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Genetic variation among and within *Uromyces* species infecting legumes

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ABSTRACT

Genetic variation of thirty different *Uromyces* isolates collected on faba bean, lentil, common
15 vetch, pea, chickpea, alfalfa, cowpea and lupin was studied. *Random Amplified Polymorphic*
DNA (RAPD) markers were used showing clear differences among *Uromyces* species. *U.*
viciae-fabae isolates clustered according to the host, with a clear cluster including all *U.*
viciae-fabae ex *Vicia faba* isolates. The *U. viciae-fabae* ex *Lens culinaris* isolate was the
nearest to the cluster of *U. viciae faba* ex *V. faba* isolates, followed by *U. pisi* from Canada
20 and *U. viciae-fabae* ex *V. sativa*. No association was found among molecular diversity and
virulence or geographic origin within *U. vicia-fabae* ex *V. faba* isolates. Among the three *U.*
pisii isolates considered, a great variability was observed and no grouping could be
established. The most different isolate from the rest of species considered was *U. striatus*,
followed by *U. vignae*. The two *U. ciceris-arietini* isolates clustered together and so did the
25 two *U. lupinicolus* isolates.

INTRODUCTION

The term rust is applied both for the disease and for the fungus that causes it. There are about 5,000 species of rust that attack an extremely wide range of crops worldwide. Several rust species can infect grain and forage legumes, most of them belonging to the genus *Uromyces*, such as *U. appendiculatus* (Pers.) Unger on common bean (*Phaseolus vulgaris* L.), *U. ciceris-arietini* Jacz. in Boyer & Jacz. on chickpea (*Cicer arietinum* L.), *U. lupinicolus* Bub. on lupine (*Lupinus* sp.), *U. pisi* ([Pers.] D.C.) Wint. on pea (*Pisum sativum* L.) and grasspea (*Lathyrus* sp.), *U. striatus* J. Schr. on alfalfa (*Medicago sativa* L.), *U. viciae-fabae* (Pers.) J. Schröt. (syn. *U. fabae* Pers.) on faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.) and common vetch (*V. sativa* L.) and *U. vignae* Barclay on cowpea (*Vigna unguiculata* (L.) Walp.). Also rust species belonging to other genera can be major problems on legumes such as *Phakopsora pachyrhizi* Sydow and *P. meibomiae* (Arthur) Arthur on soybean (*Glycine max* (L.) Merr.) or *Puccinia arachidis* Speg. on groundnut (*Arachis hypogaea* L.) (Rubiales et al., 2002).

Faba bean rust (caused by *U. viciae-fabae*) is a major disease in the Middle East, North Africa, Europe and China, where moderate to substantial yield losses can occur (Jellis et al., 1998; Sillero et al., 2006). Rust is one of the most important foliar diseases of lentil and is a limiting factor in several countries causing losses up to 100% (Negussie et al., 2005). Pea rust can be caused either by *U. pisi* (aecial stage on *Euphorbia* sp.) or *U. viciae-fabae* (Kushwaha et al., 2006) and occurs worldwide but is a problem mainly in India and China. Common bean rust is caused by *U. appendiculatus* and occurs worldwide but is most prevalent in humid tropical and subtropical areas. Yield losses can approach 100% and are directly related to earliness and the severity of infection (Statler and McVey, 1987).

Chickpea rust (*U. ciceris-arietini*) is a disease of local importance but it is present in almost every region of the world where chickpea is grown. Severe outbreaks have been reported in India, Central Mexico and Italy (Ragazzi, 1982; Jones, 1983). Lupine rust (*U. lupinicolus*) is important in the former USSR on fodder lupines (Gjaerum et al., 1986) and is of local
5 importance in areas of Europe.

The traditional criteria used to determine variation among and within fungal species are based on plant host, symptoms, colony appearance or morphological characterisation. Identification of *Uromyces* species on legumes is currently based on the morphology of telia, teliospores, uredinia and urediniospores. In many cases, however, telia are not available since
10 their formation depends on the rust species, host properties and environmental conditions. Additionally, rust species are often difficult to identify solely on the basis of urediniospore morphology; the two common features, germ pore number and arrangement, may not be easy to determine and they are often not good diagnostic tools. Host species are also used in identification, but several rust species may infect the same species of host plant. It is also
15 possible that a rust fungus may infect a plant species that was previously thought to be resistant.

U. viciae-fabae sensu lato is a species complex. Cummins (1987) stated that *U. viciae-fabae* is circumglobal on *Lathyrus*, *Pisum* and *Vicia*, with *V. faba* the host of the neotype. However, host-specialized isolates that cannot infect *V. faba* have been reported
20 (Emeran et al., 2005), suggesting that *U. viciae-fabae* may be subdivided into populations with differential pathogenicity to *V. faba*, *V. sativa*, or *L. culinaris*. More research is needed to clarify the ultimate classification of the *U. viciae-fabae* complex. A molecular tool to aid in the identification of anamorphic material would be very useful, particularly if it could be applied to dried herbarium specimens. The development of molecular technology, such as

PCR-based methods, has provided powerful tools for fungal diagnosis, genetic diversity of populations and taxonomy. PCR derived markers represent an ideal tool for the study of diversity in fungal populations since they are selectively neutral and can be generated in a large number. *Random amplified polymorphic DNA* (RAPD) assays have been extensively
5 used to differentiate fungal species, subspecies, formae speciales or isolates (Maclean et al., 1995; Kolmer and Liu, 2000; Park et al., 2000; Becerra et al., 2007).

The race phenotype of plant pathogenic fungi provides direct information concerning the effects of host selection and the potential effectiveness of resistance genes. However, virulence may represent only a small portion of the total genetic variation in a population.

10 The race phenotype of plant pathogenic fungi is controlled by relatively few loci (Michelmore and Hulbert, 1987) and the extend of other genetic differences between or within races might be unconsidered. The overall genetic differences can be assessed by comparing the background genotype of isolates at loci arbitrarily selected across the genome. In the case of *U. viciae-fabae*, although physiological races have been reported with the help
15 of a set of differential lines (Conner and Bernier, 1982; Emeran et al., 2001; Rojas-Molina et al., 2006), the genetic background has not yet been studied.

In the present study RAPD markers were selected to compare different rust isolates because of its technical simplicity and the ability to observe genetic markers at many loci. The aims of the present study were (1) to analyse the molecular diversity among 6 *Uromyces*
20 species attacking legumes, (2) to determine the genetic relationships among 3 groups of *U. vicia-fabae* isolates attacking faba beans, lentils and vetches that show host-specificity and (3) to relate the corresponding genetic variation for aggressiveness and spatial distribution in a set of *U. viciae-fabae* isolates.

MATERIAL AND METHODS

Fungal isolates

5 A total of 30 monopustular rust isolates belonging to 6 *Uromyces* species collected on different legumes have been studied (Table 1). These isolates were collected in different geographic areas: Australia, Canada, Egypt, Italy, The Netherlands, Morocco, Portugal, Spain, Syria and United Kingdom. Geographic origin, hosts and racial identity of the isolates analysed are reported in Table 1. These monopustular isolates were multiplied on very
10 susceptible cultivars of each of the host affected: faba bean (*Vicia faba* cv. Baraca), pea (*Pisum sativum* cv. Messire), lentil (*Lens culinaris* cv. Eston), vetch (*Vicia sativa* cv. Mezquita), lupine (*Lupinus albus* cv. Arthur), chickpea (*Cicer arietinum* cv. Fardón), cowpea (*Vigna unguiculata* cv. Local Egypt) and alfalfa (*Medicago sativa* cv. Baraka). The host plants with their third to fourth leaves fully expanded were inoculated by dusting freshly
15 collected urediospores (0.5 mg per plant) mixed with pure talcum powder (1:10, v/v). After inoculation plants were incubated at 100% relative humidity at 20°C in darkness for 24 hours. Since *Lupinus albus* developed necrotic lesions at this long incubation period, it was incubated for only eight hours. After inoculation plants were transferred to a growth chamber at 20°C under a 14 h light: 10 h dark photoperiod with light intensity of 148 $\mu\text{mol}/\text{m}^2/\text{s}$ at the
20 leaf canopy.

RAPD analysis

Uromyces spores were used for DNA extraction using the method proposed by Lassner et al. (1989), modified by Torres et al. (1993). For RAPD analysis, approximately 20 ng of

genomic DNA was used as a template in a 25 µl volume per PCR reaction. Mixture composition and reaction conditions were as described by Williams et al. (1990) with slight modifications (Torres et al., 1993). Reaction mixtures were amplified in a Termocycler PE Applied Biosystems GeneAmp 9700. A total of 14 RAPD primers from OPERON Technologies (Alameda, USA) were analysed (Table 2). Amplified products were electrophoresed on 1% agarose, 1 x TBE gels, and visualised by ethidium bromide staining. Bands were scored manually using the Kodak Digital Science 1D Software program.

10 Statistical analysis

Amplified fragments were scored for the presence (1) or absence (0) of homologous bands to create a binary matrix of the different RAPD phenotypes. Individual genetic distances were calculated using the Dice distance coefficient ($D_D = 1 - \text{Dice similarity index}$) (Dice, 1945; Nei and Li, 1979; Lynch, 1990). Dice similarity index was calculated as: $S_{xy} = 2n_{xy} / (n_x + n_y)$, where n_{xy} is the number of shared bands between individuals x and y, and n_x and n_y the number of bands of individuals x and y, respectively. It is an estimate of the expected portion of amplified fragments shared by two genotypes due to inheritance from a common ancestor (Nei and Li, 1979). Cluster analysis based on dissimilarity matrix was performed using the Neighbor Joining method (Saitou and Nei, 1987) as implemented in TREECON software (Van de Peer and De Wachter, 1994). The tree was rooted using *U. striatus* as the out-group. Bootstrap analysis was performed on 1000 bootstrap samples to test the reliability of branches (Felsenstein, 1985).

The average number of virulence differences between pairs of *U. viciae-fabae* isolates was determined as: $D_{SM} = d / n$, in which d = number of virulence differences

between paired isolates and n = total number of virulences. The virulence matrix and molecular dissimilarity matrix based on Dice distance coefficient were compared by cophenetic correlation coefficient followed by Mantel's test (Mantel, 1967). The randomisation procedure as implemented in NTSYS-pc version 2.02 (Rohlf, 1997) software package included 10000 permutations.

RESULTS

The 14 RAPD primers generated a total of 152 reliable fragments after excluding bands that were monomorphic for the whole data set. The size of the fragments ranged between 280 and 2500 bp and the number of bands per primer varied from 5 (OPL01) to 22 (OPL05) with an average of 10.8 bands per primer (Table 2). The estimated Dice distance coefficients varied from 0.0678 (in the case of two isolates of *U. viciae-fabae* ex *Vicia faba*) to 0.7619 (between *U. striatus* and the two isolates of *U. lupinicolus*) with a mean value of 0.3776.

The dendrogram (Fig. 1) shows clear differences among *U. viciae-fabae* ex *V. faba* and the remaining species considered with a high bootstrap value of 73. The *U. viciae-fabae* ex *Lens culinaris* isolate was the nearest to the cluster of *U. viciae faba* ex *V. faba* isolates, followed by *U. pisi* from Canada and *U. viciae-fabae* ex *V. sativa*. The separation of *U. viciae faba* ex *V. sativa* from the other three mentioned species was highly consistent with a bootstrap value of 93. Among the three *U. pisi* isolates considered, a great variability was observed and no grouping could be established. The two *U. ciceris-arietini* isolates clustered together and the *U. lupinicolus* isolates were joined in a branch that showed a bootstrap value of 86. The most different isolate from the rest of species considered was *U. striatus* showing the maximum bootstrap value (100).

All the isolates of *U. viciae-fabae* ex *V. faba* clustered together (bootstrap value of 73). The minimum and maximum Dice distance value between these isolates was 0.0678 and 0.2586 respectively with an average value of 0.157. The Dice distance between the isolates attacking faba bean and lentil was 0.3593, faba bean and vetches 0.392 and vetches and lens 0.4231. No grouping has been detected among *U. viciae-fabae* ex *V. faba*, ex *L. culinaris* and ex *V. sativa* isolates.

Out of 19 *U. viciae-fabae* ex *V. faba* isolates considered in the dendrogram, 16 of them that were previously evaluated with a set of differential lines (Emeran et al., 2001, Emeran, 2003) were also examined for differences in virulence using a simple distance coefficient. Comparing pairs of isolates, there was no differences attending to virulence between isolates 1 and 14 (Córdoba, Spain and Sicily, Italy), 9 and 15 (Logroño, Spain and Australia) and 2, 10 and 18 (Córdoba and Granada, Spain and Morocco) with a value of 0 in number of virulence differences (Table 3). The isolate from Egypt was the most different among the rest of isolates with three maximum distance values of 1 with three other isolates from Córdoba, Granada and Morocco. Considering the virulence values among isolates and the molecular dissimilarity matrix from molecular data, the Mantel test showed that the correlation between these two matrices was significant ($p = 0.002$) but low ($r = 0.31$). These results indicate poor association among considering virulence and molecular diversity.

DISCUSSION

The clustering of *U. viciae-fabae*, *U. ciceris-arietini*, *U. lupinicolus*, *U. vignae* and *U. striatus* isolates in the dendrogram confirms the utility of the method use to discern species and to study their relative evolutionary distance. Not such clear clustering was possible among *U. pisi* isolates studied. The fact that *U. pisi* is the most variable of the studied species, together with the fact that is the less specialised, with the broadest host range (Emeran, 2003) suggest that other species might have evolved from *U. pisi*. *U. ciceris-arietini* would be closer to *U. lupinicolus*, being *U. striatus* the most distant species. The great genetic variability of *U. pisi* as well as the long geographic distance among the *U. pisi* isolates considered in this study (Canada, Spain and Egypt), could explain that these isolates did not cluster together. The sexual reproduction that is frequent in *Euphorbia* spp. alternate hosts, especially *E. cyparissias* and *E. esula* (Buchheim, 1924; Jørstad, 1948) can also contribute to the wide genetic distance among them.

The study also allowed to cluster *U. viciae-fabae* isolates according to the host from which they were collected, supporting the host specialisation earlier reported (Emeran et al., 2005). All the isolates of *U. viciae-fabae* collected on faba bean (ex *V. faba*) clustered together. The isolate collected on lentils (ex *Lens culinaris*) was the nearest one, followed by the collected on common vetch (ex *V. sativa*). These isolates were found before to differ in host range and in primary infection structures and spore morphology (Emeran et al., 2005). However, more isolates collected on lentil and on common vetch should be studied to definitively support the separation of *U. viciae-fabae* into *formae specialis*.

Although clear differences in virulence exists within *U. viciae-fabae* ex. *V. faba* isolates (Emeran, 2001), the cluster analysis of RAPD markers did not yield distinct

virulence groups according to physiological specialization. In this sense, isolates belonging to the same physiological race (1, 3 and 12) can be found in different groups of the dendrogram. The lack of relationship between pathotype and molecular diversity suggest that *U. viciae-fabae* is largely clonal, evolving mainly through the mutation and selection of the fittest clones. Low correlation between virulence and molecular polymorphism has also been reported in populations of the wheat yellow rust *Puccinia striiformis* (Chen et al., 1993; Enjalbert et al., 2005; Becerra et al., 2007). However, association between molecular and race variation has been reported in *P. graminis* f.sp. *tritici* (Burdon and Roelfs, 1985a), *P. recondita* f.sp. *tritici* (Kolmer and Ordoñez, 2007) and *P. hordei* (Sun et al., 2007). The correlation in the case of *P. graminis* f.sp. *tritici*, *P. recondita* f.sp. *tritici* and *P. hordei*, but not in *P. striiformis* can be explained by the fact that although the four rust species show asexual reproduction, *P. striiformis* presents a high frequency of parasexual recombination that may allow the selection of virulence variation independently of molecular polymorphism. In this sense, Burdon and Roelfs (1985b) found that in the sexual population of *P. graminis* f.sp. *tritici* there was no association between isozyme alleles and virulence in contrast of what occurred in the case of asexual populations of the pathogen. Although *U. viciae-fabae* is known to be autoecious, completing its sexual reproduction on the same host, there is little information on occurrence of sexual reproduction as to draw conclusions of possible effects of the matting system of the species on the lack of association between the molecular polymorphisms and the virulence. Moreover, we did not find relationship between geographic and phenotypic distance. Reported geographic differentiation of isolates have been attributed not only to geographical barriers restricting movement of urediniospores between the regions (Kolmer and Ordoñez, 2007) but also to differing host selection pressures in the regions (Kolmer and Liu, 2000; Park et al., 2000; Mebrate et al., 2006;

Enjalbert et al., 2005). Additional research on virulence and molecular differentiation within different agro-ecological areas of faba bean production will be needed to ascertain the relative contributions of over summering, migration, mutation, sexual reproduction and host selection have on genetic identity and diversity of *U. viciae-fabae* populations. A more powerful technique for detection of molecular polymorphism, such as amplified fragment length polymorphism (AFLP) (Membrate et al., 2006; Becerra et al., 2007; Sun et al., 2007) or simple sequence repeats (SSR) (Kolmer and Ordoñez, 2007) might allow greater discrimination of isolates within and between different groups of *U. viciae-fabae* and different geographical regions.

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Table 1. Collection of *Uromyces* spp. monopustular isolates used in the study.

Isolate number	Species	Geographic origin	Host	Physiologic race ¹
1	<i>U. viciae-fabae</i>	Córdoba, Spain	Faba bean	Race 10
2	<i>U. viciae-fabae</i>	Córdoba, Spain	Faba bean	Race 1
3	<i>U. viciae-fabae</i>	Mengibar, Jaén, Spain	Faba bean	n.d.
4	<i>U. viciae-fabae</i>	Ubrique, Cádiz, Spain	Faba bean	n.d.
5	<i>U. viciae-fabae</i>	Medina Sidonia, Cádiz, Spain	Faba bean	Race 6
6	<i>U. viciae-fabae</i>	Trigueros, Huelva, Spain	Faba bean	Race 2
7	<i>U. viciae-fabae</i>	San Bartolomé, Huelva, Spain	Faba bean	Race 11
8	<i>U. viciae-fabae</i>	Jerez, Cádiz, Spain	Faba bean	Race 13
9	<i>U. viciae-fabae</i>	Logroño, Spain	Faba bean	Race 3
10	<i>U. viciae-fabae</i>	Granada, Spain	Faba bean	Race 1
11	<i>U. viciae-fabae</i>	Kafr El-Sheikh, Egypt	Faba bean	Race 16
12	<i>U. viciae-fabae</i>	Domiat, Egypt	Faba bean	Race 7
13	<i>U. viciae-fabae</i>	Flacognana, Italy	Faba bean	Race 15
14	<i>U. viciae-fabae</i>	Sicily, Italy	Faba bean	Race 10
15	<i>U. viciae-fabae</i>	Liverpool Plains, Australia	Faba bean	Race 3
16	<i>U. viciae-fabae</i>	Wageningen, The Netherlands	Faba bean	Race 8
17	<i>U. viciae-fabae</i>	Aleppo, Syria	Faba bean	Race 4
18	<i>U. viciae-fabae</i>	Al-Rebat, Morocco	Faba bean	Race 1
19	<i>U. viciae-fabae</i>	Elvas, Portugal	Faba bean	n.d.
20	<i>U. pisi</i>	Córdoba	Pea	n.d.
21	<i>U. pisi</i>	Kafr El-Sheikh, Egypt	Pea	n.d.
22	<i>U. pisi</i>	Winnipeg, Canada	Pea	n.d.
23	<i>U. viciae-fabae</i>	Morocco	Lentil	n.d.
24	<i>U. viciae-fabae</i>	Córdoba, Spain	Common vetch	n.d.
25	<i>U. lupinicolus</i>	Aberystwith, United kingdom	Lupine	n.d.
26	<i>U. lupinicolus</i>	Huelva, Spain	Lupine	n.d.
27	<i>U. ciceris-arietini</i>	Córdoba, Spain	Chickpea	n.d.
28	<i>U. ciceris-arietini</i>	Rome, Italy	Chickpea	n.d.
29	<i>U. vignae</i>	Kafr El-Sheikh, Egypt	Cowpea	n.d.
30	<i>U. striatus</i>	Jerez, Cádiz, Spain	Alfalfa	n.d.

5 ¹According to Emeran et al. (2001).

Table 2: Sequences of RAPD primers used in the study and number of polymorphic bands

Primer	Sequence	nº of polymorphic fragments
OPA01	CAGGCCCTTC	16
OPA02	TGCCGAGCTG	9
OPA03	AGTCAGCCAC	14
OPA05	AGGGGTCTTG	9
OPA08	GTGACGTAGG	15
OPH05	AGTCGTCCCC	9
OPI01	ACCTGGACAC	7
OPI07	CAGCGACAAG	8
OPI19	AATGCGGGAG	8
OPL01	GGCATGACCT	5
OPL05	ACGCAGGCAC	22
OPR02	CACAGCTGCC	11
OPT01	GGGCCACTCA	13
OPV09	TGTACCCGTC	6

Table 3. The average number of virulence differences and Dice's distance coefficient based on RAPD data between 16 *U. viciae-fabae* isolates

	1Vf	2Vf	5Vf	6Vf	7Vf	8Vf	9Vf	10Vf	11Vf	12Vf	13Vf	14Vf	15Vf	16Vf	17Vf	18Vf
1Vf		0.5000	0.8333	0.6667	0.6667	0.1667	0.6667	0.5000	0.5000	0.5000	0.3333	0.0000	0.6667	0.1667	0.3333	0.5000
2Vf	0.2414		0.3333	0.1667	0.5000	0.6667	0.1667	0.0000	1.0000	0.3333	0.8333	0.5000	0.1667	0.3333	0.1667	0.0000
5Vf	0.4717	0.2830		0.5000	0.1667	1.0000	0.1667	0.3333	0.6667	0.3333	0.5000	0.8333	0.1667	0.6667	0.5000	0.3333
6Vf	0.3867	0.3425	0.4118		0.6667	0.5000	0.3333	0.1667	0.8333	0.5000	1.0000	0.6667	0.3333	0.5000	0.3333	0.1667
7Vf	0.3636	0.3538	0.2542	0.2593		0.8333	0.3333	0.5000	0.5000	0.1667	0.3333	0.6667	0.3333	0.8333	0.3333	0.5000
8Vf	0.3333	0.3333	0.3929	0.2308	0.3623		0.8333	0.6667	0.3333	0.6667	0.5000	0.1667	0.8333	0.3333	0.5000	0.6667
9Vf	0.2941	0.2727	0.2131	0.3012	0.1892	0.3803		0.1667	0.8333	0.1667	0.6667	0.6667	0.0000	0.5000	0.3333	0.1667
10Vf	0.4839	0.3226	0.3818	0.3506	0.3529	0.3538	0.2857		1.0000	0.3333	0.8333	0.5000	0.1667	0.3333	0.1667	0.0000
11Vf	0.2667	0.3220	0.4138	0.3784	0.3333	0.3651	0.3134	0.4516		0.6667	0.1667	0.5000	0.8333	0.6667	0.8333	1.0000
12Vf	0.4035	0.2982	0.2941	0.3611	0.2698	0.3000	0.2615	0.2542	0.4138		0.5000	0.5000	0.1667	0.6667	0.1667	0.3333
13Vf	0.2537	0.2923	0.3333	0.2683	0.2055	0.2571	0.2267	0.4203	0.2121	0.3125		0.3333	0.6667	0.5000	0.6667	0.8333
14Vf	0.2812	0.3548	0.3684	0.3671	0.2857	0.3731	0.2500	0.3846	0.3016	0.4098	0.2676		0.6667	0.1667	0.3333	0.5000
15Vf	0.2778	0.2286	0.2923	0.1954	0.2308	0.2533	0.1750	0.3243	0.2676	0.3043	0.1899	0.2895		0.5000	0.3333	0.1667
16Vf	0.3333	0.3704	0.3600	0.4783	0.3667	0.4737	0.3226	0.5000	0.4286	0.4118	0.3770	0.3448	0.3939		0.5000	0.3333
17Vf	0.3143	0.2941	0.3437	0.3176	0.2368	0.4247	0.2051	0.3611	0.3333	0.3433	0.2468	0.2162	0.2683	0.3750		0.1667
18Vf	0.2857	0.2647	0.3333	0.2706	0.2632	0.3151	0.1795	0.1944	0.2857	0.3134	0.2468	0.2162	0.1951	0.3750	0.2000	

Above diagonal: average number of virulence differences

Below diagonal: Dice's distance coefficient based on RAPD data

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Figure 1. Cluster analysis based on molecular dissimilarity matrix of *Uromyces* populations. Neighbour Joining method (Saitou and Nei, 1987) was used as implemented in TREECON software (Van de Peer and De Wachter, 1994). Bootstrap analysis was performed on 1000 bootstrap samples to test the reliability of branches (Felsenstein, 1985). Bootstrap values greater than 50% are indicated above the respective internodes.

