USA] were used.

Primers

All primers used in this study were synthesized by VBC-Biotech Service GmbH (Vienna, Austria) and their nucleotide sequences are presented in Table 1.

DNA extraction

Total DNA from young leaves was extracted using the method of Doyle and Doyle [29], modified as follows: Approximately 1 g of leaf tissue was frozen with liquid nitrogen and ground using cold (-20 ^{o}C) mortar and pestle, in the presence of 0.15 g of polyvinylpyrolidone 40 (PVP-40). The resulting white powder was suspended in 5-7.5 ml of a preheated (60 ^{o}C) modified extraction buffer, containing: all other components as described by Doyle and Doyle [29], except that the concentration of Cetyl-*tri*methyl ammonium bromide (CTAB) was doubled to 4% and was further supplemented with 1.5% PVP-40 and 20 mg/ml Proteinase K. The resulting suspension was incubated at 60 ^{o}C, for one hour. The procedure followed after this point was the one described originally by Doyle and Doyle [29].

RAPD reactions

RAPD reactions were performed in a thermocycler (Mastercycler gradient, Eppendorf®, Germany) using approximately 100 ng of DNA, [1X] *Taq* DNA polymerase reaction buffer, 3.0 mM $MgCl_2$, 0.27 mM of each deoxynucleotide triphosphate (dNTP) and 1 U *Taq* DNA polymerase, in a final volume of 50 μL. In the course of a hot-start procedure at 85 °C, each primer was added to a final concentration of 0.2 μM. The cycling profile used was as follows: an initial denaturation step of the template at 94 °C for 2 min, followed by 30 cycles consisting of a denaturation step at 94 °C for 45 sec, an annealing step at 40 °C for 1 min and an extension step at 72 °C for 2 min. The reaction was completed with a single final extension step at 72 °C for 7 min. For each individual plant triplicate RAPD reactions were performed. Reaction products were separated in a 1.8% agarose gel in 1X *Tis*-acetate electrophoresis buffer (1X TAE), using

published procedures [30] and documented using a Kodak EDAS 290 (© Eastman Kodak Company, Rochester, NY, USA) documentation system.

PCR amplification, cloning and sequencing of the ITS regions

PCR reactions [31] were performed using approximately 25-125 ng of total DNA, 2.5 μL of each primer (each primer's final concentration: 1 μM) and 1 U of Phusion DNA polymerase, in a final volume of 25 μL, according to the manufacturer's recommendations. The ITS regions were amplified using primers ITS 18S and ITS 26S (Table 1), designed on the basis of sequences available in the GeneBank. Cycling profiles used consisted of one cycle at 94 ^{o}C for 10 min and 30 cycles of three steps each as follows: 94 ^{o}C for 30 sec, 63 ^{o}C for 1 min and 72 ^{o}C for 2 min. The final extension cycle was at 72 ^{o}C for 10 min. Produced amplicons were electrophoresed in a 1.0% agarose gel, bands were cut from the gel and the contained DNA was extracted using the GenecleanÒKIT BIO 101 Systems kit (Solon, OH, USA) according to the manufacturer's instructions. Gel-extracted amplicons were ligated to plasmid vector pBlueScript II SK (+) (Stratagene, West Cedar Creek, TX, USA; Table 2), digested with the restriction enzyme *Sma*I, using standard procedures [30]. Ligation products were introduced into *Escherichia coli* (*E. coli*) DH5a cells (Table 8) by electroporation. Five recombinant bacterial colonies from each clone studied were purified and examined for the inclusion of an insert of the correct size by restriction analysis. Both strands of the inserts were sequenced by VBC-Biotech Service GmbH (Vienna, Austria), using plasmid specific primers. The ITS1 and ITS2 sequences obtained from each individual plasmid clone were deposited in the GeneBank and their deposition numbers are presented in Table 2.

Table 8. Plasmids and bacterial strains used.

Plasmids/Bacterial strains	Properties/Genotype	Source
Plasmid: Bluescript II (SK+)	*bla,* f1 origin in + orientation	Stratagene
Bacterial strains: *E.coli* DH5α	*supE44 ΔlacU*169 (φ80 *lacZ* ΔM 15) *hsdR17 recA1 endA1 gyrA96 thi*-1 *relA1*	[30]

Bioinformatic and statistical analyses of the ITS1 and ITS2 data

Comparison of inserts' sequences with sequences deposited in the data banks was performed using algorithm Basic Local Alignment Search Tool (BLAST; [32]). The intervening, between ITS1 and ITS2, *5.8S rRNA* gene sequences of all *Pistacia* species were deleted prior to the alignment of the ITS sequences, since they all are 100% identical and therefore, they would mask the extent (percentage) of variability among aligned ITS sequences. The data of the ITS1 and ITS2 sequences were analyzed by genetic analysis in Excel with the cross-platform package GenAlEx 6.502 [33]. Similarity of qualitative data was calculated using the Nei and Li [34] similarity index. Similarity estimates were analyzed using Unweighted Pair Group Method using Arithmetic Averages (UPGMA) and the resulting clusters were expressed in form of a dendrogram (Fig. 5). The matrices of mutual coefficients of similarity calculated by GenAlEx 6.502 and converted to MEGA 4 v.4.1 software [35]. Furthermore, the above similarity coefficients were presented by the PCoA analyzed by GenAlEx 6.502 [33].

To study how morphology-based groups reflect the inter-individual similarity, genetically homogeneous populations were identified by an *ad hoc* designed clustering approach implemented in the software Structure 2.3.4 [36]. This software places plants in *K* clusters that have distinct marker frequencies, where *K* is chosen in advance and can be varied across different runs. Plants can have memberships in several clusters, with membership coefficients equaling 1 across clusters. ITS1 and ITS2 data were analyzed by treating each class of genotypes, according to the software

documentation. A no-admixture ancestry model was used and allele frequencies were correlated, with a burn-in length of 30,000 followed by 100 000 runs at each *K* [37, 38].

Abbreviations

BLAST: Basic Local Alignment Search Tool

IRAP: Inter-retrotransposon Amplified Polymorphism

ISSR: Inter Simple Sequence Repeat

ITS: Internal Transcribed Spacer

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

RAPD: Random Amplified Polymorphic DNA

SSR: Simple Sequence Repeat

UPGMA: Unweighted Paired Group Method Analysis

Declarations

Authors' Contributions

EH and IS conceptualized the study. EH designed the work. IS and TV assessed and collected the plant material. PK, CZ-P, KP, AM and PM executed the laboratory experiments. SK and AV conducted bioinformatic and statistical analyses. EH, AE, A-SA and SH interpreted the data. PK drafted the work. A-SA and SH revised the manuscript. EH and AE updated and edited the final manuscript. EH, AE, A-SA, SH and SK were involved in funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets in this paper are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

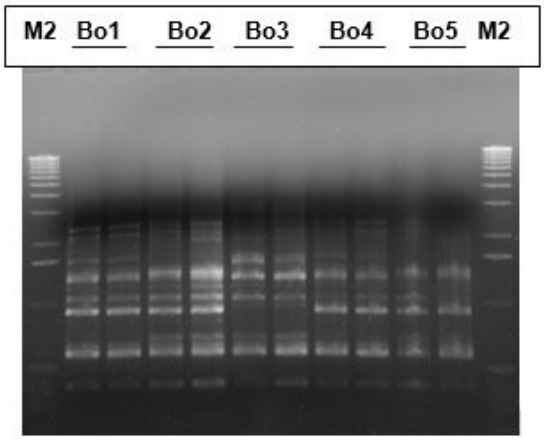

Figure 1

RAPD amplification profiles of five individual plants belonging to “Votomos” using primer OPG2 and a hot-start procedure. Duplicate samples from each individual were electrophoresed in a 1.8% agarose gel (1X TAE buffer). Lanes: M2, molecular mass marker 1.0 kb- DNA ladder. Bo1, Bo2, Bo3, Bo4 and Bo5 are individual “Votomos” plants.

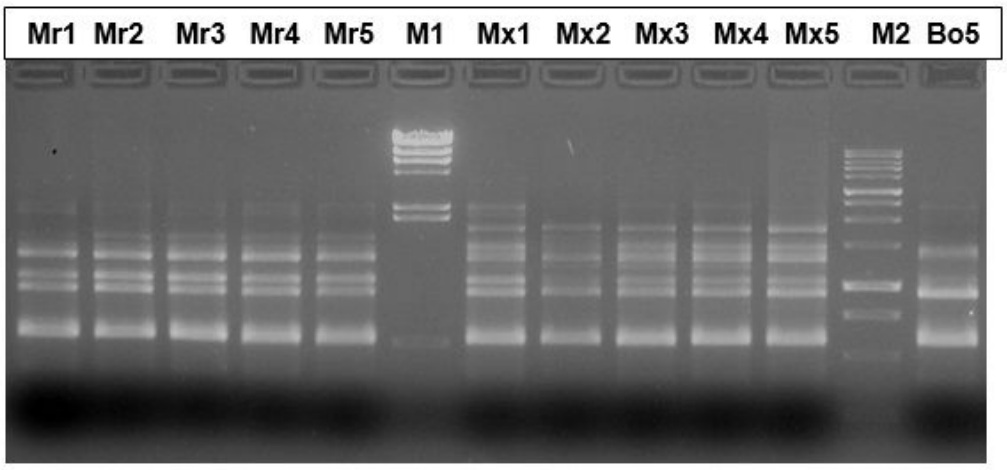

Figure 2

Comparative RAPD amplification profiles of individual plants belonging to “Maroulitis” (5 samples), “Mavroschinos” (5 samples) and “Votomos” (1 sample) using primer OPG2 and a hot-start procedure. Samples were electrophoresed in a 1.8% agarose gel (1X TAE buffer). Lanes: M1, DNA λ/HindIII. M2, 1.0 kb- DNA ladder. Mr1 to Mr5, are five “Maroulitis” plants. Mx1 to Mx5 are five “Mavroschinos” plants. Bo5 is a “Votomos” plant.

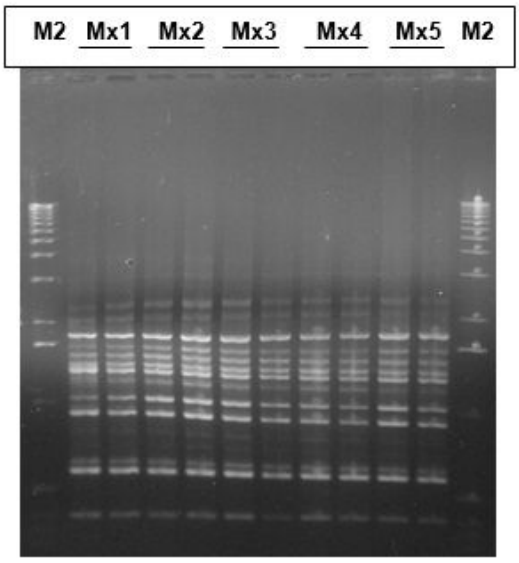

Figure 3

RAPD amplification profiles of five individual plants belonging to “Mavroschinos” using primer OPG2 and a hot-start procedure. Duplicate samples from each individual were electrophoresed in a 1.8% agarose gel (1X TAE buffer). Lanes: M2, 1.0 kb- DNA ladder. Mx1, Mx2, Mx3, Mx4 and Mx5 are individual “Mavroschinos” plants.

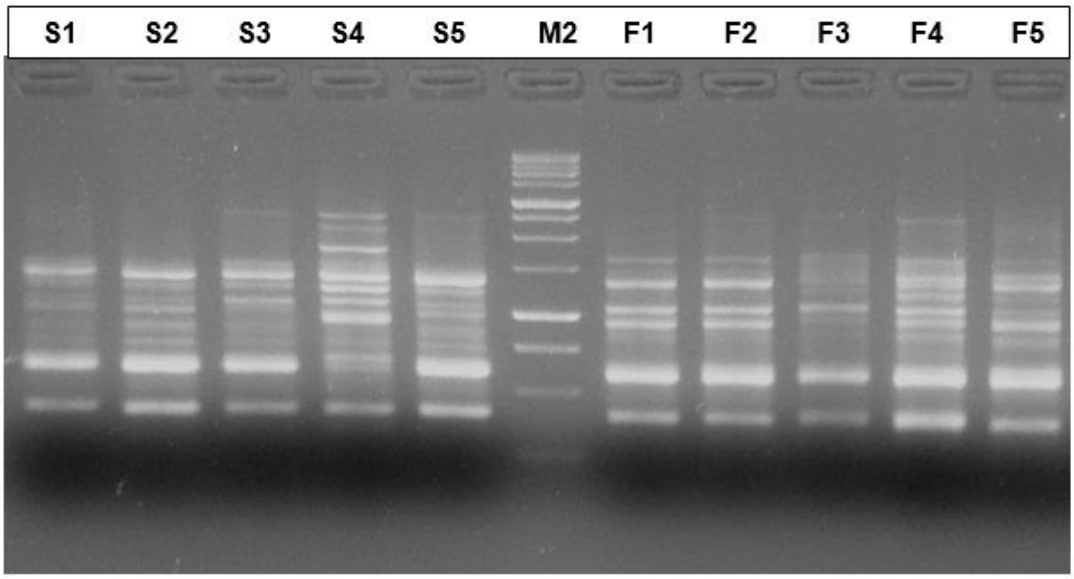

Figure 4

Comparative RAPD amplification profiles of individual plants belonging to “Stenophyllos” (samples S1 to S5) and “Fardyphyllos” (samples F1 to F5) using primer OPG2 and a hot-start procedure. Samples were electrophoresed in a 1.8% agarose gel (1X TAE buffer). Lanes: S1 to S5 five plants belonging to “Stenophyllos”. M2: 1.0 kb-DNA ladder. F1 to F5, are five plants belonging to “Fardyphyllos”.

Figure 5

Genetic relationships of five cultivated, morphologically determined varieties of mastic tree [“Stenophyllos” (S), “Mavroschinos” (Mx), “Fardyphyllos” (F), “Maroulitis” (Mr) and “Votomos” (Bo)], based on ITS1 and ITS2 markers. Unweighted pair group method using arithmetic average (UPGMA) unrooted tree illustrating genetic relationships among 28 individuals of mastic tree and four reference plants (P. lentiscus, P. terebinthus, P. vera and P. x saportae). In this alignment, the ITS sequences of P. terebinthus subsp. palaestina and P. vera have served as out-groups.

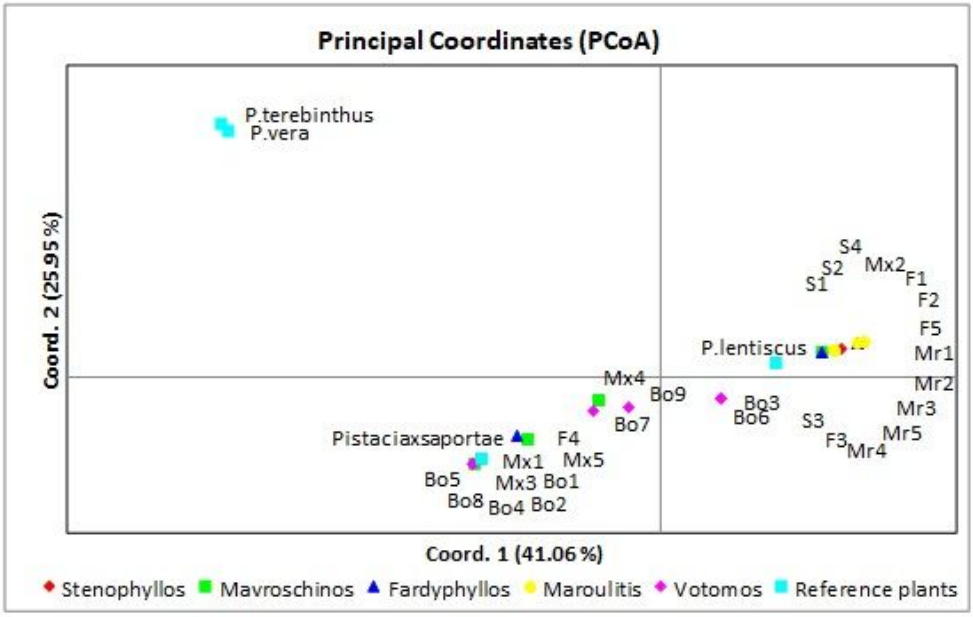

Figure 6

Principal coordinates analysis (PCoA) of 28 individuals in five phenotypes of P. lentiscus based on ITS1 and ITS2 data, compared on four indicator plants. 63.82% of total variance accumulated on the first two components (axis 1 = 38.39%; axis 2 = 25.43%).

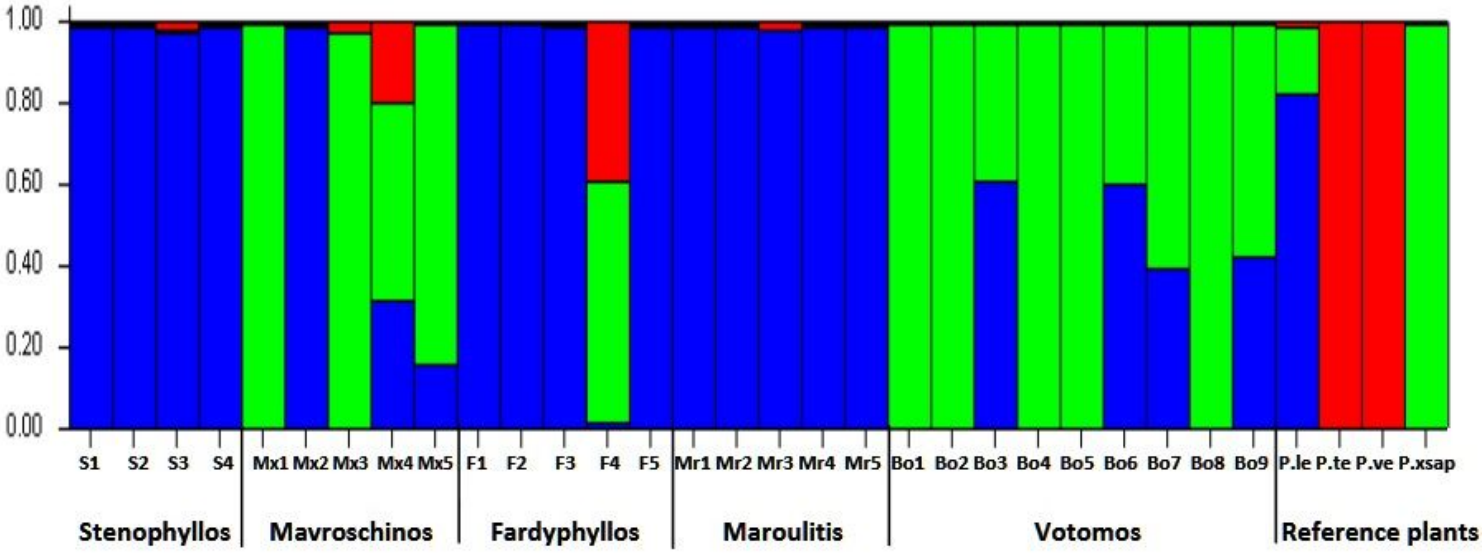

Figure 7

STRUCTURE analysis of the data from the five morpho-varieties of mastic trees based on ITS1 and ITS2 data, with K = 3 clusters. Each vertical bar represents a single plant (n = 32) and the probability of its membership in four clusters. The five morpho-varieties “Stenophyllos”, “Mavroschinos”, “Fardyphyllos”, “Maroulitis” and “Votomos”, compared genetically with the 4 reference plants P. lentiscus (P.le), P. terebinthus (P.te), P. vera (P.ve) and P. x saportae (P.xsap).