Phytophthora and gummosis of pistachio in Iran

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For most people in the West, pistachios are a delicious snack normally consumed at parties - green kernels locked temptingly, and sometimes frustratingly, in a slightly opened thin shell. Elsewhere in the world, especially Iran, the cultivated pistachio (*Pistacia vera*) is an important staple crop, a source of food and cooking oil. Iran has about 250,000 ha of pistachio orchards producing 210,000 tonnes of nuts per annum and worth *c.* US$ 400M. Nearly 90% of Iranian production is concentrated in Kherman province, where wild *P. vera* also grows; members of the genus occur principally in an area stretching from the Mediterranean to W. China, but there are also native species in Mexico and USA.

Gummosis is one of the most important diseases of pistachio trees. It is characterised by a gummy or tarry exudate from the crown and lower trunk, whilst the underlying tissues are usually stained brown or dark brown and rotted. This, and severe root rotting, often kills the tree with average tree mortality in Kherman *c.* 10-12% or higher (Mirabolfathy, 1988 and unpublished observations).

Phytophthora species cause gummoses of a number of crop plants and several species have been isolated from affected pistachio trees: *P. citrophthora*, *P. cryptogea,* and two described originally as *P. megasperma* and *P. drechsleri*. All are pathogenic to pistachio seedlings and scions. In particular, the last two species above represent more than 90% of all *Phytophthora* isolates recovered from pistachio and both produced typical symptoms of gummosis and decline when re-inoculated onto seedlings and young trees[1,2].

P. megasperma and *P. drechsleri* are poorly resolved species and many *Phytophthora* isolates have been assigned wrongly to them. This is a common problem in *Phytophthora* taxonomy as there is a dearth of reliable morphological and physiological characters for identification. For example, one of the main characters used to identify *P. drechsleri* is the ability to grow at 35°C, a character not confined to this species.

ITS identification of *Phytophthora* isolates from pistachio SCRI has been at the forefront of developing rapid and reliable molecular methods for identifying *Phytophthora* species[3,4,5]. Principal among these has been a PCR-based system based on the sequence and restriction digests of the ITS region of the genomic ribosomal RNA gene repeat (rDNA)[5]. Using this system, we were able to compare the pistachio isolates of *P. megasperma* and *P. drechsleri* with isolates from international mycological collections that had been well characterised by classical and molecular criteria, including ITS. DNA fingerprints, generated by amplified fragment length polymorphism (AFLP), provided further comparisons among isolates.

ITS identification of *Phytophthora* Obtaining PCR products containing the ITS sequence of the *Phytophthora* isolates from pistachio was straightforward[3,4,5]. Small fragments of hyphae, picked off from cultures with sterile toothpicks, were added to the PCR mixture with primer ITS6, a version of the universal primer ITS5, which gives good amplification of Oomycetes, and ITS4. Thereafter, the amplicon was either sequenced or digested with restriction enzymes to yield ITS-RFLPs. Sequences were aligned against the database containing the ITS sequences of many isolates of nearly all described species of *Phytophthora*[3]. Likewise ITS-RFLP patterns could be compared with the patterns in a similar database[4,5]. A phylogram (Fig. 1) based on a sequence alignment shows the relationship of the pistachio isolates to the rest of the genus.

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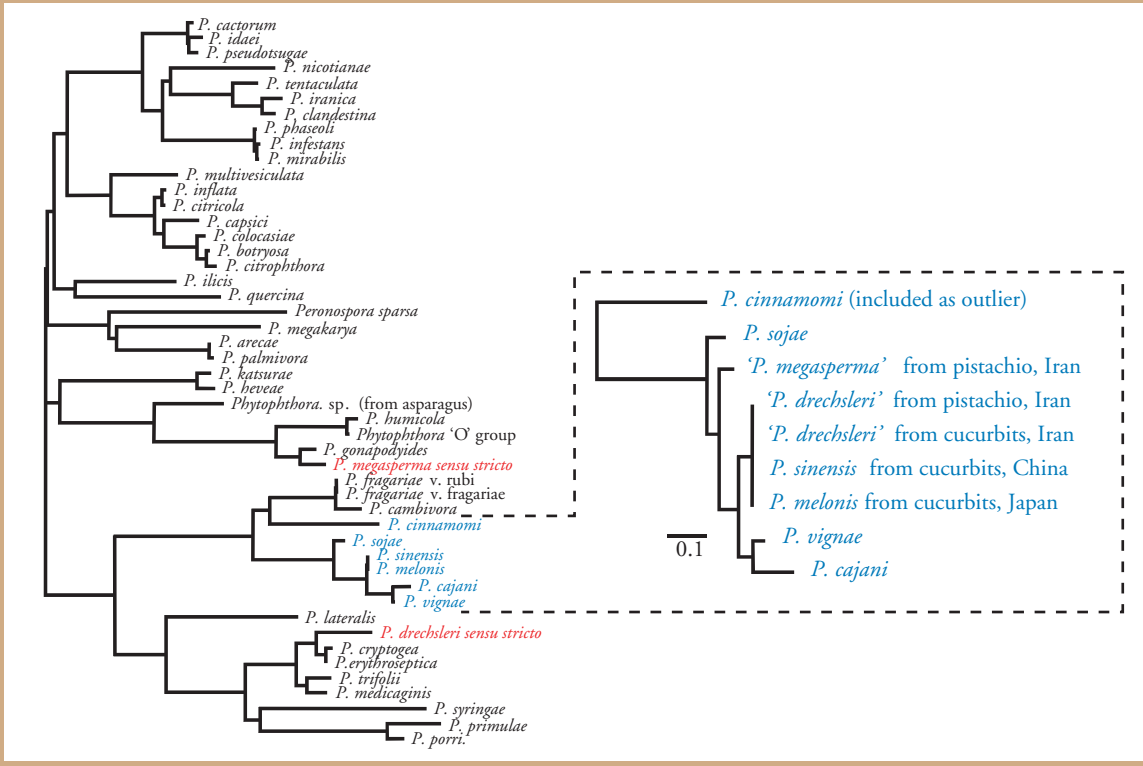

Figure 1 Neighbour-joining DNA-distance phylogenetic tree based on the ITS sequences of *Phytophthora.* Part of the tree has been expanded to include the *Phytophthora* species from pistachio trees and isolates, supposedly of *P. drechsleri,* from cucurbits in Iran. *P. cinnamomi,* as one of the most closely related species not within the clade, has been included as an outlier.

The pistachio isolates that had been assigned previously to *P. megasperma* and *P. drechsleri* belonged to neither of these species. Instead they aligned within a closely related group of non-papillate species that comprised *P. cajani, P. melonis, P. sinensis, P. sojae,* and *P. vignae* (Fig. 1). The *'P. megasperma'* isolates obviously share a common ancestor with *P. sojae,* a species that causes a severe root rot of soya world-wide and one that was also once designated as *P. megasperma*[6]. In fact, neither is closely related to *P. megasperma sensu stricto* (Fig. 1). All the sequences or restriction digests of the ITS region of a large collection of *'P. megasperma'* pistachio isolates were identical. Their AFLP patterns were also very similar (Fig. 2), indicating that they all belonged to the same species. AFLP is time-consuming and expensive but it gives reproducible and detailed DNA 'fingerprints' and, because they are well dispersed throughout the genome, is an ideal counterweight to a single molecular marker like ITS.

Further support for the isolates belonging to a single species was their similarity in appearance on a range of agars and in other classical characteristics in culture. All molecular, morphological and physiological evidence marked them out as different from all other described *Phytophthora* species and, therefore, the name of the species *P. pistaciae* sp. nov. was erected for the *'P. megasperma'* isolates causing gummosis of pistachio[1].

Similarly, isolates of *'P. drechsleri'* from pistachio were not related to *P. drechsleri sensu stricto* but had identical ITS sequences (Fig. 1) and RFLPs and very similar AFLP fingerprints (Fig. 2) to *P. melonis* and *P. sinensis*, species that attack cucurbits in Japan and China respectively. Pathologists had long thought that *P. melonis* and *P. sinensis* might be the same species[7] and the present study confirmed it, while extending the host and geographical ranges of what should now be called *P. melonis,* the name with priority.

This finding promoted a re-examination of other so-called *P. drechsleri* isolates from cucurbits from Iran. These also belonged to *P. melonis,* a result that has important implications for control of pistachio gummosis in Iran, where cucurbits are widely grown, often in close proximity to pistachio trees. *Phytophthora* root

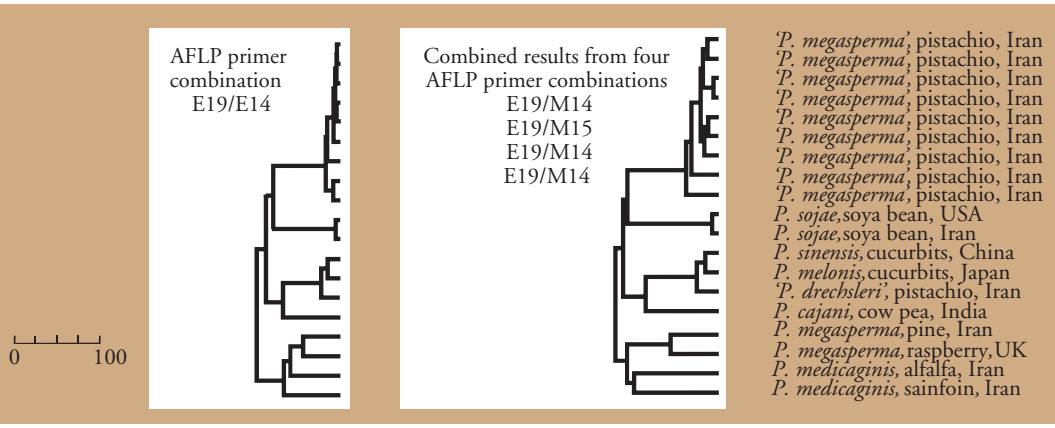

Figure 2 AFLP-based dendrograms and respective images of banding patterns from which they were derived indicating the level of relatedness of the *P. megasperma*-like and *P. drechsleri*-like isolates from pistachio to other *Phytophthora* taxa. The top phylogenetic tree has been constructed form the results of a single AFLP primer combination (E19/M14); the bottom from combined results of four primers combinations (E19/M14, E19/M15, E19/M14, E19/M14). The scale bar indicates percentage similarity.

rot of cucurbits is common in Iran, as it is elsewhere in the region. Again the findings were confirmed by AFLP but, interestingly, the similarity among isolates in classical morphology and physiology seen in *P. pistaciae* was not seen in *P. melonis*. There were marked difference in these characters, although all isolates from whatever host and origin grew at temperatures >35°C.

So far, all described species belonging to the clade containing *P. pistaciae* and *P. melonis* attack hosts of Old World origin[8]: cowpea *(P. cajani)* in Asia; pigeon pea *(P. vignae)* in Africa and Asia; cucurbits *(P. melonis)* in Asia and world-wide; and pistachio *(P. pistaciae* and *P. melonis)* in Asia. Thus, it may be that this clade of *Phytophthora* is also of Old World origin, although an isolate that clearly belongs to the same group has recently been isolated from cassava in S. America (Alvarez, Lokke, Williams and Duncan, unpublished results). Whatever their origins, it is clear that *P. melonis*, at least, may be a much more common and more economically important pathogen than previously suspected.

A PCR diagnostic for *P. pistaciae* and *P. melonis*

One aim of the project was to develop a diagnostic that could be used to identify and detect the main *Phytophthora* spp. causing pistachio gummosis and possibly detect them in tree nurseries on rootstocks. Many, if not most *Phytophthora* diseases, are spread through planting infected material and, where grafts are involved, it is logical to test the rootstocks for disease. In apples for instance, a survey within the USA showed that hardly any apple rootstocks were free of infection by *P. cactorum*, and infection by other species, such as *P. cambivora*, was also very common[9].

SCRI has developed molecular diagnostics for a number of *Phytophthora* diseases, most notably for red core (*P. fragariae*) of strawberries[10]. The approach taken has been a generic one based on a two-round nested PCR. In the first round, which is the same for all Phytophthoras, DNA extracted from plants (also soil and water) is amplified with primers DC6 and ITS4. These primers amplify only DNA of the Peronosporales of the Oomycetes (all *Phytophthora* spp. and their allies such as *Pythium*, the downy mildews and white blister rusts – *Albugo*). The product of the first round of PCR is then used as a starting point for a second, in which a forward primer located in ITS1 and a reverse primer in ITS2 amplify the DNA of a particular *Phytophthora* species, or a small group of closely related species.

In the case of the pistachio *Phytophthora*, all the species in the clade had very similar ITS sequences, making difficult the design of a set of unique primers for each species. A different approach, combining PCR and ITS-RFLP, was therefore adopted. A set of second-round primers was developed that amplified all the species in the clade but none from any other clade. Individual species then could be identified by digesting the resultant PCR product with restriction enzymes, which exploited small, single base-pair differences between the species of interest. The restriction enzymes that differentiated all the species in the clade from one another are given in Table 1. Note that *P. pistaciae* was differentiated from all others by digestion with restriction enzyme *Bsl*I (Fig. 3).

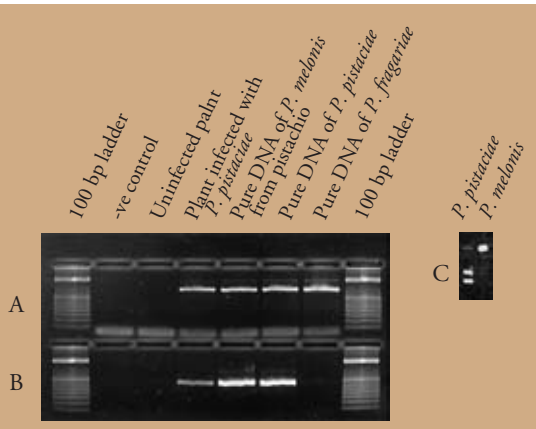

Figure 3 *Right:* pistachio seedlings nearly 3 weeks after inoculation with a zoospore suspension of an isolate of *P. pistaciae* (formerly *'P. megasperma'*). On the right of this picture is a pot of un-inoculated seedlings (control). The inoculated seedlings which have collapsed (left) had badly rotted roots. Roots from inoculated and un-inoculated seedlings were tested by nested PCR.
Left: Nested PCR on infected and healthy roots from inoculated pistachio seedlings
A. - First round nested PCR with primers DC6 & ITS. B. - Second round nested PCR with primers PISfwd1 and PISrev1.
Centre: C. - Digestion of PCR amplicon generated by primers with restriction enzyme BslI.

Although the new diagnostic could not be tested on naturally infected pistachio trees at SCRI, it was tested on artificially inoculated pistachio seedlings in growth cabinets (Fig. 3). The test worked well in this environment and there is no obvious reason why it should not work under more practical conditions in Iran and elsewhere where gummosis of pistachio is a problem. It should also be applicable to any other disease caused by a *Phytophthora* species belonging to the same clade as *P. pistaciae*. Some of the diseases caused by other species in the clade are very serious: root rot caused by *P. sojae* is of world-wide economic importance and cowpea blight (*P. cajani*) and root rot of pigeon pea are locally important in India and Australia. The work therefore could have much wider application than originally intended, an unforeseen but welcome bonus!

Restriction Enzyme	Phytophthora species				
	P. melonis	*P. vignae*	*P. cajani*	*P. pistaciae*	*P. sojae*
AatII			+		
AgeI		+	+		
AluI				+	+
BsaW1		+	+		
BslI				+	
BsiE1					+
DrdI	+			+	+

Table 1 Digestion by restriction enzymes of the PCR product obtained with primers ITS6 (forward sense) and ITS4 (reverse sense) from *Phytophthora* species within the same clade as *P. pistaciae*.

Acknowledgements

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