A molecular survey on the genetic variation and mating type of *Erysiphe necator* Schw. isolates found in Iran, using RAPD technique

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Abstract
Powdery mildew caused by *Erysiphe necator*, is one of the most economically damaging diseases of grapevine throughout the world. This is the first molecular biology study on this fungus in Iran. The aims of the present study were (a) to analyse genetic diversity between isolates of *E. necator* fungus in four main grape production regions in Iran, *i. e.*, Qazvin, Alamoot, Takestan and Shahriar and (b) to obtain an overview on the probable mating types of *E. necator* isolates infecting grapevines in the mentioned regions. In total, 11 RAPD primers were used, amongst which 7 primers showed high level of polymorphism. Out of 20 isolates, only 4 isolates were of mating type + and the rest were of mating type –. For statistical analysis, bands with molecular size between 180-2900 bp and high degree of polymorphism were chosen for analysis. Cluster analysis was carried out using Jaccard's similarity coefficient, based on UPGMA that classified isolates into 7 groups.

Key words: *Erysiphe necator*, genetic variation, grapevine, RAPD, UPGMA.

Introduction

Grapevine powdery mildew casual agent is an obligate, biotrophic parasitic fungus of the phylum Ascomycota and tribe *Erysiphaseae*. At the sexual stage it is named *Erysiphe necator* Schw (*Uncinula necator*) and at non sexual stage it is named *Oidium tuckeri* (Saenz and Taylor, 1999). This is one of the major diseases of grapevine throughout the world (Rumbolz *et al*., 2000; Hajjeh *et al*., 2005; Péros *et al*., 2005; Crisp *et al*., 2006). The disease is common, widespread, and easily recognizable. It can also negatively affect wine pH, aroma, and flavour and can predispose berries to infection by other pathogens such as *Botrytis sp.* (Falacy, 2003). Infection caused by this fungus is developed in high humidity conditions but not by free water. Also, the release of ascospores has always been associated with rainy periods where cumulative rainfall ranged between 2 and 58.5 mm. Therefore, rain is necessary for ascospore release that is a primary inoculums source (Jailloux *et al*., 1999). *E. necator* has been reported to overwinter as mycelium or conidia in dormant buds and/or as cleistothecia on infected tissues, on the bark of vines or in the soil (Delye *et al*., 1997; Miazzi *et al*., 2003; Cortesi *et al*., 2005).

Since *E. necator* is naturally an obligate biotrophic fungus, little information is available about its genotypic diversity, epidemiology or the importance of sexual reproduction (Delye *et al*., 1997). The fungus cannot be grown on artificial media and observations in the field can be carried out only in a limited period of the year (Miazzi *et al*., 2003). Consequently, few molecular studies have concerned mildew

fungi, with the exception of the highly sporulating powdery mildew of cereals, *Erysiphe graminis*, for which conidia can be easily collected from host leaves by tapping or blowing (Newton *et al*., 2004). Many powdery mildews are not as highly sporulating as *E. graminis*, and the collection of adequate quantities of fungal material is a tedious work (Delye *et al*., 1995). Genetically distinct but morphologically similar groups have been identified in Australian and European populations of *E. necator* (Delye *et al*., 1995, 1997; Evans *et al*., 1997; Delye and Corio-Costet, 1998; Stummer *et al*., 2000; Miazzi *et al*., 2003; Péros *et al*., 2005). RAPD technique has proved very useful for studying genetic variation in a number of fungal species including the biotrophic fungi that cause barley powdery mildew and wheat leaf rust (Delye *et al*., 1997; Newton *et al*., 2004). RAPD markers are dominant, but the fact that *E. necator* is a haploid, uninucleate fungus greatly facilitates analyses (Delye *et al*., 1997).

This study was carried out on *E. necator* single spore isolates from vineyards in 3 sites of Qazvin province and 1 site around this province. The importance of this study is that this is the first study on genetic variation and mating type of this pathogenic fungus in Iran, using a molecular marker.

Materials and methods

Fungal isolates were collected from vineyards in Qazvin, Shahriar, Alamoot and Takestan areas, from several vineyards (sites) within each area and from several different vines in each site. A total of 24 single-spore isolates were obtained, 12 isolates from Qazvin, 4 isolates from Shahriar, 4 isolates from Takestan and 4 isolates from Alamoot were collected. All isolates were collected from the leaves of one seedless cultivar (Table 1).

Plant material

A seedless cultivar (*Vitis vinifera*) was used as a host in this study. Cuttings were taken in winter, from one-year-old vine branches, planted in pots containing a mixture of soil and humus (2:1), kept in a greenhouse under a 16/8 (light/dark) and normal humidity at 25˚C. Subsequently, rooted cuttings were transferred into new pots and grown in the greenhouse under the conditions as described. Plants were trimmed during spring and summer to allow the development of new shoots with young glossy leaves suitable for inoculation.

The second expanded youngest leaves of greenhouse-grown plants were collected, washed in tap water and disinfected in a bleach solution (0.24 % active chloride) for 1 min and 30 s. After three rinses in distilled sterile water, the leaves were dried between several layers of sterile filter paper. The petioles were then reduced to a size of 1.5-2 cm. Leaves were placed upper surface up on 8 g/L agar medium in 9 cm diametre petri dishes, the remaining part of the petiole being inserted into the medium and single-spores were inoculated on the leaves (see below).

Single-spore isolation

Within 2 days after collection from the vineyard, infected tissues were brushed onto detached grape leaves (Péros *et al*., 2005).

The inoculated leaves were incubated for 10-15 days at 25 °C under 16 h d^{-1} of illumination (40 µE /m^2 s). Either a single or a few conidia were then removed from the same conidial chain, using a glass needle under a binocular microscope (Optika, Scientific, Italy, LAB2). Conidia were spotted onto newly detached leaves. The process was repeated once. To maintain fungal clones onto leaves, they were subcultured on fresh leaves every 4-5 weeks. All inoculated material was cultured under the temperature and light regimes as described above.

Table 1. Location and collection date of *Erysiphe necator* isolates.

Isolates	*Region*	*Date of sampling*
Sh1	Shahriar	September 2008
Sh2	Shahriar	September 2008
Sh3	Shahriar	September 2008
Sh4	Shahriar	September 2008
Al1	Alamoot	August 2008
Al2	Alamoot	August 2008
Al3	Alamoot	August 2008
Al4	Alamoot	August 2008
D1	Qazvin	September 2008
D2	Qazvin	September 2008
D3	Qazvin	September 2008
D4	Qazvin	September 2008
Q1	Qazvin	September 2008
Q2	Qazvin	September 2008
Q3	Qazvin	September 2008
Q4	Qazvin	September 2008
Kh1	Qazvin	October 2008
Kh2	Qazvin	October 2008
Kh3	Qazvin	October 2008
Kh4	Qazvin	October 2008
T1	Takestan	August 2008
T2	Takestan	August 2008
T3	Takestan	August 2008
T4	Takestan	August 2008

DNA extraction

The mass production of conidia of each isolate was performed on detached leaves. Leaves were inoculated by brushing infected tissues and incubated for 15 days. Leaves were then carefully checked under the binocular microscope and those having contaminating fungi were discarded. Conidia were harvested using a glass needle, then DNA was extracted following the method described by Abbasi *et al*., (2003).

One side of two microscope slides were coated by sigmacote, and conidia were harvested using a glass needle, then transferred on the coated side of one slide and a few drops of extraction buffer (Tris-base 0.089 M, Boric acid 0.045 M, EDTA 0.05 µM and β-mercaptoethanol 1%) was added. Then, the coated side of the other slide was placed on the top of the first slide, containing fungal mix and extraction buffer and gently was stroke on the slide using a pen. The two slides were separated and 30 µl of extraction buffer was added onto each slide. Fungal mix was transferred into a 1.5 ml microcentrifuge tube and incubated at 75 °C for 15 min. Samples were centrifuged for 10 min at 5000 x g at room temperature. Supernatant containing fungal DNA was then transferred into a new tube. The concentration of the extracted DNA was then made to approximately 100 ng/µl using deionised water. To make sure that fungal DNA was not contaminated by the host plant DNA, DNA was extracted from grapevine leaves, using the method of Doyle and Doyle (1990) and used in PCR as a control.

RAPD amplification

Each reaction contained 10 µl of PCR master kit (Cinna Gen Tehran-Iran.), including all components for PCR (PCR buffer, $MgCl_2$ and dNTPs), except DNA template and primers (Table 2). Seventy five ng of primers and approximately 100 ng of template DNA was then added to each reaction mixture. The final volume was adjusted to 20 µl using deionised water. The reaction was overlaid with a drop of mineral oil. A thermal cycler (Thechne, England. TC-512) was programmed for an initial step of 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 35 °C, 2 min at 72 °C, and a final extension step of 5 min at 72 °C. Amplified products were analysed by electrophoresis in 1.6% agarose gels at 85 V for 60 min. A molecular size marker (1-kb ladder, Fermentas, Germany) was run on each gel, alongside the PCR products. DNA from young sterile grape leaves was treated the same as fungal DNA in the RAPD assay A negative control reaction with no DNA template was used in each run. Eleven decamer primers (Fazapajouh Co., Tehran, Iran) were used (Table 1).

Polymorphic bands were scored for the presence or absence of bands. Genetic dissimilarity (D) between all pairs of isolates was calculated using the coefficient of similarity given by Jaccard: a/(a+b+c) and D = 1- S, where a is the number of fragments shared by the two isolates, and b and c are the number of fragments observed in each isolate (Péros *et al*., 2005). The data were subsequently used to construct a phenogram using the unweighed pair-group method with arithmetical averages (UPGMA). Distance calculations and construction of a phenogram, were performed using SPSS 12 software.

Table 2. Sequences of the primers used for RAPD analysis.

Primer	*Primer sequence*	*Length (bp)*	*Annealing temp. (°C)*
E07	AGATGCAGCC	10	35
C08	TGGACCGGTG	10	35
P06	GTGGGCTGAC	10	35
P14	CCAGCCGAAC	10	35
U19	GTCAGTGCGG	10	35
J20	AAGCGGCCTC	10	35
D	TGGGCTCGCT	10	35

Results

RAPD experiments were carried out on 24 fungal single spore isolates, using 11 primers, amongst which only 7 primers gave well-amplified, polymorphic and reproducible bands. Primers E07, C08, P06, P14, U19, J20 and D showed high level of polymorphism and generated between 3 to 17 amplicons that were used to compare the fungal isolates (Table 2). Only repetitive bands were considered in all runs. The molecular sizes of PCR products ranged from 200 to 2900 bp. The seven primers generated 97 amplicons, among which, 91 amplicons (>93%) were polymorphic, easily scorable and reproducible (Fig. 1). These amplicons were used to compare the genetic relatedness of all 24 isolates of *E. necator*. No contamination by grape DNA was detected in RAPD amplifications (Fig. 1).

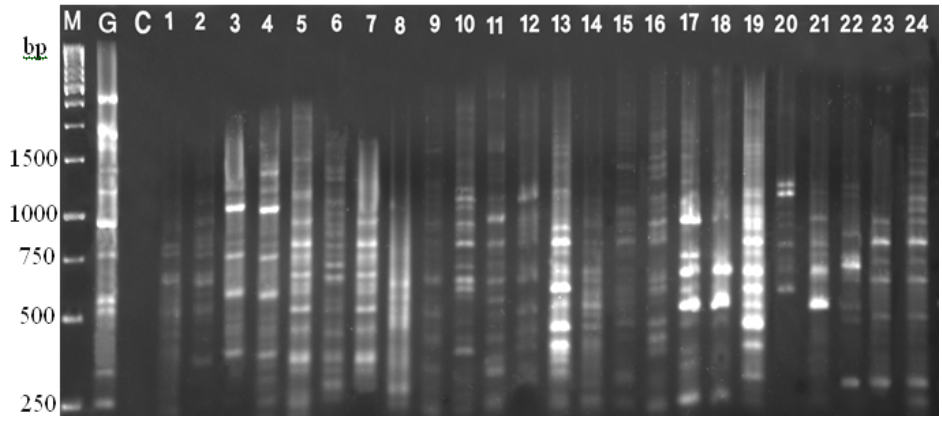

Fig. 1. An example of RAPD banding pattern generated by primer E07. lane M: molecular weight marker (1 kb-ladder); lane G amplification of DNA extracted from grape leaves; lane C control with no DNA template; lanes 1-4: Shahriar isolates, 5-8 Alamoot isolates, 9-20 Qazvin isolates and 21-24 Takestan isolates.

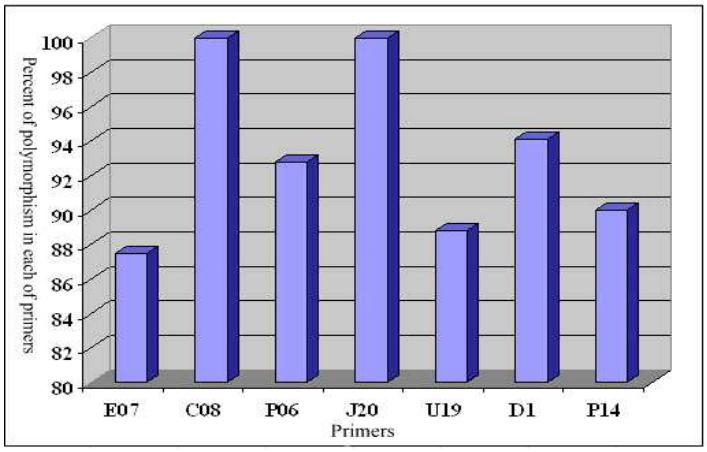

Fig. 2. The percentage of polymorphism detected by each primer.

Primers C08 and J20 showed the highest, whereas primer E07 showed the lowest percentage of polymorphism (Fig. 2). *E. necator* isolates were classified based on the observed variation in banding pattern with each primer and the constructed phenogram, using UPGMA. The average variability of banding pattern was 13.85 % for each primer. The highest and lowest number of bands were produced by primers D and U19, respectively. All polymorphic amplicons obtained were used to establish a matrix of genetic distance. Isolates fell into 7 groups, Group A comprised of four isolates from Shahriar (Sh1, Sh2, Sh3 and Sh4), group B comprised of three isolates from Alamoot region (A1, A2 and A3) and five isolates from Qazvin (D1, D2, D3, Kh3 and Kh4). Group C comprised of four isolates from Qazvin (Q2, Q3, Kh2 and Kh1). Group D comprised of one isolate from Alamoot (A4) and one isolate from Qazvin (Q1). Group E comprised of four isolates from Takestan (T1, T2, T3 and T4). Groups F and G each comprised of one isolate from Qazvin (Q4) and (D4), respectively (Fig. 3).

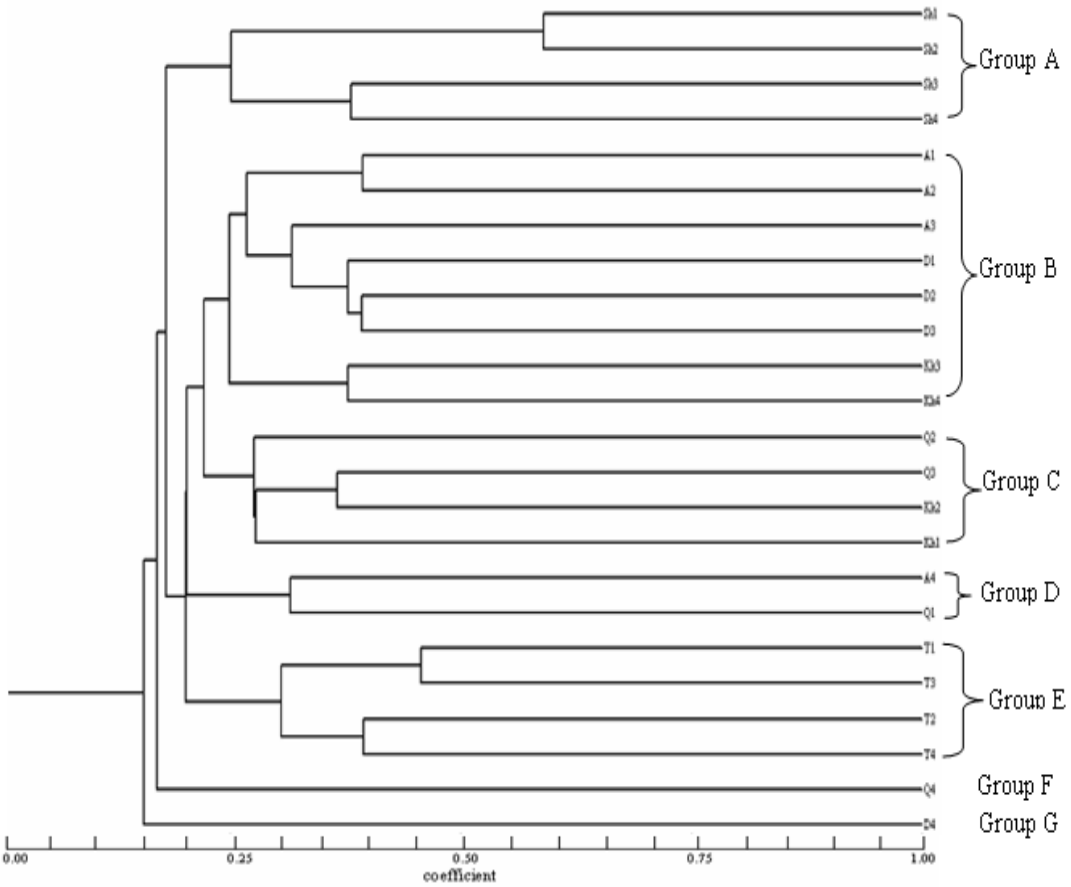

Fig. 3. Phenogram generated by the analyses of RAPD results on the 24 isolates of *Erysiphe necator*, using UPGMA method

Table 3. The presence (+) and absence (-) of the 1000 bp amplified fragment by primer E07.

Isolates	Presence of 1000 bp band	Isolates	Presence of 1000 bp band
1	-	13	-
2	-	14	-
3	-	15	-
4	-	16	-
5	-	17	+
6	-	18	-
7	-	19	+
8	-	20	-
9	-	21	+
10	-	22	-
11	+	23	-
12	-	24	-

The highest value obtained for similarity was between isolates Sh1 and Sh2 from Shahriar (0.59) and the lowest similarity value (0.094) was obtained between Sh1 and Q1 isolates from Qazvin. Péros *et al*., (2005) based on RAPD results classified the *E. necator* isolates found in southern France into two groups, A and B. They showed that all isolates in group A were of mating type +, but those in group B could be either + or -. Group A reproduced asexually and group B, reproduced sexually or asexually and a higher variation was observed in group B compared to Group A. The basis for considering isolates as + or – was the amplification of a 1000 bp fragment using E07 primer. As our isolates were tested with primer E07, only isolates 11, 17, 19 and 21 showed the 1000 bp fragment and considered +, and the rest were – (Fig. 1 and Table 3).

Discussion

Genetic diversity has been evaluated in *E. necator* using different techniques, such as transposon-PCR (Bouscant and Corio-Costet, 2007), ISSR (Cortesi *et al*., 2005), nested allele-specific (NAS) PCR (Deyle and Corio-Costet 1998) and SCAR primers (Hajjeh *et al*., 2005). For the first time, Delye *et al*., (1995) described a RAPD assay, using DNA extracted from the mycelium of the grapevine powdery mildew fungus, *E. necator*. They used 95 primers, among which 21 primers were polymorphic (4%). Delye *et al*., (1997) using RAPD analysis, distinguished two biotypes in European powdery mildew populations, having different overwintering modes. They obtained a large number of genetic markers suitable for epidemiological studies of 90 isolates of grape powdery mildew in the vineyards. However, other studies on the population genetic of *E. necator*, have shown conflicting results to those of Délye and colleagues (1997). For instance, Miazzi *et al*., (2003) used 23 primers on a collection of 374 isolates of *E. necator*. Each primer generated from 1 to 10 amplicons. The mean value of genetic similarity in the whole population was 0.87. They also showed that Italian populations of *E. necator* posses a very high level of genotypic diversity. According to the results of Péros *et al*., (2005), most of our isolates lie in group B, i.e. they reproduce sexually and/or asexually. This assumption is strengthened by the high genetic variation observed between the isolates.

Our results also showed that *E. necator* isolates were genetically distant in the studied geographical regions. This high level of genotypic diversity in *E. necator* isolates is also similar to those of Miazzi *et al*., (2003) and Cortesi *et al*., (2005). Isolates within one region had higher genetic similarity with each other than isolates from other areas. In groups A and E only isolates from Shahriar and Takestan and in groups C, F and G only Qazvin isolates were placed, respectively. Isolates in groups A (Shahriar) and E (Takestan) seem to be genetically distant from the other isolates. In groups B and D Alamoot and Qazvin isolates were placed together.

As a hypothesis, the similarity observed between Alamoot and Qazvin isolates could be interpreted as a result of exchange and plantation of vine cuttings between the vineyards in these two areas. Consequently, some fungal isolates were moved and spread between those areas. This hypothesis is supported by the findings of Deley *et al*., (1997), who mentioned that the conidia of *E. necator* are not easily disseminated by wind. Therefore human interference such as trade of infected vine cuttings could cause its dispersal.

In conclusion, some points may be noticeable: a) genetic similarity of *E. necator* isolates seems to be low in the 4 areas studied b) The isolates between 2 areas (Qazvin and Alamoot) out of 4, are relatively closer to each other. This could be due to artificial man interference and c) a possibility for a high degree of genetic

distance between fungal isolates observed here may be the type of reproduction mode in *E. necator* in different areas or climates; as sexual reproduction enforces more variation than asexual reproduction (Deley *et al*., 1997; Peros *et al*., 2005; Glawe, 2008).

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