RAPD Analysis Provides Insight into the Biology and Epidemiology of *Uncinula necator*

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ABSTRACT

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Ninety isolates of grape powdery mildew (*Uncinula necator*) from Europe (sixty-two) and India (twenty-eight) were collected. Ten of the sixty-two European isolates originated from mycelium overwintering in dormant buds ("flagshoots"). Mating types were determined, and genetic variation was assessed by random amplified polymorphic DNA (RAPD). Forty-one European isolates, including all "flagshoot" isolates, were mating type +, and twenty-one were mating type -. All Indian isolates were mating type -. Phenetic analysis based on 414 amplicons revealed three main groups. Most European isolates (53) clustered together. Nine

flagshoot isolates clustered in a second distinct group. These isolates, which coexisted with other isolates in the field, may represent a genetically isolated biotype of *U. necator*. Indian isolates clustered into two groups. The first group (15 isolates) was a subgroup of the group containing European nonflagshoot isolates. The second group (12 isolates) was distinct from the other groups. These two groups of Indian isolates may represent genetically isolated populations with different climatic tolerances. A polymerase chain reaction primer pair, derived from a RAPD fragment specific to the Indian isolates, proved to be suitable for field studies.

Additional keywords: cleistothecia, heterothallism, UPGMA, *Vitis vinifera*.

Powdery mildew, caused by the biotrophic ascomycete fungus *Uncinula necator* (Schwein.) Burrill, is one of the major diseases of grape (*Vitis vinifera* L.). This fungus is now present in vineyards throughout the world, but it is believed to originate from North America (26,33). The first outbreak of *U. necator* in Europe occurred in England in 1845 and was related to increased trade with America. The fungus spread extremely rapidly: in only 7 years, grape powdery mildew caused heavy losses in vineyards throughout Europe and the Mediterranean Region (6,33). This spread was attributed to commercial exchange and/or wind dispersal (6,33), although a recent study (36) has suggested that *U. necator* is incapable of being disseminated over long distances by wind.

Grape powdery mildew can overwinter as mycelium in dormant buds on leaf primordia (5,23,25,37). The mycelium resumes activity at budbreak, causing the appearance of heavily infected sporulating shoots called "flagshoots," early in the growing season (5). *U. necator* is a heterothallic fungus (16,28) that also can overwinter as cleistothecia (the ascigerous stage of this fungus) retained on the corky bark of grapevines (7,13,22). In this case, the primary source of inoculum consists of ascospores released during spring (11,14,15). In Europe, cleistothecia were not observed between 1845 and 1892 (8), suggesting that mycelium in dormant buds was possibly the only overwintering form of *U. necator* for more than 40 years. Currently, cleistothecia and/or flagshoots have been reported in vineyards throughout Europe; cleistothecia represent the most common way the fungus overwinters in Europe (6,36).

Due to the obligate biotrophic nature of *U. necator*, little is known about its genotypic diversity, its epidemiology, or the importance of sexual reproduction. It is still unknown whether a given isolate is capable of overwintering both as cleistothecia and mycelium in buds or whether different biotypes of grape powdery mildew may exist. Because of the lack of epidemiological data concerning grape powdery mildew in Europe, no risk-assessment model has been developed for this disease. This has led to the use of systematic fungicide treatments, mainly with sterol biosynthesis-inhibiting compounds. As a consequence, development of resistance to these compounds was observed in field populations of *U. necator* in Portugal in 1988 (31). Resistance to these fungicides now has been detected in many European vineyards (30). A better knowledge of the biology and epidemiology of grape powdery mildew is required to develop integrated disease management strategies.

Epidemiological studies of *U. necator* require population or individual markers. Because there is no major resistance gene known in grape, no virulence/avirulence pattern can be established for characterization of *U. necator* populations. Recently, we developed a random polymorphic amplified DNA assay (RAPD [35]) suitable for genetic studies of grape powdery mildew (10). This technique has proved very useful for studying genetic variation in a number of fungal species (9,17), including the biotrophic fungi that cause barley powdery mildew (19) and wheat leaf rust (18). RAPD markers are dominant (35), but the fact that *U. necator* is a haploid, uninucleate fungus greatly facilitates analyses. The purposes of the current study were (i) to obtain a large number of genetic markers suitable for epidemiological studies of grape powdery mildew in the vineyard and (ii) to determine whether significant genetic differences exist between isolates of *U. necator* overwintering in the asexual (mycelium) or sexual (cleistothecia) form. Insight into population biology is important, because genetic recombination occurring

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during sexual reproduction is likely to have consequences for the evolution of fungicide resistance in grape powdery mildew field populations.

MATERIALS AND METHODS

***U. necator* isolates.** The 90 isolates of *U. necator* used in this study are listed in Table 1. It was possible to collect European isolates only in vineyards where grape powdery mildew was not fully controlled by fungicide treatments. This resulted in an unbalanced sampling scheme. Except for “flagshoot” isolates, which were collected at the beginning of April, most European isolates were collected between May and September. To determine whether the same isolates of *U. necator* were capable of overwintering both in the dormant buds and as cleistothecia, FCP isolates were collected in the same vineyard and on the same grape plants on which both flagshoots and cleistothecia were present. Isolates FCP11 to -15 and FCP17 to -113 were collected immediately after the appearance of flagshoots (April) and 2 months after the ascospore release peak (August), respec-

tively. Grape powdery mildew isolates from India were included in this study for the following reasons: (i) the outbreaks of *U. necator* in Europe and India seem to have occurred independently but in a similar way. Grape powdery mildew was reported in India in the early 1900s (32) and spread rapidly throughout the country. (ii) The environmental conditions in India are extremely different from those in Europe. In India, there is no dormancy, or only partial dormancy, of grape plants. Moreover, climatic variations between seasons and geographic areas are extremely wide. This may have influenced the evolution of *U. necator* populations. Indian isolates, therefore, were collected during the winter and summer in northern (Punjab) and southern India.

Isolates were taken from grape leaves, shoots, or green berries covered with sporulating powdery mildew. Young leaves from grape cv. Cinsaut were decontaminated by a 10-min immersion in 50 g of calcium hypochlorite per liter, rinsed, and placed on agar medium at 20 g/liter. To obtain clonal isolates, a single powdery mildew conidium was picked from contaminated samples, using an eyelash fastened to a holder, under a binocular microscope under a laminar-flow hood. The conidium was placed on the upper

TABLE 1. Geographic origins of the 90 isolates of *Uncinula necator* used in this study

Isolates	Geographic origin		Year of isolation ^a	Grape cultivar ^b	Mating type
	Country	Location			
FBO11 ^c , -12 ^c , -13 ^c	France	Bordeaux	1992	Cabernet-Sauvignon	+
FBO21	France	Bordeaux	1993	Cabernet	+
FBO31	France	Bordeaux	1994	Cabernet	+
FCP11 ^d -15 ^d	France	Case de Pène	April 1994	Carignan	+
FCP18, -19, -110	France	Case de Pène	1994	Carignan	+
FCP16, -17, -111, -112, -113	France	Case de Pène	1994	Carignan	-
FIO11-14	France	Île d'Oléron	1994	SO4	+
FIO21 ^c , ^d	France	Île d'Oléron	April 1994	Ugni blanc	+
FMA11 ^c	France	Madiran	1989	Tannat	+
FMA21 ^c	France	Madiran	1990	Tannat	+
FMA31, -32	France	Madiran	1992	Tannat	-
FMA41	France	Madiran	1994	Tannat	-
FPE11 ^c , -12 ^c	France	Perpignan	1993	Carignan	-
FPE21 ^c , -31 ^c , -32	France	Perpignan	1992	Chardonnay	+
FPE22	France	Perpignan	1992	Chardonnay	-
FPO11 ^c , -12 ^c	France	Poussan	1993	Carignan	-
GNE11 ^d , -12 ^d , -13 ^d , -14 ^d	Germany	Neustadt	April 1994	Kerner	+
GUN11-14	Germany	Ungstein	1994	Portugieser	-
IBA11, 12	India	Bangalore	1995	29/7 hybrid	-
IHY11-13	India	Hyderabad	1996	Madhir Angow	-
IHY21-24	India	Hyderabad	1996	Perlette	-
IJA11	India	Jassian	1994	Perlette	-
IJA21	India	Jassian	1995	Perlette	-
INA11	India	Naruana	1994	Perlette	-
IPA11-14	India	P. A. U. ^e	1994	Perlette	-
IPA21	India	P. A. U.	1995	Perlette	-
IPA31	India	P. A. U.	1996	Perlette	-
IPU11, -12	India	Puna	1995	Thompson seedless	-
IRA11, -12	India	Rampura	1995	Perlette	-
ISA11, -12, -21, -22, -31, -32	India	Sangali	1995	Thompson seedless	-
PAL11 ^c	Portugal	Alenquer	1991	Cardinal	+
PAZ11	Portugal	Azambuja	1988	Carignan	-
PER11 ^c	Portugal	Ereira	1991	Carignan	+
PPV11 ^c	Portugal	Portela do Vado	1993	Loureiro	+
PTV11 ^c , -12 ^c	Portugal	Torres Vedras	1993	Carignan	+
PTV21 ^c	Portugal	Torres Vedras	1993	Carignan	-
PVA11 ^c , -12 ^c , -21 ^c , -22 ^c	Portugal	Varatojo	1993	Loureiro	+
SLE11	Switzerland	Leytron	1994	Gamay	+
SNO11	Switzerland	Noutze	1994	Gamay	+
SNO21	Switzerland	Noutze	1994	Gamay	-
SSI11 ^c , -12 ^c , -13 ^c	Switzerland	Sion	1993	Chasselas	+
SSI21 ^c	Switzerland	Sion	1993	Chasselas	-

^a Unless mentioned, European isolates were collected between May and September.

^b Cultivars from which the isolates were taken.

^c Isolates used for preliminary random amplified polymorphic DNA amplifications with 95 primers.

^d “Flagshoot” isolate.

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surface of a decontaminated leaf. After 14 days of incubation at 22°C under 16 h of illumination (1,000 lux) per day, powdery mildew isolates were inoculated under sterile conditions onto the upper surface of four decontaminated grape leaves by blowing 600 to 800 conidia per cm² of leaf with an air pump. They were allowed to grow for 14 days. The isolates were named with three letters and two numbers, indicating the country (first letter), the location in the country (second and third letters), the grape field (first number), and the sample (second number) from which each was isolated.

Mating studies. Isolates PTV11 (mating type +) and FPE11 (mating type –) were used as testers to determine the mating type of all other isolates. Isolates of unknown mating type were inoculated on four leaves together with tester isolate PTV11 or FPE11 and incubated at 22°C under 16 h of illumination (1,000 lux) per day for 14 days. The presence of cleistothecia was checked under the binocular microscope every 3 days for a period of 30 days. Sixty days after formation, the fertility of cleistothecia was assessed by squashing them in a drop of cotton blue on a microscope slide and checking for the presence of asci.

DNA extraction and RAPD amplifications. DNA from total fungal material (conidia plus mycelium) was extracted and amplified as previously described (10). Mycelium and conidia were harvested by scraping with a disposable scalpel 14-days after inoculation of leaves. Fungal material was subsequently freeze-dried. DNA was extracted by DNA-Now monophasic reagent (Biogenex, Seabrook, TX) and dissolved in sterile distilled water. DNA solutions were kept at –20°C. RAPD amplifications were performed in 20- μ l reaction mixtures containing 70 mM Tris-HCl, 2 mM MgCl₂, 17 mM (NH₄)₂SO₄, 10 mM β -mercaptoethanol, 0.05% (wt/vol) polyoxyethylene-ether W1 (Sigma Chemicals, St. Louis), 0.2 mg of bovine serum albumin per ml, 200 μ M each of dATP, dCTP, dGTP, and dTTP, 0.4 μ M a single 10-mer oligonucleotide, ~10 ng of template DNA, and 0.2 units of Goldstar DNA polymerase (Eurogentec S.A., Seraing, Belgium). A Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus Instruments Division, Norwalk, CT) was used with a program consisting of 37 cycles of 30 s at 94°C, 30 s at 37°C, and 1 min at 72°C. Ninety-five-decamer primers (Operon Technologies, Alameda, CA) were used for the amplification of DNA from a first set of 26 isolates (Table 1). Primers that revealed polymorphism between

these isolates were used subsequently to amplify DNA extracted from the remaining isolates (a list of primers that reveal polymorphism in *U. necator* is available from the authors upon request). At least two independent amplifications, using two different DNA samples, were performed for all isolate and primer combinations. The RAPD products were separated by electrophoresis at 110 V for 3 h on ethidium bromide-stained (0.4 μ g/mm), 1.3% agarose gels run in 0.5 $\times$ Tris-borate EDTA buffer and visualized under UV light.

Data analysis. Each isolate was scored for the presence or absence of each amplicon. When a primer revealed polymorphism, all detectable amplified DNA fragments were used for analysis and treated as binary characters. Genetic similarities were computed between all pairs of isolates using the formula given by Nei and Li (20): $S = 2b_{ij}/(b_i + b_j)$, where b_{ij} is the number of amplicons shared by two isolates, i and j , and b_i and b_j are the total number of amplicons found in isolates i and j , respectively. Genetic distances (or dissimilarities) were computed as genetic distance = $1 - S$, and the data were used to construct a phenogram, using the unweighted pair-group method with arithmetic average (UPGMA [29]) or the Fitch-Margoliash method (12). Distance calculations and construction of a phenogram, using UPGMA, were performed using Splus 3.1 software (Statistical Sciences, Seattle). To check the robustness of the phenogram, the programs Kitsch and Drawgram contained in the PHYLIP software package (Phylogeny Inference Package, version 3.5.2, J. Felsenstein, University of Washington, Seattle) were used to construct a phenogram according to the Fitch-Margoliash method (12). A bootstrap analysis also was used to support major branches of the phenogram. One hundred bootstrap samples of the same size as the original RAPD data set were generated to compute one hundred genetic dissimilarity matrices, using the Splus software. The programs Neighbor and Consense of the PHYLIP software package were used to construct the consensus UPGMA phenogram.

RESULTS

Mating studies. All isolates were paired with both tester strains and formed abundant cleistothecia within 14 to 18 days when paired with isolate PTV11 or FPE11. The isolates consequently were assigned a + or – mating type (Table 1). No isolate produced cleistothecia alone or with both tester isolates. All 10 flagshoot isolates were mating type +. Among the 52 remaining European isolates, 31 were mating type +, and 21 were mating type –. All 28 Indian isolates were mating type –.

RAPD amplifications. Of the 95 primers tested, 46 revealed polymorphism among a first set of 26 isolates (Table 1). These primers were retained for RAPD amplification of DNA extracted from the 64 remaining isolates to obtain as many polymorphic amplicons specific to one isolate or group of isolates as possible. Such amplicons are of interest for the development of molecular epidemiological tools, although primer selection leads to an overestimation of genetic dissimilarities computed between isolates of *U. necator*. All RAPD profiles, including amplicons of weak intensity, were fully reproducible for at least two independent assays using two independent DNA extractions. Furthermore, RAPD profiles were unchanged when DNA samples from the same isolates were prepared 18 months apart, which corresponded to 36 asexual generations (Fig. 1). No contamination by grape DNA was detected in RAPD amplifications, as was reported previously (10).

Data analysis. The 46 RAPD primers tested on the 90 *U. necator* isolates yielded 414 amplicons (9.1 per primer), 216 (52.3%) of which were polymorphic. The sizes of the amplified DNA fragments ranged from 0.2 to 3 kb. All amplicons obtained were used to establish a matrix of genetic distances. The mean genetic dissimilarity obtained for the 90 isolates was 0.0955. UPGMA (Fig. 2) and Fitch-Margoliash (12) (data not shown) methods pro-

Fig. 1. Random amplified polymorphic DNA (RAPD) profiles of seven isolates of *Uncinula necator* obtained with primer OPE-7 (5'-AGATGCAGCC). RAPD patterns are shown for DNA extracted from asexual generation A, 1 and B, 36 of isolates IPA11 (lane 1), FBO11 (lane 2), SSI11 (lane 3), PTV11 (lane 4), INA11 (lane 5), GNE11 (lane 6), and FIO21 (lane 7). Lane G, amplification of DNA extracted from grape leaves following the same protocol used for fungal DNA extractions. Lane H, H₂O negative control (no DNA). Lane M, molecular weight marker (1-kb DNA ladder; Gibco-BRL, Gaithersburg, MD).

duced very similar phenograms. In both cases, cluster analysis revealed three distinct groups (groups I, II, and III). Bootstrap analysis with 100 repetitions showed that these groups were present in 100% of the generated trees. With respect to the geographic origin of the isolates, group III was split into two subgroups (subgroups III-1 and III-2). These subgroups appeared as distinct groups in only 35% of the trees, but all isolates in subgroup III-1 clustered in a specific clade in 78% of the trees. Group I included 9 of the 10 European flagshoot isolates and the Indian isolate IBA11. Group I was clearly distinct from the other groups. The

isolates in this group displayed 295 amplicons, 20 (6.8%) of which were polymorphic. Isolate IBA11 differed from the other isolates in group I by 4 additional and 11 missing amplicons. Group II contained 12 closely related Indian isolates that displayed 290 amplicons, 13 (4.5%) of which were polymorphic. Mean genetic dissimilarity between groups I and II was high (0.2041; Table 2) compared to the general mean genetic dissimilarity (0.0955). The two subgroups in group III were closely related, exhibiting a between-group genetic dissimilarity of 0.0374 (Table 2). Subgroup III-2 contained 53 isolates, all from Europe. Isolates clustered in

Fig. 2. Phenogram generated by unweighted pair-group method with arithmetic average cluster analysis of random amplified polymorphic DNA (RAPD) data from 90 isolates of *Uncinula necator*. Genetic dissimilarities were calculated using Splus software, based on 414 RAPD markers generated by 46 primers. The mating types of isolates are shown between brackets. Numbers at major nodes indicate the number of times the group of isolates to the left of the node occurred among a sample of 100 bootstrap-generated phenograms.

subgroup III-2 displayed 337 amplicons, 75 (22.2%) of which were polymorphic. Within this group, isolates from Switzerland, Portugal, and Madiran (France, FMA isolates) generally clustered in neighboring clades. The remaining French isolates clustered with the German isolates. Isolates originating from the same vineyard or vine-production area generally clustered together. Isolates in subgroup III-2 also had a tendency to cluster according to their mating type (Fig. 2). Subgroup III-1 contained the 15 remaining Indian isolates. They displayed 299 amplicons, 14 (4.7%) of which were polymorphic.

Distribution of amplicons exclusively present or absent in the RAPD patterns of all isolates clustered in one or two groups was as follows. Isolates in group I shared 24 specific amplicons. They did not exhibit 17 of the amplicons present in all other isolates. Isolates in group II shared 27 specific amplicons and did not exhibit 11 of the amplicons present in all other isolates. All isolates in groups I and II shared seven amplicons that were absent in the remaining isolates. None of these isolates displayed 21 of the amplicons present in isolates in group III. One specific amplicon was displayed by all Indian isolates in group II and subgroup III-1, but not by Indian isolate IBA11, which clustered in group I. Two amplicons seemed to be related to mating type. They were displayed exclusively by all mating type + isolates. A total of 13 amplicons exclusively found in one isolate or one group of isolates with identical RAPD patterns were obtained. These "rare" amplicons are of interest for the development of molecular tools for epidemiological studies.

To determine whether polymerase chain reaction (PCR) primers derived from specific RAPD amplicons may be suitable for detection of particular isolates in the vineyard, the two extremities of the 1-kb RAPD fragment specific to Indian isolates were sequenced. The sequence characterized amplified region (SCAR)-PCR (21) primers deduced from the sequence were SU12 (5' TCTGGAGCGCTGGTCTAG) and SU12R (5' TAGCCGCTCTCACTTCTTC). PCR amplifications were performed with DNA extracted from the 90 *U. necator* isolates, grape, *Plasmopara viticola* (grape downy mildew), and various saprophytic fungi that grew on potato dextrose agar plates in which nondecontaminated grape leaves collected from the vineyard were placed. These fungi were mostly *Botrytis* sp., *Trichoderma* sp., *Penicillium* sp., *Rhizopus nigricans*, and unidentified yeasts. The same reaction mixture was used as was used for RAPD amplifications. PCR primers were used at a final concentration of 0.1 μ M each, and the annealing temperature was 60°C. No amplification was obtained with DNA extracted from grape, downy mildew (*P. viticola*) (data not shown), or other fungi (Fig. 3). All Indian isolates, except isolate IBA11, displayed the expected 1-kb PCR fragment. This fragment was not amplified from the remaining *U. necator* isolates. However, some of them (25 isolates, including isolate IBA11) exhibited an unexpected 1.5-kb PCR fragment when their DNA was subjected to PCR with primers SU12 and SU12R (Fig. 3).

To estimate the detection threshold of this assay, freeze-dried fungal material from Indian grape powdery mildew isolates and saprophytic fungi from the vineyard were mixed in $1/1$, $1/10$, $1/100$, $1/300$, $1/500$, $1/700$, and $1/1,000$ (wt/wt) proportions before DNA extraction and PCR amplification (Fig. 3). Amplifications were performed three times with three different DNA extractions. Ampli-

fication of the 1-kb PCR fragment was obtained for all mixtures ranging from proportions of $1/1$ to $1/500$. Amplification of this fragment in $1/700$ proportion mixtures was not obtained in all assays. The lower limit for detection of an Indian isolate of *U. necator*, thus, was 1 mg dry weight of powdery mildew for 500 mg dry weight of other fungi.

DISCUSSION

Genetic dissimilarity among 90 clonal isolates of *U. necator* was evaluated using the RAPD technique. Phenetic analysis revealed three distinct groups of isolates of *U. necator* (Fig. 2). Group III was split into two subgroups: subgroup III-1 contained only Indian isolates, whereas subgroup III-2 contained only European isolates.

European isolates. The major subgroup in group III, subgroup III-2, included all of the European isolates, except 9 of the 10 flagshoot isolates. The low percentage of polymorphic amplicons found among isolates in this group (22.2%) was consistent with our preliminary study, in which 13 European isolates (FPO11 and -12; FPE11, -12, and -21; SSI11, -12, and -13; PVA11, -12, -21, and -22; and PPV11) were studied using 16 primers (10). These primers yielded 119 amplicons, 21 (17.6%) of which were polymorphic. As was found previously, most Portuguese and Swiss isolates clustered together. Isolates originating from Madiran (FMA isolates) also clustered with Swiss and Portuguese isolates. Isolates originating from the same grape field generally were found in the same clusters (isolates PVA21 and -22 or FCP16, -17, -111, -112, and -113, for instance) or displayed identical RAPD patterns (isolates SSI11 to -13 or FIO11 to -14, for instance) and were probably of the same clonal line.

A previous study reported that conidia of *U. necator* require high wind speed and/or substantial leaf vibrations to be released (36). The relative agreement found between geographic origin and the genetic dissimilarities of isolates, as well as these epidemiological data suggests that the conidia of *U. necator* are not easily nor massively disseminated by wind. Thus, human activity, such as trade of contaminated grape plants, could be a major cause of inoculum dispersal.

European isolates clustered into two distinct groups (group I and subgroup III-2). Group I contained all flagshoot isolates, except isolate FCP15. Despite diverse geographic origins, isolates in group I displayed small between-isolate dissimilarities (Table 2). All flagshoot isolates collected in the same grape field displayed identical RAPD patterns and were probably of the same clonal line. All flagshoot isolates were mating type +, whereas European isolates in group III included 32 mating type + and 21 mating type - isolates.

Isolate FCP15, which was collected during April, together with isolates FCP11 to -14 (group I), displayed RAPD patterns similar to isolates FCP17 to -113 (subgroup III-2, collected during August on the same plants from which isolates FCP11 to -15 were collected). Both flagshoots and cleistothecia were present on these plants. This suggests that isolate FCP15 probably originated from an ascospore that was released early. It is known that, although most ascospore discharge occurs from May to July in Europe (36), ascospores are released from budburst to bloom of grapevines (14).

All group I-type European isolates (isolates FCP11 to -14, GNE11 to -14, and FIO21) were collected during April (Table 1). Group I-type isolates were not found in samples collected from May to September throughout Europe. The absence of group I-type isolates in the second sampling, collected during summer, suggests that flagshoot isolates were no longer actively developing in the vineyards or that they represented a small proportion of the powdery mildew population. It is possible that flagshoot isolates survive inside buds until the next grape budbreak, when new bud contamination occurs. This is consistent with previous

TABLE 2. Arithmetic mean genetic dissimilarities within and between the groups of isolates of *Uncinula necator* identified by phenetic analysis

	Group I	Group II	Subgroup III-1	Subgroup III-2
Group I	0.0089	0.2041	0.2293	0.2232
Group II		0.0045	0.1856	0.1867
Subgroup III-1			0.0106	0.0374
Subgroup III-2				0.0193

data suggesting that bud infection by *U. necator* flagshoot isolates occurs early in the season (23).

The high genetic dissimilarity found between flagshoot isolates and the remainder of European isolates (Table 2), as well as the number of amplicons exclusively present (33 amplicons) or absent (35 amplicons) in flagshoot isolates, may be due to selection in relation to the overwintering stage or the absence of genetic recombination, and thus of sexual reproduction, between these two populations. This may be caused either by the early disappearance of flagshoot isolates in the vineyard or by sexual incompatibility. The second hypothesis is strengthened by pairing experiments. Cleistothecia formed abundantly and matured when flagshoot isolates were paired with tester isolate FPE11. However, these cleistothecia were devoid of ascogenous content 70 days after formation. This seems to be a case of "delayed incompatibility," which agrees with previous findings suggesting that heterothallism in *U. necator* is rather complex (16). Flagshoot isolates may have started to lose their mating capacities, possibly because of their asexual method of overwintering. Two "overwintering" biotypes of *U. necator*, thus, may exist in Europe: the "flagshoot" biotype, overwintering inside dormant buds infected early in the grape-growing season, and the "cleistothecium" biotype, overwintering as cleistothecia formed at the end of the grape-growing season. A more extensive study, conducted on large samplings of isolates collected during spring and summer in vineyards where cleistothecia and flagshoots are both present, is required before definitive conclusions can be drawn.

The presence of two biotypes of *U. necator* in Europe may be related to the historical pattern of cleistothecia production observed after the disease was introduced. For more than 40 years after the introduction of grape powdery mildew into Europe, no cleistothecia were reported in vineyards (6). After first being observed in 1892 (8), cleistothecia occasionally were observed late in the season (1,4). During the early 1940s, cleistothecia appeared in large numbers in several European vineyards (2,3,27,34). Peyronel (24) proposed that these events were due to independent introductions of the two mating types into Europe 40 years apart. Gadoury and Pearson (16) argued that the transition between the sparse and abundant production of cleistothecia was the consequence of the introduction of isolates with "delayed compatibility," followed by the introduction of readily compatible isolates. However, the only isolates they reported as having delayed compatibility originated from *V. labrusca* and *V. labruscana*, not from *V. vinifera*. In addition, no delayed compatibility was observed among European isolates in our mating studies. Thus, we suggest that flagshoot isolates may have been introduced first into Europe. Thereafter, group III-type isolates, mainly of a single mating type, would have been introduced, causing occasional sexual reproduction of grape powdery mildew. The second mating type may have been introduced during the late 1930s, causing locally abundant production of cleistothecia. The fact that significant cleistothecia production did not occur across Europe is consistent with a short-range natural dissemination of *U. necator*. A study of genotypic diversity of *U. necator* in North America would be useful to check the hypothesis of successive introductions of *U. necator* isolates from this origin.

Indian isolates. Indian isolates clustered in two groups (groups II and III), except isolate IBA11, which clustered with the European flagshoot isolates in group I. This particular isolate, which did not exhibit the Indian isolate-specific 1-kb PCR fragment but, instead, displayed the 1.5-kb PCR fragment in common with isolates in group I, could be a flagshoot-biotype isolate. Should this be true, the existence of the mating type – in the flagshoot biotype would be proved. Pairings of isolate IBA11 with tester isolate PTV11 yielded sterile cleistothecia. Pairings of this isolate with European flagshoot isolates currently are being performed.

Indian isolates clustered in subgroup III-1 are closely related to subgroup III-2 European isolates, suggesting a common origin for

these two populations. No geographic clustering was observed within subgroup III-1: isolates IBA12 and IPU11 and -12 were collected in vineyards more than 900 km apart and displayed identical RAPD patterns. Conversely, isolates IJA11 and IPA11 to -14, which were collected in two locations only 30 km apart, displayed distinct RAPD patterns. The same was observed for isolates clustered in group II: isolates ISA11 and INA11, which displayed identical RAPD patterns, were collected in vineyards more than 1,500 km apart, whereas isolates INA11 and IRA11 and -12 were obtained from vineyards only 20 km apart. It is likely that human activity, rather than wind dispersal, was involved in long-range dispersal of *U. necator* inoculum, because no grape fields were present between the vineyards in northern and southern India, which were more than 1,500 km apart. Introduction of exogenous inoculum by human activity, thus, may be the reason identical isolates have been found in vineyards 1,500 km apart.

The occurrence of two very distinct groups of isolates in India (Table 2) may be related to environmental factors. In tropical southern India, there is no dormancy of grape plants. Receptive grape shoots, thus, are present all year round, and the fungus does not need an overwintering stage. In northern India, grape plants do not bear leaves from December to February. However, dormancy is never completely established, and shoots and leaves continue to develop during this period. Grape powdery mildew, thus, may overwinter either in buds or on shoots. The sole significant difference between the periods of collection of group II- and subgroup III-1-type isolates was climatic. Subgroup III-1 isolates all were collected during the cold season in tropical southern India (isolates ISA, IBA, and IPU) or during spring in northern India (isolates IJA11 and IPA). Group II isolates, except isolate ISA11, all were collected under high-temperature conditions during the hot monsoon season in southern India (isolates IHY) or during summer in northern India (isolates IJA21, INA11, and IRA). Subgroup III-1-type isolates, thus, may be better adapted to cold-season conditions, whereas development of group II-type isolates, which are still present during the cold season (isolate ISA11), may be favored by high temperatures.

Sexual reproduction seems to be rare in India. It was observed only occasionally in the northern portion of the country. Because all 28 Indian isolates studied were mating type –, it is possible that

Fig. 3. Polymerase chain reaction (PCR) amplifications performed with primers SU12 and SU12R with DNA extracted from mixtures of fungal material obtained from Indian grape powdery mildew (*Uncinula necator*) isolate IPA11 and from saprophytic fungi from the vineyard (*Botrytis* sp., *Trichoderma* sp., *Penicillium* sp., *Rhizopus nigricans*, and unidentified yeasts) in $1/1,000$, $1/700$, $1/500$, $1/300$, $1/100$, $1/10$, and $1/1$ (wt/wt) proportions (lanes 1 to 7, respectively). Lane 8, DNA extracted from fungi growing on potato dextrose agar plates. Lane G, grape DNA. Lane H, H₂O negative control (no DNA). Lane 9, 1.5-kbp PCR fragment amplified from isolate FCP11. Lane 10, 1-kbp PCR fragment specific to Indian downy mildew isolates amplified from isolate ISA11. Lane M, molecular weight marker (1-kb DNA ladder; Gibco-BRL, Gaithersburg, MD).

mating type + isolates may be absent from the southern and rare in the northern portions of the country. The alternate development of group II- and subgroup III-1-type isolates in Indian vineyards, as well as the rarity of mating type + isolates, may result in the genetic isolation of these two populations. It would be interesting to determine the temperature ranges and RAPD patterns of a large sample of Indian isolates collected at different periods in the same grape fields, because *U. necator* isolates with different climatic adaptation, to our knowledge, have never been described.

Conclusions. Data obtained from pairing experiments suggest that sexual incompatibility in *U. necator* may result either in the absence of formation of cleistothecia in pairing with one mating type (most isolates) or in the formation of sterile cleistothecia in pairing with the apparently compatible mating type (flagshoot isolates). Cloning and sequencing the two amplicons specific to mating type + isolates may lead to a better understanding of genetic control of sexual reproduction in *U. necator*.

The relative agreement found between geographic origin and genetic polymorphism of the European group III-type isolates of *U. necator*, as well as epidemiological data, suggests that human activity rather than wind transportation is responsible for the dissemination of grape powdery mildew. RAPD has proven useful in differentiating individual (clonal) grape powdery mildew isolates, and derived molecular tools, such as specific SCAR-PCR primers SU12 and SU12R, may be suitable for epidemiological field studies. Such molecular tools, thus, may be developed to assess the degree of gene flow between local *U. necator* populations. For this purpose, particular amplicons, such as amplicons specific to one isolate ("rare" amplicons) or to one population, are potentially of great interest. Using a range of SCAR primer pairs will make it possible to follow a "tagged" isolate and monitor its dispersal, survival, and the consequences of possible sexual reproduction with indigenous powdery mildew populations. Assessing the importance of sexual reproduction of *U. necator* in the field will provide a better understanding of the genetic control and evolution of the polygenic resistance of this fungus to sterol biosynthesis-inhibiting fungicides.

Amplicons particular to flagshoot isolates are also of great interest. Flagshoot isolates are very distinct genetically from other European isolates and may represent a particular, genetically isolated, biotype of *U. necator*. Should this be true, flagshoots and cleistothecia would have to be considered two independent sources of inoculum. Flagshoot-specific amplicons will facilitate development of molecular tools for gathering data concerning the biology of these isolates and monitoring powdery mildew population succession in the field. Molecular tools also may be used in the vineyard to gain a better understanding of the population genetics and epidemiology of *U. necator*. This, ultimately, will lead to more efficient disease control strategies for this important disease.

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