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Short communication

Molecular characterization of the interspecific hybrid *Pistacia vigros* (*P. vera* L. × *P. atlantica* Desf.)

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ABSTRACT

A new *Pistacia* variety, VIGROS, has been characterized here using nuclear and chloroplastidial DNA sequences, which are suitable tools for genetic improvement and cultivar identification. The nuclear DNA sequences (ribosomal ITS1, 5.8S, ITS2) determined that VIGROS is an interspecific hybrid between *Pistacia vera* L. and *Pistacia atlantica* Desf. The chloroplastidial DNA sequences (trnC-D, trnL-F regions) demonstrated that VIGROS bears a haplotype 100% coincident with that of *P. vera*, which would then be regarded as the female parent, while *P. atlantica* would be the male one. Due to its vigorous phenotype VIGROS has the potential to be used as a rootstock. This characterization may benefit producers and contribute to improved grafting of pistachios. Also, this paper demonstrates the validity of this set of molecular markers to characterize interspecific *Pistacia* hybrids.

1. Introduction

Like many other fruit trees, *Pistacia vera* L. is hard to root and thus requires a rootstock for vegetative propagation. The choice of an appropriate tandem bud-rootstock is the most decisive factor in pistachio cultivation, and needs to be carefully considered for any given area (Ferguson et al., 2005). *Pistacia atlantica* Desf., *Pistacia integerrima* Stewartson, *P. vera* L., *Pistacia terebinthus* L. are rootstocks used worldwide. Significantly, UCB-1 and PGII (two interspecific hybrids of *P. atlantica* × *P. integerrima*) are also fairly common (Couceiro et al., 2013; Ferguson et al., 2005). The occurrence of hybrids in the genus *Pistacia* is frequent and well documented (Zohary, 1952), with no evidence of crossing barriers between its species (Parfitt, 2003). These often exhibit improved traits with respect to the parents, making them suitable rootstocks. Vigour, in terms of trunk perimeter and circumference, as well as other factors more connected to particular conditions of the cultivation area, such as cold resistance and diseases tolerance, have been demonstrated to be crucial features affecting the success of the grafts (Guerrero, 2011).

More in detail, UCB-1 comes from the close pollination between a selected female tree of *P. atlantica* and a male tree of *P. integerrima*, performed at the University of Berkeley (California, USA) in 1960 (Ferguson et al., 2008). PGII (Pioneer Gold II) comes from the same parent but resulted from open pollination between a population of female trees of *P. atlantica* and a population of male trees of *P. integerrima*. Both varieties have increased vigour compared to *P. atlantica*, and generally have equal or greater vigour compared to *P. integerrima*. UCB-1 is highly resistant to *Verticillium dahliae* K. infection (Ferguson et al., 2005).

In Castilla-La Mancha (Spain), the Centro Agrario el Chaparrillo (CAC) has developed different interspecific hybrids by crossing *P. terebinthus* with *P. integerrima*, *P. vera*, and *P. atlantica* by close pollination. All of the crosses have vigorous phenotypes, give good rates of grafting success, and are cold resistant except for *P. terebinthus* × *P. integerrima* (Couceiro et al., 2013). Also, hybrids *P. vera* × *P. atlantica*, with large seeds and good vigour, and *P. vera* × *P. terebinthus*, with a good initial vigorous period, have been produced by open pollination (Couceiro et al., 2013).

Despite having favourable climatic conditions to produce pistachios, Spain (where this research is focused) is still behind the main producers, with only around 5000 Ha cultivated (Couceiro et al., 2013). We propose that any effort at characterizing and testing new rootstocks will benefit the producers, whose main concern is the low rate of grafting success, i.e. 60% on average for Castilla la Mancha with *P. terebinthus* as the rootstock

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Fig. 1. VIGROS seeds without exocarp. Bar represents 1 cm.

(Couceiro et al., 2013). In this context, using nuclear and chloroplastidial DNA sequences, we here characterize VIGROS, a naturally occurring variety of *Pistacia* that, due to its vigorous phenotype, has the potential to be used as a rootstock. The aim of this study was to identify the provenience of this variety which might facilitate its establishment.

2. Materials and methods

2.1. Plant material and genomic DNA isolation

During a routine classification of *P. terebinthus* L. material collected from Sierra de Baza, Granada (Spain) to be used as rootstock, the presence of a group of dissimilar seeds in size and shape, with respect to average *P. terebinthus* seed, was detected (Fig. 1). These were considered a new variety, named VIGROS. To confirm this status, a molecular characterization was carried out. For that purpose, VIGROS seeds were germinated in autoclaved fine sand soaked in distilled water until roots emerged. Then, plants were grown in 6 × 4 seedling pots. Genomic DNA from fresh leaves was isolated using the Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) following the manufacturer instructions. The quality was measured by Infinite® 200 PRO NanoQuant (Tecan, Switzerland) and confirmed by running the extracted DNA in a 1% agarose gel.

2.2. Cloning, sequencing, and DNA analyses

Nuclear ribosomal ITS1, 5.8S, and ITS2 sequences, and the plastid trnL-F and trnC-D regions were amplified using the pairs of primers described in Yi et al. (2008). Restriction products were excised from agarose gel and purified using ilustra™ GTX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). After purification, they were ligated to pGEM®-Teasy cloning vector (Promega), and transformed into competent cells of *Escherichia coli* JM109. The transformant colonies, screened by blue/white selection, were grown in 1 ml of LB medium at 37 °C overnight.

PCR reactions for sequencing, in an ABI PRISM 3100 – Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), were made under the following conditions: 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 5 min.

Using the software Geneious 6.1.7 (http://www.geneious.com/, Biomatters), sequences were trimmed, BLASTed against GenBank, and aligned with positive matches found in other 12 *Pistacia* species

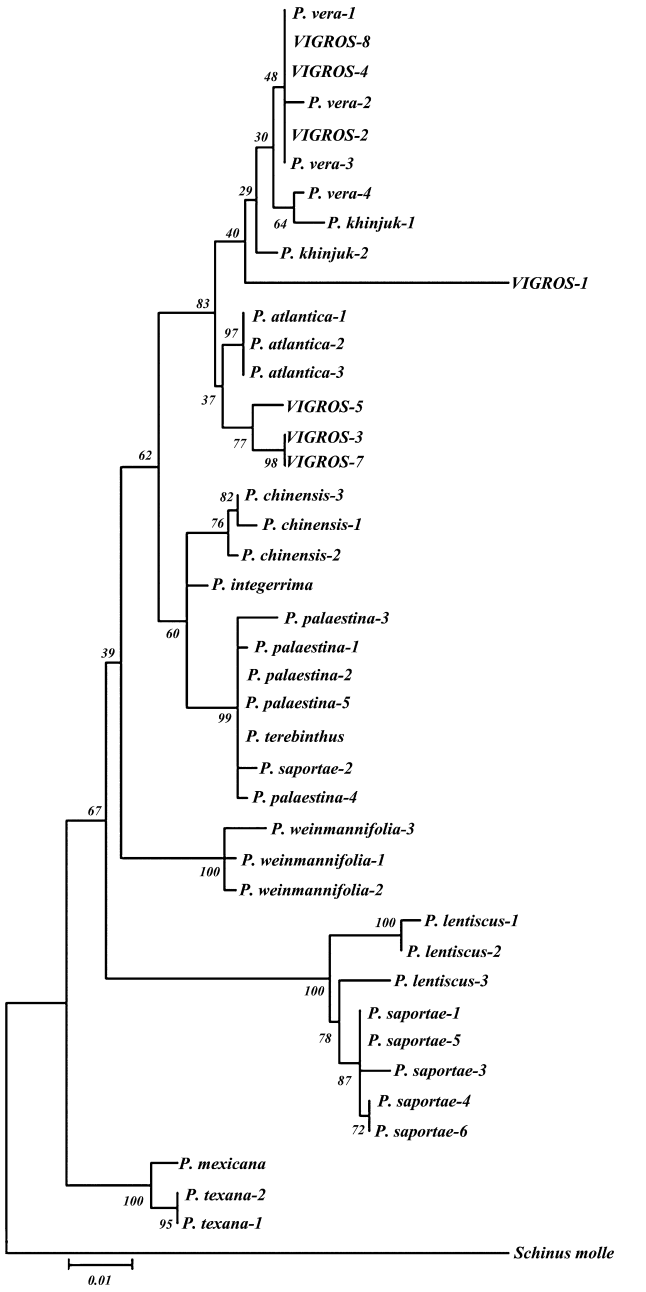

Fig. 2. Maximum likelihood tree based on ribosomal DNA sequences. Numbers at each node indicate bootstrap support.

and the outgroup *Schinus molle* L. New sequences were deposited in GeneBank, and accession numbers assigned (Table 1).

Phylogenetic reconstructions based on ribosomal DNA were conducted using the maximum likelihood method as implemented in MEGA 5.2 (Tamura et al., 2011). The DNA-substitution model was Kimura 2 parameters; a uniform rate of substitution and the nearest-neighbour-interchange heuristic search strategy were considered. For gaps, a *complete deletion* option was chosen. Internal branch support was estimated with 500 bootstrap replicates (Felsenstein, 1985).

Plastid markers were used to assign the female parent. For that task, the haplotypes isolated from our variety were aligned with those in GenBank belonging to the species mentioned above (Table 1). Thus, diagnostic positions were determined by visual inspection of the alignment.

Table 1
List of species analyzed, and accession numbers of the sequences.

	Ribosomal DNA	trnC-D region	trnL-F region
P. integerrima	EF193081	EF193145	EF193128
P. khinjuk	EF193104-05	EF193146	EF193129
P. lentiscus	EF193082-83, DQ390467	EF193147, DQ400561	EF193130, DQ390471
P. palaestina	EF193084-85, EF193095-97	EF193148-50	EF193131-33
P. terebinthus	EF193086	EF193153	EF193136
P. vera	AY677201, EF193089-91	EF193156, DQ400564	EF193139, DQ390473
P. mexicana	DQ390468	DQ400562	DQ390472
P. texana	EF193087-88	EF193154-55	EF193137-38
P. chinensis	EF193079-80, DQ390466	EF193143-44, DQ400560	EF193126-27, DQ390470
P. weinmannifolia	EF193092-94	DQ400564	DQ390473
P. atlantica	EF193076-78	EF193140-42	EF193123-25
P. saportae	EF193098-103	EF193151-52	EF193134-35
VIGROS	HE652101-07	HE652108	HE652109-11
Schinus molle (outgroup)	AY641512	DQ400565	AY640463

3. Results and discussion

3.1. Phylogenetic analyses using ribosomal DNA sequences

A total of 34 ribosomal sequences belonging to 12 *Pistacia* species, plus 7 sequences of VIGROS, and a sequence of *S. molle* L. (outgroup) were aligned (Table 1). The distance matrix of the ITS1, 5.8S, and ITS2 regions had a length of 682 bp due to the inclusion of indels, ranging from 646 and 659 bp of *Pistacia* × *saportae* and *Pistacia weinmannifolia* Poisson, respectively. The mean divergence among all the sequences was 0.042 (0.039 excluding the outgroup).

Maximum likelihood analyses showed trees with a topology that not only supports the monophyly of the group, but draw sequences together according to their taxonomic affiliation (Fig. 2). In fact, sequences can be classified into two large clades: Lentiscus and Terebinthus, as pointed out elsewhere (Yi et al., 2008). Within the Lentiscus group, sequences are divided into three subclades corresponding to *Pistacia mexicana*/*Pistacia texana*, *Pistacia lentiscus L.*, and *P. weinmannifolia*. Within the Terebinthus group, four subclades can be found, *P. terebinthus*/*Pistacia palaestina*, *Pistacia chinensis*/*P. integerrima*, *P. atlantica*, and *P. vera*/*Pistacia khinjuk* (Fig. 2).

Notably, 4 VIGROS sequences (1, 2, 4, and 8) grouped together with the sequences of *P. vera*/*P. khinjuk* subclade; the other 3 VIGROS sequences (3, 5 and 7), fell into *P. atlantica* subclade (Fig. 2). VIGROS-2, 4, and 8 had a 100% identity with sequences 1–3 of *P. vera*. In turn, VIGROS-3, 5, and 7 sequences showed 99.1% identity with *P. atlantica* sequences 1–3 (Fig. 2). Hybrids can maintain copies of both parental alleles in their genomes which can be detected by cloning and sequencing. Under this assumption, Yi et al. (2008) determined the hybrid nature of *Pistacia* × *saportae* (*P. lentiscus* × *P. terebinthus*), isolating different variants of ribosomal sequences. As shown in Fig. 2, some *Pistacia* × *saportae* sequences showed identity with *P. lentiscus* (98.3% identity), while others grouped into the *P. terebinthus*/*P. palaestina* subclade (99.7% identity). This confirmed previous observations using RAPDs by Werner et al. (2001).

On these grounds, VIGROS can be regarded as an interspecific *P. vera* × *P. atlantica* cross. Morphological features of the seeds of the open-pollination hybrid *P. vera* × *P. atlantica* obtained in the CAC (Spain) (Couceiro et al., 2013) would support this contention. Next, we sought to determine the gender of the species involved in the cross that produced VIGROS. We have been unable to find this information for the CAC hybrid.

3.2. Determining the female parent using chloroplastidial DNA sequences

Chloroplastidial DNA sequences were used to determine the gender of the VIGROS parent. For that task, trnC-D and trnL-F regions of VIGROS were sequenced and compared to sequences

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Pistacia x vigros          TTCCAAAGAAAAGTGGGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia vera-1            TTCCAAAGAAAAGTGGGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia vera-2            TTCCAAAGAAAAGTGGGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 494
Pistacia khinjuk           TTCCAAAGAAAAGTGGGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 486
Pistacia atlantica-1       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia atlantica-2       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia atlantica-3       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia chinensis-1       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia chinensis-2       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 487
Pistacia chinensis-3       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia integerrima       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia palaestina-1      TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia palaestina-2      TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia palaestina-3      TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia terebinthus       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia saportae-1        TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia saportae-2        TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia lentiscus-1       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia lentiscus-2       TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia weinmannifolia    TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia mexicana          TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 494
Pistacia texana-1          TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 500
Pistacia texana-2          TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 499
Schinus molle              TTCCAAAGAAAAGTGTGACTGCGTATTTTGTTTAATGTTTCCCGATTCTA 497
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Fig. 3. Partial alignment of the trnC-D region showing the diagnostic position (466) for *P. x vigros* and *P. vera* (shaded).

of other 12 *Pistacia* species and the outgroup (*S. molle*) (Table 1). VIGROS showed a haplotype 100% coincident with that of *P. vera*. Additionally, a diagnostic site (466 position of trnC-D region) differentiated *P. vera*, *P. khinjuk* and VIGROS from the rest of species (Fig. 3). This, together with the data shown above, supports the contention that *P. vera* is the female parental, while *P. atlantica* is the male one. VIGROS has been protected by the CPVO (Community Plant Variety Office) under the file number 2012/1925 (CVPO Gazette, 2012). Unfortunately, with the data available, it is not possible to determine whether the cross was produced naturally or the hybrid naturalized from an established plantation.

Preliminary field studies have demonstrated that VIGROS is a highly vigorous variety with significantly high rates of germination and rapid growth, and its potential to serve as a rootstock is currently being evaluated (Viveros Zuaime, personal communication). Pistachio requires a rootstock for vegetative propagation because, like many other fruit trees, it is a hard-to-root crop. Furthermore, the percentage of successful grafting is surely the most decisive factor in the viability of pistachio cultivation (Ferguson et al., 2005), and therefore increasing the number of choices by the characterizing and field testing of new potential rootstocks may greatly benefit producers. Also, the results presented here demonstrate that the combined use of ribosomal and chloroplastidial DNA sequences constitutes a useful tool to characterize hybrids in this group of species.

4. Conclusions

By using different molecular markers, we have characterized VIGROS, a new variety of *Pistacia*. Ribosomal DNA sequences (ITS1, 5.8S, ITS2) demonstrated that this variety has arisen from an interspecific cross between *P. vera* and *P. atlantica*. Chloroplastidial DNA sequences (trnC-D, trnL-F regions) show that the VIGROS haplotype is identical to that of *P. vera*, which also shares a diagnostic position that differentiates them from the other *Pistacia* species. This suggests that *P. vera* is the female parent, while *P. atlantica* is the male one. This molecular characterization also allows close pollination to propagate the variety, which, due to its vigorous phenotype, is being tested as a rootstock for pistachio cultivation.

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