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Research Article

Intraspecific differentiation of Chilean isolates of the entomopathogenic fungi *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers

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Abstract

The genus *Metarhizium* consists of a diverse group of asexual entomopathogenic fungi, which have a wide geographical distribution. The Chilean National Agricultural Research Institute (Instituto de Investigaciones Agropecuarias - INIA, Quilamapu Chile) has collected about 350 isolates of *Metarhizium anisopliae* var. *anisopliae* from central and southern Chile. These isolates have been partially characterized using morphological traits such as conidia size and shape, colony color, growth pattern and the efficiency of the isolates in controlling specific pests. However, further characterization with molecular markers could detect differences in DNA which could help to better understand the genetic diversity and structure of Chilean populations of this fungus. We analyzed approximately 10% of the INIA collection (39 isolates selected at random) collected from different geographical origins using the polymerase chain reaction (PCR)-random amplified polymorphic DNA (RAPD) method, simple sequences repeat (SSR or microsatellites) analysis and the PCR-restriction fragment length polymorphism (RFLP) assay of internal transcribed spacer (ITS)-rDNA sequences. The RAPD data revealed high genetic diversity in this fungus and an average of 41% of similarity while SSR analysis detected 45.2% similarity and the ITS markers 70.2% similarity. For the three molecular markers, this diversity was not associated with the geographical origin of these isolates.

Key words: Metarhizium, genetic diversity, RAPD, SSR, ITS markers.

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Introduction

Entomopathogenic fungi are an attractive alternative for the biological control of insect pests, mainly because they are safer for plants, animals and the environment relative to conventional pesticides (Khetan, 2001). Furthermore, such fungi have also been evaluated as biological control agents for more than 200 economically important insects species (Tanada and Kaya, 1993; Maurer *et al.*, 1997; Zurek and Keddie, 2000). Since 1996, the Insect-Pathology Program at the Chilean National Agricultural Research Institute (Instituto de Investigaciones Agropecuarias - INIA, Quilamapu, Chile) has collected over 1000 isolates of entomopathogenic fungi from throughout the Chile, with about 350 isolates having been classified as *Metarhizium anisopliae* var. *anisopliae* (Guerrero *et al.*, 1999; France *et al.*, 2000) according the parameters described by Humber (1997) and Alves (1998). This germplasm is important, since Chile's ecological conditions show contrasting climates varying from hot and dry areas in the North to the very wet and cold areas near the Antarctic in the South. Preliminary research has indicated that many of these isolates show promising pathogenicity against important Coleoptera insect pests (France *et al.*, 2000).

Several kinds of molecular techniques have been used to study genetic diversity, for example, labeled probes to detect restriction fragment length polymorphism (RFLP) (Hegedeus and Khachatourians, 1993; Maurer *et al.*, 1997) and methods based on the polymerase chain reaction (PCR) such as the use of random amplified polymorphic DNA

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(RAPD) (Fegan *et al.*, 1993; Bidochka *et al.*, 1994; Piatti *et al.*, 1998; Freire, 2001; Jensen *et al.*, 2001; Gaitan *et al.*, 2002), simple sequences repeat (SSR or microsatellites) analysis (Kretzner *et al.*, 2000; Enkerli *et al.*, 2001, 2005) and internal transcribed spacer (ITS)-rDNA sequence RFLP analysis (Rakotonirainy *et al.*, 1994; Buscot *et al.*, 1996; Coates *et al.*, 2002a). Even though they have different and complementary discriminatory power RAPD, SSR and RFLP analysis have been very effective in detecting genetic diversity in several species (Couteaudier *et al.*, 1998; Glare and Inwood, 1998; Coates *et al.*, 2002b; Enkerli *et al.*, 2005). In Chile, however, there has been no previous molecular characterization of entomopathogenic fungi populations.

The PCR technique combined with RAPD analysis (Welsh and McClelland, 1990; Williams *et al.*, 1990) has been extensively used to determine the genetic diversity of various entomopathogenic fungi (Cobb and Clarkson, 1993; Bidochka *et al.*, 1994; Neuvéglise *et al.*, 1994; Neuvéglise and Brygoo, 1994; Bidochka *et al.*, 1995; Hodge *et al.*, 1995; Fungaro *et al.*, 1996; Maurer *et al.*, 1997) including *Metarhizium* (Fegan *et al.*, 1993; Leal *et al.*, 1994) and to relate genetic diversity to pathogenicity index (Bridge *et al.*, 1997) and to identify strains (Milner *et al.*, 2002).

More recently, SSR markers have been used as powerful molecular markers for the study of the population genetics of fungi (Coates *et al.*, 2002a; Enkerli *et al.*, 2001; 2005), SSR markers being particularly useful because they are highly polymorphic (multi-allelic) between strains due to the variation of the number of repeats, co-dominant and highly reproducible compared with other markers. The main disadvantage of SSR markers is that the development of primers is time-consuming and expensive, but this can be offset by the fact that transferability of primer sequences within genera is high and allows primers to be shared between laboratories.

Nuclear ribosomal RNA sequences, especially the two internal transcribed spacers (ITS1 and ITS2), have demonstrated differential rates of nucleotide changes that allow intra-specific comparison of fungi (Neuveglise *et al.*, 1994; Buscot *et al.*, 1996). The treatment of PCR-amplified DNA fragments with restriction endonucleases forms the basis of the RFLP technique which has been used to investigate the inter and intraspecific diversity of the ITS sequences of entomopathogenic fungi (Coates *et al.*, 2002 b). Shih *et al.* (1995) reported a moderate level of mutation in the ITS1 region of *Beauveria bassiana*, although ITS2 was invariant for in this specie. However, *Beauveria brongniartii* isolated from a single host showed higher ITS2 sequence variation (Neuveglise and Brygoo, 1994) and at least 5 RFLPs have been detected among *Beauveria* species (Neuveglise *et al.*, 1994; Glare and Inwood, 1998) for the ITS regions, considered to be a well-conserved sequence.

The objectives of this study were to determine the level of genetic diversity in a core sample of Chilean isolates of *Metarhizium anisopliae var anisopliae* using several molecular markers and to evaluate different molecular markers which are suitable for the genetic fingerprinting of this enthomopathogen.

Material and Methods

Fungal strains and DNA extraction.

We investigated 39 of the approximately 350 *Metarhizium anisopliae* var. *anisopliae* (France *et al.*, 2000) isolates collected from different geographic hosts and regions of Chile and maintained in the Chilean National Agricultural Research Institute (Instituto de Investigaciones Agropecuarias - INIA, Quilamapu, Chile) collection, details of the strains investigated being shown in Table 1.

Monosporic cultures of the fungi were obtained by plating multisporic isolates onto Petri plates containing potato dextrose agar (PDA: containing (gL^{-1}) Dextrose, 20; Potato extract, 20; agar, 20) and incubating the plates at 27 °C for 3 days and then cultivating single spore colonies in Sabouraud media: containing (gL^{-1}) Peptone, 10; Dextrose, 40; agar, 20; supplemented with 1% (w/v) of yeast extract (Difco, France) to produce mycelium for DNA extraction.

For each fungal isolate, about 50 mg of mycelium was homogenized in DNA extraction buffer (100 mM Trizma; 1.4 M NaCl; 20 mM EDTA; 1% polyvinylpyrrolidone; 2% cetyltrimethylammonium bromide (CTAB); 1% mercaptoethanol; 10 mg/mL proteinase K; pH 8.0) and incubated for 45 min at 65 °C. After incubation the mixture was extracted twice with 24:1 chloroform:isoamyl alcohol, centrifuged at 3500 revs min^{-1} for 15 min and the DNA precipitated with isopropanol and stored at -20 °C overnight, after which the DNA was washed with 70% and 95% alcohol, dried at room temperature and re-suspended in 150 µL of TE buffer pH 8.0. The DNA concentration was measured with a fluorometer and the DNA stock solutions kept at -80 °C until needed. All percentages w/v.

RAPD analysis

For each isolate, PCR-RAPD of the extracted DNA was performed in a PTC-220 thermocycler (Dyad MJ Research) for 40 cycles of 35 s at 94 °C, 35 s at 40 °C and 1 min at 72 °C, and a final additional 10 min cycle at 72 °C. Reactions were carried out in a total volume of 25 µL containing 0.2 µM of dNTPs, 0.2 µM of the primer, 1x PCR amplification buffer, 0.3 mM of $MgCl_2$, 1 U *Taq* DNA Polymerase (Invitrogen) and 25 g of DNA template and ultrapure water. Reactions were done twice to evaluate the consistency of the banding patterns for all isolates studied.

Products were separated on a 1.5% (w/v) agarose gel in 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at 100 V for 3.5 h. The gels were stained with ethidium

Table 1 - The source of the 39 Chilean *Metarhizium anisopliae* var. *anisopliae* isolates investigated and their random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and internal transcribed spacer (ITS) genotypes.

N.	Accession number	Source of isolates	Isolation location	Latitude (South)	Longitude (West)	Chilean \one (Region)	SSR genotype	ITS genotype
1	Qu- M142	Soil. prairie	Futaleufú	43°10' 22.66"	72°51'53.61"	South (X)	1	1
2	Qu- M145	Soil. piedmont	Chaitén. Lago negro	42°38' 49.30"	72°42' 39"	South (X)	4	1
3	Qu- M150	Soil	Lago Chapo	41°24'28.11"	72°31'52.73"	South (X)	7	9
4	Qu- M151a	Soil. prairie	Desembocadura Río Chamiza	41°29'47.91"	72°50'39.83"	South (X)	4	1
5	Qu- M151b	Soil. prairie	Desembocadura Río Chamiza	41°29'47.91"	72°50'39.83"	South (X)	4	4
6	Qu- M155	Soil. native forest	Puerto Piedra	43°22'41.06"	72°17'33.66"	South (X)	15	13
7	Qu- M159	Soil. prairie	Quemchi	42°08'10.30"	73°29'44.02"	South (X)	8	5
8	Qu- M171	Soil. prairie	Pumanzano. Chiloé	42°28'30.52"	73°46'00.60"	South (X)	17	13
9	Qu- M173a	Soil. piedmont	Villa Sta. Lucía	43°27'12.42"	72°21'05.44"	South (X)	4	1
10	Qu- M180	Soil. native forest	Lago Yelcho. Pto. Ramírez	43°16'04.81"	72°23'22.53"	South (X)	_	1
11	Qu- M202	Soil. organic crop	Osorno. Cerro Puschel	40°33'59.96"	73°09'15.46"	South (X)	2	1
12	Qu- M203	Soil. prairie	Osorno. Cerro Puschel	40°33'59.96"	73°09'15.46"	South (X)	4	1
13	Qu- M205	Soil. prairie	Precordillera de la costa. Osorno	40°32'52.34"	73°37'40.03"	South (X)	6	3
14	Qu- M213	Soil. river	Forrahue	-	-	South (X)	16	14
15	Qu- M221b	Soil. native forest	Bahía Mansa	40°36'28.64"	73°44'20.33"	South (X)	2	11
16	Qu- M247	Soil	Cañete	37°52'19.09"	73°25'47.25"	Central (VIII)	8	5
17	Qu- M253	*Phytoloema hemanii larvae*	Osorno. Entre Lagos	40°41'23.41"	72°36'07.90"	South (X)	8	5
18	Qu- M270	*Hylamorpha elegans larvae*	Osorno	40°33'59.96"	73°09'15.46"	South (X)	19	13
19	Qu- M271	*Curculionidae larvae*	Paso Alejo. Coihueco	36°33'59.67"	71°41' 51.95"	Central (VIII)	1	1
20	Qu- M283	Soil. prairie	Pacific Flower. Puyehue	40°39'03.37"	72°19'05.86"	South (X)	1	1
21	Qu- M326	Soil	San Vicente. Melipilla	33°41'33.86"	71°12'40.37"	Central (RM)	9	5
22	Qu- M354	Soil	Quidico. Cañete	37°50'58.65"	73°27'11.51"	Central (VIII)	11	5
23	Qu- M363	*Sericoides viridis*	Pinto	36°42'04.03"	71°53'16.45"	Central (VIII)	21	12
24	Qu- M371	Soil	Chanco	36°15'43.24"	72°42'44.94"	Central (VII)	12	5
25	Qu- M430	Soil	Osorno	40°33'59.96"	73°09'15.46"	South (X)	10	5
26	Qu- M436	Soil	Huape	36°37'48.66"	72°19'26.62"	Central (VIII)	10	5
27	Qu- M481	Soil	Monte Patria	30°41'31.56"	70°57'23.31"	Central (IV)	10	6
28	Qu- M489a	*Otiorhynchus salcatus larvae*	Río Tea. San Pablo. Osorno	40°24'47.53"	73°00'38.63"	South (X)	9	5
29	Qu- M490	*Forticola auricularia*	La Fama	38°41'15.39"	72°13'32.29"	South (IX)	3	2
30	Qu- M501	Organic berry fruit	Bulnes	36°39'29.77"	72°19'00.72"	Central (VIII)	10	5
31	Qu- M549	*Sericoedes larvae*	Agrícola Santa Fe. Los Angeles	37°27'48.50"	72°21'38.03"	Central (VIII)	18	13
32	Qu- M551	Pellinida	Puerto Octay	40°58'46.86"	72°53'23.06"	South (X)	5	1
33	Qu- M553	Soil	Puerto Varas	41°23'03.25"	72°51'54.10"	South (X)	10	5
34	Qu- M558	Soil	Purranque	40°54'34.80"	73°09'59.74"	South (X)	3	8
35	Qu- M559	Soil	Chipin. Río Negro	40°47'39.79"	73°13'41.21"	South (X)	20	15
36	Qu- M594	Organic berry fruit	Santa Rosa	36°34'34.60"	72°07'22.42"	Central (VIII)	14	7
37	Qu- M608	Soil	Paso Argentina	38°36'42.11"	70°59'34.36"	South (IX)	8	7
38	Qu- M633	Soil	La loma	-	-	South (IX)	22	10
39	Qu- M982	*D. maculipennis*	Cachapoal	36°28'27.07"	73°29'44.02"	Central (VI)	13	5

bromide and visualized with UV light using a Cole Palmer FLUO-LINK FLX apparatus and photographed for later evaluation.

A pre-screening of eight *M. anisopliae* isolates with 64 primers from the OPA-A, OPA-B, OPA-M, OPA-C, OPA-AD and OPA-AB series (Operon Technologies, California, USA) was performed to select the most informative primers. For the final evaluation of the 39 isolates we selected 20 primers (OPA-03, 04, 08, 09, 10, 11, 12, to 13; OPC-01; OPD-02, 03, 07, 08, 13, 17 and18; and OPAB-03, 04, 09 and 11).

SSR analysis

The same 39 isolates were analyzed with 12 SSR primers (*Ma097*, *099*, *142*, *145*, *164*, *165*, *195*, *210*, *307*, *325*, *327* and *375*) (Enkerli *et al.*, 2005). The PCR reactions were performed in a PTC-220 thermocycler using 10 g of genomic DNA from each isolate, 1x PCR buffer, 0.2 μM of dNTPs, 2.5 mM $MgCl_2$, 200 μM of forward and reverse primers, and 0.5 U *Taq* DNA polymerase (Invitrogen). Amplification started with an initial step of 4 min at 95 °C, followed by 38 cycles of 1 min at 95 °C, 1 min at 50 °C (44 ° or 56 °C depending on the primer set) and 1 min at 72 °C, with a final 4 min extension at 72 °C. Products were separated on 6% denaturing polyacrylamide gel in 1 x TBE buffer at 1800 V for about 2 h. The DNA fragments were stained with silver nitrate and the fragment sizes determined based on internal and external size standards. The *Ma307* and *Ma325* primers did not amplify.

ITS analysis

Oligonucleotide primers ITS1, ITS2, ITS3, ITS4 and ITS5 were used for ITS PCR-amplification (White *et al.*, 1990). The PCR reactions were carried out in a PTC-220 thermocycler using a total volume of 25 μL containing 0.2 μM of dNTPs, 0.2 μM of the ITS forward and reverse primers, 1xPCR amplification buffer, 0.25 mM of $MgCl_2$, 1 U *Taq* DNA Polymerase (Invitrogen) and 25 g of DNA template and an initial amplification step of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 40 s at 57 °C, and 20 min at 72 °C. Restriction of the PCR products was carried out according to the manufacturer's instructions using the following endonucleases: *Acc* I, *Alu* I, *Dde* I, *Eco* RI, *Eco* RV, *Hae* III, *Hha* I, *Hinf* III, *Hpa* II, *Mbo* I, *Mse*I, *Msp* I, *Nde* II, *Rsa* I, *Sin* I, *Tru* 9I and *Xho* I. Restriction fragments were separated on 6% polyacrylamide gel (20x20) in 1x TBE buffer at 100 V for 2 h. Ethidium bromide gel staining was performed and the DNA fragments visualized under UV light, the molecular weights of the fragments being determined by comparison with a 100 bp DNA ladder using the 1D Image Analysis program (Kodak digital system).

Data analysis

For each marker, polymorphic bands were considered as binary characters and scored as present (1) or absent (0). Genetic similarity between pairs was estimated using Jaccard's coefficient with the SIMQUAL option. The similarity matrix was run on sequential, agglomerative, hierarchical and nested clustering (SAHN) (Sneath and Sokal, 1973) using the unweighted pair-group method with arithmetic average (UPGMA) clustering algorithm to generate a dendrogram. The COPH option was used to generate a matrix of cophenetic values which was used in the MXCMOP option to calculate the correlation between the cophenetic matrix and the original SIMQUAL matrix. This analysis measured goodness-of-fit under 1000 permutations and provided a cophenetic correlation value (r). A cophenetic correlation value of r 0.9 is considered a very good fit according to Mantel (1967). All computations were undertaken using the NTSYSpc 2.1 program (Rohlf, 2000).

For SSR analysis, based on the allelic profile, the PowerMarker program (Liu and Muse, 2005) was used to calculate the number of alleles per locus, gene diversity (He) and the polymorphic information content (PIC). For ITS analysis, genotypes were determined by using gel fragment patterns of different primer-restriction enzyme combinations. Finally, the specific genotypes for strain identification were analyzed.

Analysis of molecular variance (AMOVA) and fixation index(Fst, θ)(Weir and Cockerham 1984; Weir 1996; Excoffier *et al.*, 2005) was calculated to clarify molecular patterns of within-population variation for the 39 isolates, collected in two main geographical areas (central and southern Chile, Table 1). All calculations were performed using Arlequin 3.01 software (Excoffier *et al.*, 2005). The total variance was partitioned into genetic variance between/among geographical areas and among isolates within geographical areas.

Results and Discussion

RAPD analysis

The 20 RAPD primers used to analyzed the 39 isolates of *M. anisopliae* generated a total of 189 bands ranging from 200 bp to 1500bp, but only bands that amplified consistently over two DNA extractions and PCR experiments were used for the analysis. We found that 96.8% of the bands were polymorphic and 5 to 13 bands were obtained for each RAPD marker depending on the primer used, with an average of 9.5 bands per primer. These results agree with other RAPD studies on *M. anisopliae* (Leal-Bertioli *et al.*, 2000). In our study seven primers (OPA-04, 03, 09 and 13; OPAB-09; OPC-01; and OPD-02) were the most informative, since they detected the greatest number of polymorphic bands and produced a high number of banding patterns. These primers detected 43.4% of the total polymorphic bands. The OPA-03 primer was important because it was able to discriminate the commercially available Qu-M633 isolate from the other isolates (Figure 1).

The similarity data dendrogram clustered the 39 isolates into three main clades with a general average similarity coefficient of 41.3%. Clades I and III clustered the isolates mostly collected in the southern X region, while clade II clustered isolates collected in a wider area. The dendrogram was statistically supported by a high cophenetic correlation value (r = 0.96).

Our results showed that the PCR-RAPD analysis was useful in detecting genetic variability within and between groups of isolates collected from different geographical areas. No identical genotypes were detected by these analysis (Figure 1).

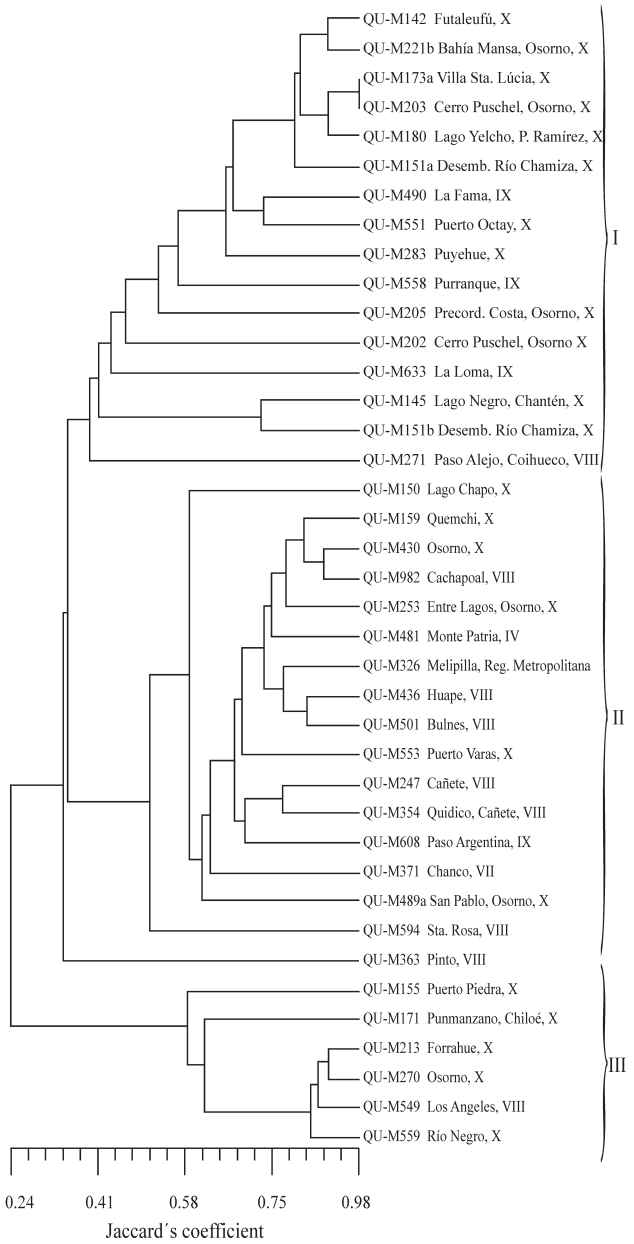

Figure 1 - Dendrogram based on random amplified polymorphic DNA (RAPD) analysis of 39 Chilean isolates of *Metarhizium anisopliae* var. *anisopliae.*

The AMOVA results (Table 2) indicated that the within population variance accounted for 89.8% of the total genetic variation while the between population variance accounted for the remaining 10.2% of the variation. However, RAPD analysis did not detect any association between the genetic diversity of the isolates and the location from which the isolates were collected. The fixation index of 0.1025 suggested that little genetic differentiation had occurred between central and southern populations based on geographical distribution (Table 1 and 2).

SSR analysis

Analysis of the same 39 Chilean *M. anisopliae* isolates using microsatellites markers also indicated a high level of diversity in this entomopathogenic fungi. Only a couple of primers amplified the QuM-180 isolate so this isolate was not included in the analysis of the SSR data, which means that the number of isolated analyzed was 38. Of the 12 SSR primers used only ten amplified with our isolates and since 52 alleles were detected the mean number of alleles per locus was 5.2, with a range of from 2 to 9 (Table 3). These values are higher than those obtained by Enkerli *et al.* (2005) in populations of *M. anisopliae* collected in Switzerland. In our study, the *Ma375* locus was the most frequent allele found in every isolate (Table 3).

The gene diversity (He) value estimates the probability that two alleles at any randomly chosen locus are different from each other, in our case the 39 isolates showed a mean He value of 0.27. The polymorphic information content (PIC) is a measure of the probability that two randomly sampled genotypes have different allelic profiles, the mean PIC value for our isolates being 0.52 (Table 3). This information indicates that our Chilean *M. anisopliae* isolates had a high degree of biodiversity. We detected 10 distinct *M. anisopliae* genotypes in populations from the Chilean central zone, of which only isolates Qu-M501, QuM-481 and QuM-436 were identical (Table 1). In populations from the Chilean southern zone we detected 16 distinct genotypes, six of which we detected in several isolates. However, in both geographical areas the number of identical genotypes were low but more diversity might have been detected if more isolates had been investigated, remembering that 38 isolates represented only about 10% of the *M. anisopliae* isolates in the INIA collection.

Jacard's genetic similarity coefficient gave an average similarity value of 45.2%, and separated the Chilean isolates into three major clades (Figure 2). Clades I and III clustered the isolates mostly collected in region X of the southern zone, while Clade II included isolates collected from different regions. It is important to note that both the RAPD and SSR data generated dendrograms with three well-defined clades that clustered almost the same isolates in each clade, although they had different pair-wise similarity coefficients. The dendrogram generated with the SSR

Table 2 - Analysis of molecular variance (AMOVA) results and fixation index (F_{ST} θ) of the random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), internal transcribed spacer (ITS) and combined (RAPD-SSR-ITS) data for Chilean isolates (n = 39. degrees of freedom (df) = n-1) of *M. anisopliae* var. *anisopliae*.

Source of variation	df	Sum of squares	Variance components	Percentage variation
RAPD				
Among populations	1	66.758	2.6313 Va	10.25
Within populations	37	852.370	23.0370 Vb	89.75
Total	38	919.128	25.6684	
Fixation index	F_{ST}	0.1025		
SSR				
Among populations	1	27.560	1.3105 Va	18.47
Within populations	37	214.056	5.7853 Vb	81.53
Total	38	241.615	7.0958	
Fixation index	F_{ST}	0.1847		
ITS				
Among populations	1	16.643	0.6590 Va	10.38
Within populations	37	210.639	5.6929 Vb	89.62
Total	38	227.282	6.3519	
Fixation index	F_{ST}	0.1038		
Combined data				
Among populations	1	110.961	4.6009 Va	11.76
Within populations	37	1277.065	34.5153 Vb	88.29
Total	38	1388.026	39.1162	
Fixation index	F_{ST}	0.1176		

Table 3 - Allele size range. allele number. genetic diversity (He) and polymorphic information content (PIC) of some simple sequence repeat (SSR) microsatellite markers detected in Chilean isolates of *Metarhizium anisopliae* var. *anisopliae*.

	Metarhizium anisopliae var. *anisopliae*			
SSR marker	Size range	Allele number	He	PIC
Ma097	160-180	5	0.34	0.54
Ma099	150-160	4	0.18	0.24
Ma142	100-130	8	0.45	0.73
Ma145	100-120	9	1.00	0.70
Ma164	100-120	3	0.00	0.41
Ma165	120-150	3	0.41	0.54
Ma195	100-120	7	0.05	0.68
Ma210	90-150	6	0.13	0.53
Ma327	200-230	5	0.05	0.71
Ma375	130-160	2	0.05	0.13
Mean		5.2	0.27	0.52

data was supported statistically by a high cophenetic correlation value (r = 0.97).

The AMOVA results for the SSR data (Table 2) indicated that the within population variance accounted for 81.5% of the total genetic variation while the between population variance accounted for the remaining 18.5% of the variation. This was also reflected by the wide distribution of the isolates in the dendrogram. This low differentiation could be due to the presence of a large number of common alleles, either because the alleles co-evolved independently by mutation or they were identical by descent and distributed through gene flow as has been reported for *B. brogniartti* (Enkerli *et al.*, 2001). However, this mechanism is still unclear in *M. anisopliae* (Enkerli *et al.* , 2005).

The RAPD and SSR analysis indicated that isolates Qu-M173a and Qu-M203 (both collected in the region X of the southern zone) were almost identical genetically. However, compared to RAPD analysis, SSR analysis showed a higher number of isolates to be genetically identical, probably due to the low average number of different alleles in the population studied. The SSR analysis did not detect any association between the genetic diversity of the isolates and the location from which the isolates were collected. The fixation index (Fst = 0.1847) showed that little genetic differentiation had occurred between the Central and Southern populations (Table 2).

Approximately 80% of the isolates were collected from soil samples and about 20% from larval and adult insects. Clade I contained isolate Qu-M490 collected from an adult *Forticola auricularia* (L.) and isolate Qu-M271 from a curculionid larva. Clade II clustered three insect-derived

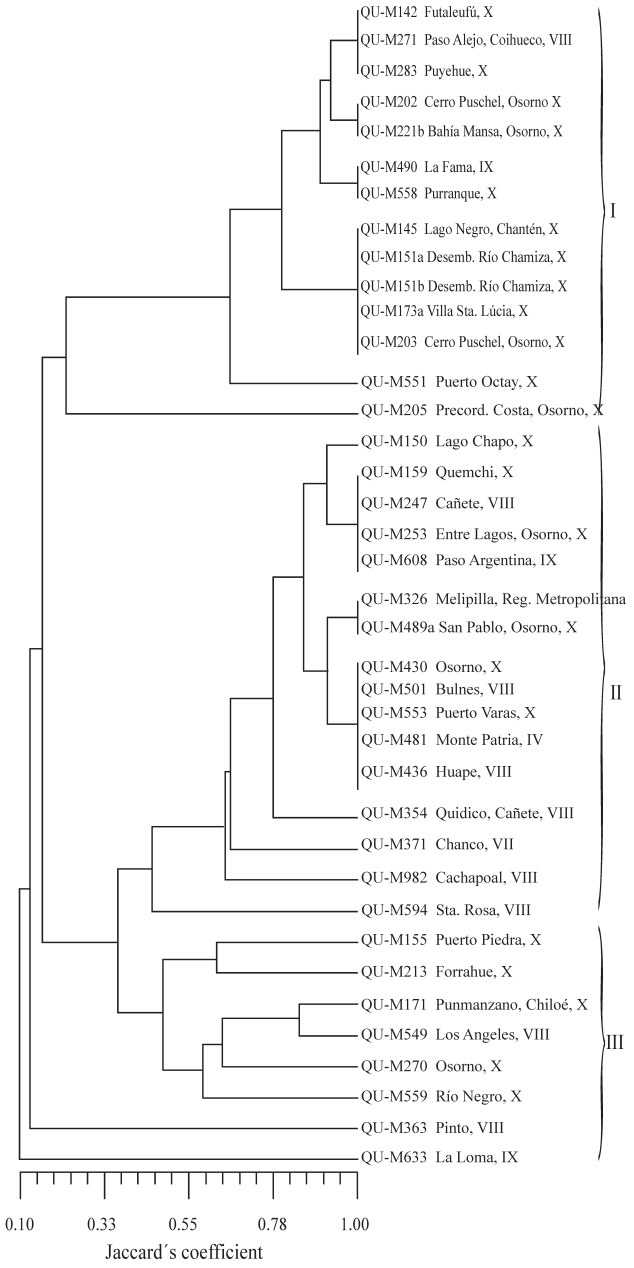

Figure 2 - Dendrogram based on simple sequences repeat (SSR) analysis of 39 Chilean isolates of *Metarhizium anisopliae* var. *anisopliae.*

isolates, Qu-M982 from an adult *Dichroplus maculipennis* (Bl.), Qu-M253 from a *Phytoloema* hermanni (Herm) larva and Qu-M489a from a *Otiorhynchus sulcatus (*Boh.) larva. Clade III contained three insect-derived isolates, Qu-M270 from a *Hylamorpha elegans* (Burm.) larva, Qu-M363 from an adult *Sericoides viridis* and Qu-M549 from a *Sericoides* sp. This agrees with a previous PCR-RFLP study of *B. bassiana* that concluded that there is a weak association between isolate genotype and insect preference (Coates *et al.*, 2002a), while studies on *Metarhizum* have shown a weak associationbetween insect host range and isolate genotype (Cobb and Clarkson, 1993; Bidochka *et al.*, 1994).

ITS analysis

Both ITS regions resulted in a single PCR-amplification product for all isolates, while digestion of the ITS1 and ITS2 PCR-amplification products with several restriction enzymes detected several molecular differences between our *M. anisopliae* isolates (Figure 3). The restriction enzymes *Acc* I, *Dde* I, *Eco* RI, *Eco* RV, *Nde* II, *Sin* I, and *Xho* I used in this study were not able to detect restriction sites.

Digestion of ITS1 yielded the following fragments (in bp): *Hha* I, 170 to 90; *Hinf* III, 260 to 50; *Msp* I, 180 to 100; *Alu* I, 300 to 230; *Hae* III, 300 to 180; *Rsa* I, 290 to 220; and *Tru* 9I = 220 to 30 (Figure 3a). Digestion of ITS2 yielded the following fragments (in bp): *Hinf* III, 270 to 40; *Msp* I, 290 to 100; *Tru* 9I, 390 to 150; *Alu* I, 410 to 400; *Hae* III, 230 to 150; and *Hha* I, 180 to 70 (Figure 3b).

A total of 12 genotypes were identified by digesting the ITS1 PCR products with *Hha* I, *Hinf* III, *Msp* I, *Tru* 9I, *Alu* I, *Hae* III and *Rsa* I while nine genotypes were identified by digesting the ITS2 PCR products with *Hha* I, *Hinf* III, *Msp* I, *Tru* 9I, *Alu* I and *Hae* III. The higher number of restriction sites within ITS1 has been reported in other species (Kuninaga *et al.*, 1997; Zare *et al.*, 1999). Considering both regions together a total of 15 genotypes were identified in the 39 isolates, which were thus reduced to 15 different groups (Figure 4, Table 1). This indicates that genetic discrimination between isolates using ITS was more difficult than with RAPD or SSR. This is corroborated by a lower pair-wise similarity coefficient, which averaged 70.9% compared to those obtained by RAPD and SSR, but even so the dendrogram in Figure 4 shows four clades, with a high percentage of identical isolates within clades I, II and IV. For example, clade I contained ten isolates (25% of the total isolates) with the same genotype, mostly collected from region X of the southern zone, while clade II contained 12 isolates with identical genotypes (Figure 4). For the ITS data, the cophenetic correlation coefficient between the cophenetic matrix and the ITS1-ITS2 data matrix was 0.98, higher than for the RAPD and SSR data.

When the data was analyzed by geographical origin the central zone isolates showed six distinct genotypes while the southern zone isolates contained 13 distinct genotypes, only three of which represented multiple isolates (Table 1).With the ITS markers, as with the RAPD and SSR markers, we were, in general, unable to find any association between isolate diversity and collected location, the exceptions being the ITS Clade I isolates QU-M202 and QU-M203 collected in the X region of the southern zone (Table 1). The differences in the ITS regions detected by PCR-RFLP resulted in a fixation index (Fst = 0.1038) which indicated a low level of genetic differentiation in our *M. anisopliae* sample. Moreover, AMOVA showed that 89.6% of the within isolate molecular variance was and 10.4% of the total variation occurred between central and southern populations, suggesting a weak geographic origin influence on the genotype of the isolates studied.

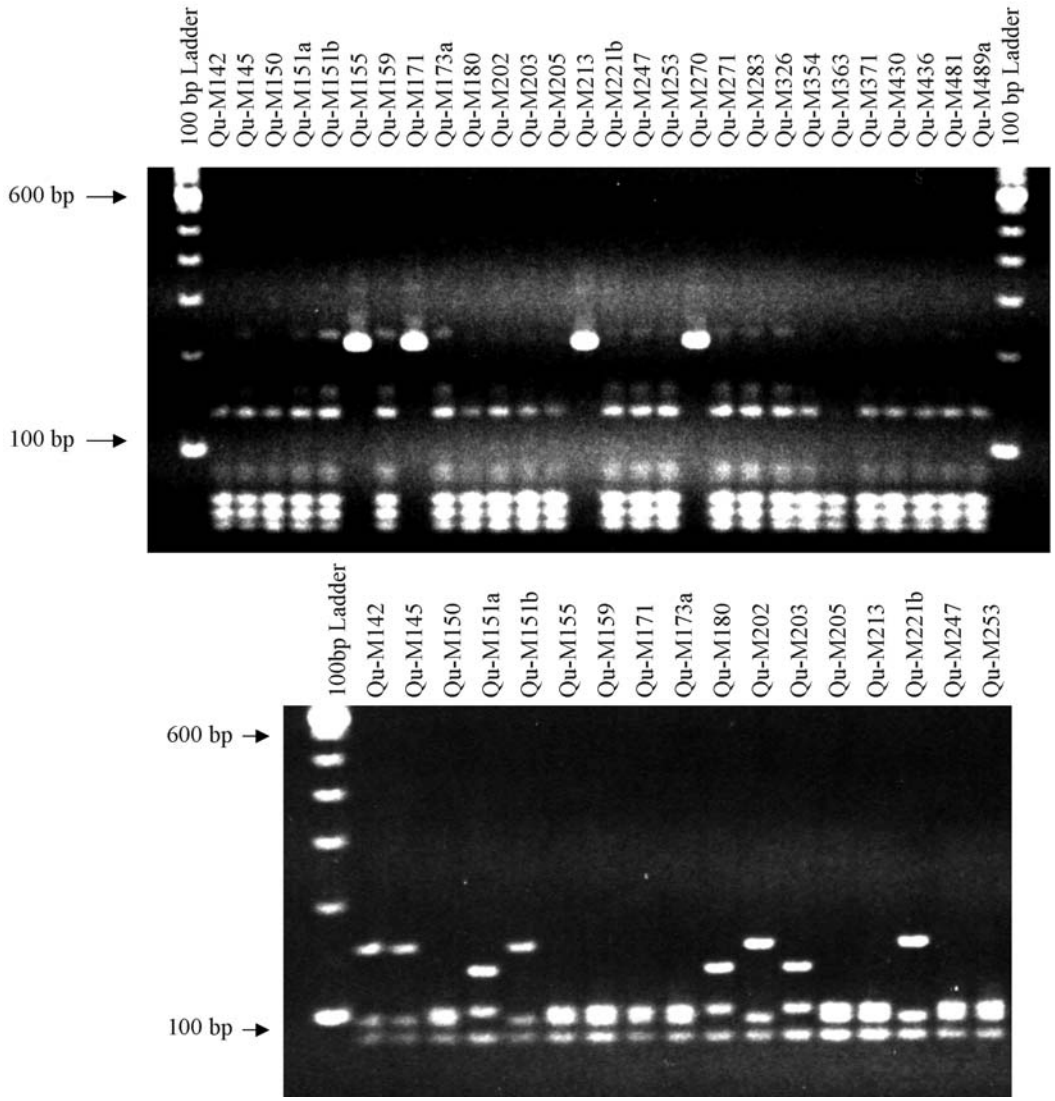

Figure 3 - Fragment length polymorphism (RFLP) of the rDNA internal transcribed spacer (ITS) 1 (a) and ITS 2 (b) regions, ITS 1 was digested with the *Tru* 9I restriction enzyme and ITS 2 with the *Hha* I restriction enzyme.

The dendrogram generated with the combined RAPD, SSR and ITS similarity data clustered the 39 isolates into three main clades with a general average similarity coefficient of 25.1% (Figure 5). Clades I, II and III clustered the isolates from all regions. The dendrogram was supported statistically by a high cophenetic correlation value (r = 0.91). Results from AMOVA (Table 2) indicated that the within population variation accounted for the 88.2% of the total genetic variation and between population accounted for the remaining 11.8%. As was the case when the RAPD, SSR and ITS data was analyzed separately, the fixation index (Fst = 0.1176) suggested that there was little genetic differentiation between geographic areas (Table 2).

Genotype identification

When implementing biological control using entomopathogenic fungi it is very important to have an efficient system of identifying the fungal isolates used (Coates *et al.*, 2002b) in order to measure their efficiency and detect multiple-isolate infections in the host.

In this study we found that PCR-RAPD detected a higher level of genetic diversity and was able to distinguish different entomopathogenic fungi isolates using at least four primers, but in some cases it was possible to fingerprint an isolate with only one primer. For instance, OPA-03 discriminated QU-M633 from other isolates very clearly. These results agree with previously published work in which PCR-RAPD markers were able to find a large amount of genetic variability between isolates of *Entomophaga grylli* pathotypes (Bidochka *et al.*, 1995).

Allelic profiling with ten SSR markers detected 22 genotypes among the 38 isolates, isolate QuM-180 was not included in the analysis because only a few primers amplified with this isolate. The following isolates showed identi-

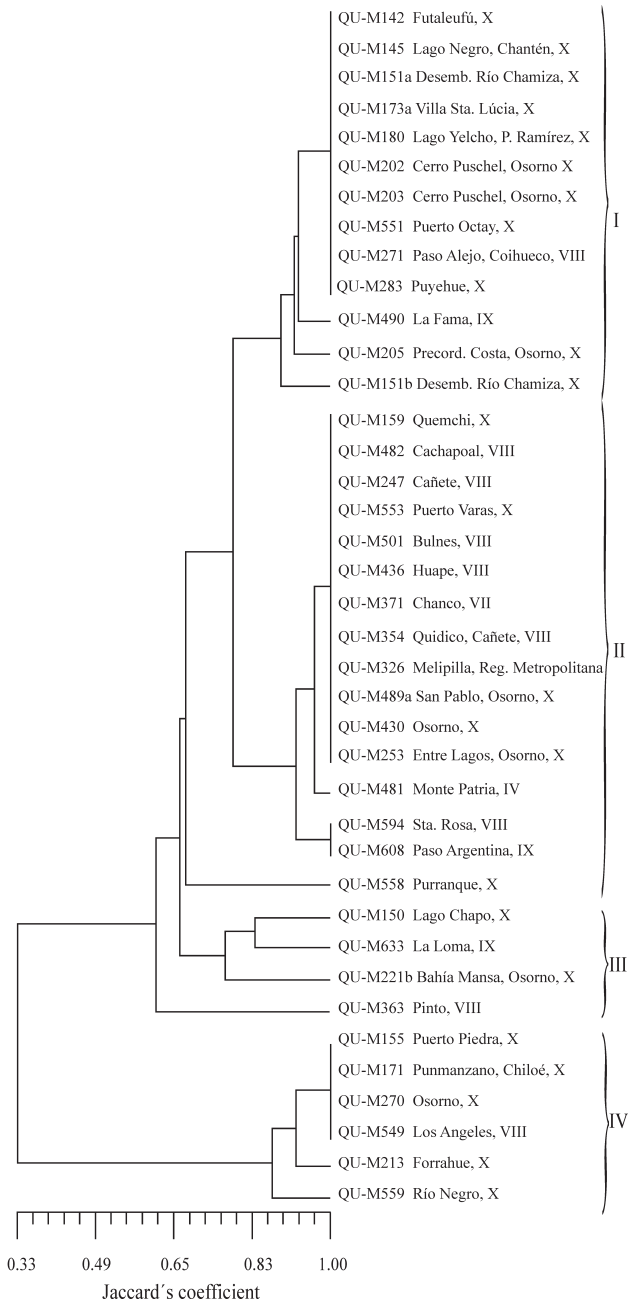

Figure 4 - Dendrogram based on Internal transcribed spacer (ITS) analysis of 39 Chilean isolates of *Metarhizium anisopliae* var. *anisopliae.*

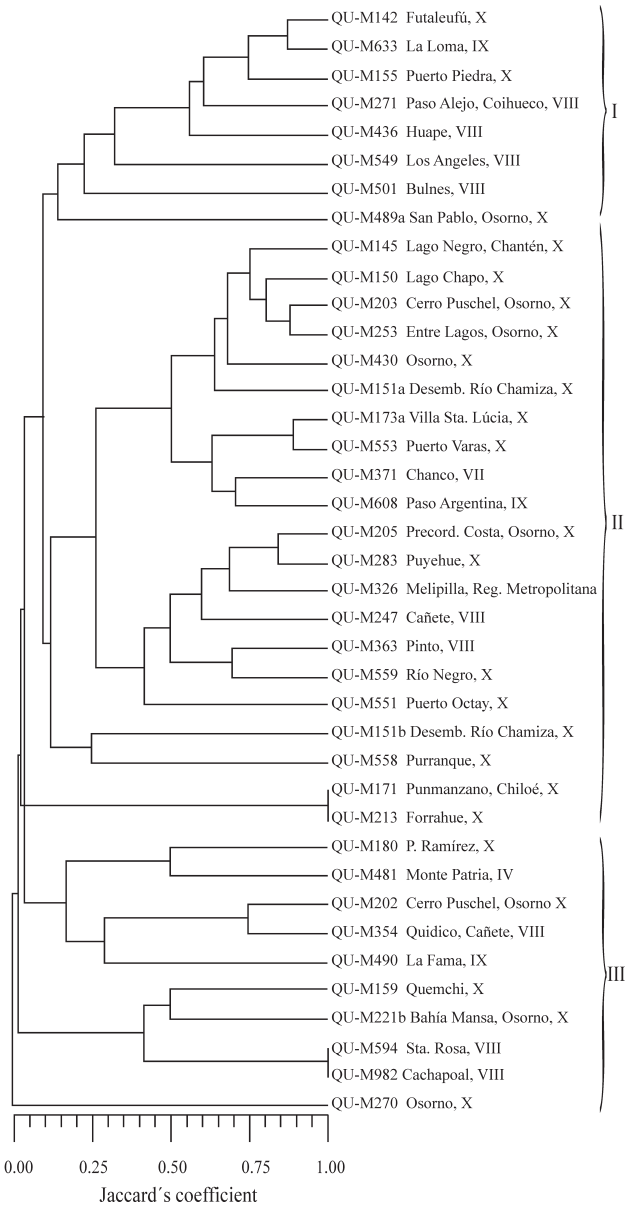

Figure 5 - Dendrogram based on the combined data from the RAPD, SSR and ITS-RFLP analysis of 39 Chilean isolates of *Metarhizium anisopliae* var. *anisopliae*.

cal genotypes as regards the ten SSRs analyzed: Qu-M142, Qu-M271 and Qu-M283; Qu-M202 and Qu-M221b; Qu-M490 and Qu-M558; Qu-M145, Qu-M151a, Qu-M151b, Qu-M173a and Qu-M203; Qu-M159, Qu-M247, Qu-M253 and Qu-M608; Qu-M326 and Qu-M489a; and Qu-M430, Qu-M501, Qu-M553, Qu-M481 and Qu-M436. Since these loci displayed less polymorphism more SSR primers may be required to discriminate between all the genotypes. One of the reasons for this could be that the number of alleles per locus detected in the Chilean *M. anisopliae* isolates were not enough to have a good discrimination among isolates. In this case, a good PCR-RAPD protocol could allow us to discriminate among individuals.

The conservative nature of our ITS fingerprinting analysis meant that it was very difficult to discriminate between isolates because many of the isolates analyzed shared the same genotype. However, it is interesting to note that isolate QU-M633 was discriminated with RAPD, SSR and ITS markers.

Our results indicate that RAPD, SSR and ITS markers could be used in a complementary manner to identify specific isolates, although SSR markers have the advantage of being more reliable and easier to score than RAPD markers and therefore easier to use. However, because the cross-

species transferability of SSR markers is limited (Enkerli *et al.*, 2001) methods such as RAPD, PCR-RFLP or group I intron analysis of 28S rDNA may be advantageous over SSR markers for studies of distantly related isolates at the genus level.

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