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Analysis of AFLP markers associated to the Mex-1 resistance locus in Icatu progenies

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ABSTRACT *-The root-knot nematode Meloidogyne exigua is a parasite which attacks the Arabica* coffee *tree (*Coffea *sp.) and its eradication from infested areas is practically impossible. The wide dissemination of this nematode across coffee plantations in the south of the state of Minas Gerais has been causing great damage to the coffee producers of the area. Previous studies showed that the simple inheritance gene present in* C. canephora, *designated Mex-1, controls M. exigua resistance. Some genetic breeding programs have developed resistant Arabica coffee lines through the introgression of this gene. To confirm the introgression, twenty-one Icatu lines were analyzed and compared to two resistant ("Iapar 59" and "Híbrido de Timor") and one susceptible cultivar (Catuaí). Among the AFLP markers used, five confirmed the presence of the introgressed fragment associated to Mex-1 resistance, showing that this marker can be used in marker-assisted selection.*

INTRODUCTION

Nematodes associated to coffee form a large group of species, among which the genus *Meloidogyne* accounts for the greatest harm done to coffee in Brazil (Gonçalves et al. 1998). Of the over 15 *Meloidogyne* species that attack coffee (*Coffea arabica* L.) roots in the diverse coffee producing regions around the world (Campos et al. 1990), six occur in Brazil. One can not exclude the possibility of the appearance of at least three more *Meloidogyne* species, whose occurrence on Brazilian coffee plantations has not been described so far.

The species of the genus *Meloidogyne* cause characteristic symptoms such as root swelling with the typical gall formation. The symptoms in the aerial part of a plant attacked by *Meloidogyne* spp. are plant decadence, including chlorosis, early leaf drop and nutritional deficiency which can cause plant death in stress situations of long drought or cold (Campos and Lima 1986).

Among the species of this genus that attack coffee, *M. exigua* Goeldi (1892) undoubtedly has the widest dissemination in Brazil, and was first identified and described as nematode species (Gonçalves and Silvarolla 2001). Attacks of this nematode were observed in coffee plantations in the states of Minas Gerais, São Paulo, Paraná, Espírito Santo, Rio of Janeiro, Bahia, and Ceará (Campos and Lima 1986), as well as in Central and South America (Bertrand et al. 1997, Campos et al. 1990). Despite less aggressive than other nematode species, *M. exigua* is responsible for considerable production losses. Effects on the root system are easily evaluated, whereas the damage done direct and indirectly to the coffee yield is not estimated appropriately in Brazil. It is one of the dominant species in Costa Rica (Flores and López 1989), where the production drop caused by the nematode attack was estimated in a dimension of 10 to 20% (Bertrand et al. 1997).

Earlier studies showed that *M. exigua* resistance is

controlled by a dominant simple inheritance gene, designated *Mex*-1, present in the species *Coffea canephora*. Fourteen AFLP (Amplified fragment length polymorphism) markers associated with the resistance to *M. exigua* were found and a genetic map locating locus *Mex*-1 was constructed (Noir et al. 2003). Some Arabica coffee lines presenting resistance to this nematode have been developed in breeding programs by introgression of the gene (Bertrand et al. 1999, Bertrand et al. 2001, Bertrand et al. 2002).

The use of resistant cultivars is an ecologically correct and inexpensive option to control nematodes. Opposite to *C. arabica* where all genotypes, whether wild or cultivated, are susceptible (Bertrand et al. 2001), other species of the genus *Coffea* sp., such as *C. canephora* and *C. racemosa* (Curi et al. 1970a, Anthony et al. 2003) for example, are used as resistance source against the *Meloidogyne* nematodes (Anthony et al. 2002). *M. exigua* resistance was further detected in some introgressed lines derived from an interspecific natural hybrid (Híbrido de Timor) of *C. arabica* x *C. canephora* (Bertrand et al. 1997, Gonçalves and Pereira 1998, Silvarolla et al. 1998), with similar resistance levels to those observed in *C. canephora* (Bertrand et al. 2001).

In simple terms, resistance can be defined as the trait of the plant that inhibits nematode reproduction. In the case of nematodes of the genus *Meloidogyne*, it can be race and/or species-specific. The species *C. canephora*, *C. congensis* and *C. dewevrei* can be used as resistant root stock for improvement studies, because the nematode resistance is linked to a more developed root system in these species and/or because these species present resistance to nematodes as well as to other pathogens. Other authors have described *M. exigua* resistance in *C. canephora*, *C. congensis*, *C. dewevrei*, *C. liberica*, *C. racemosa*, and *C. salvatrix* (Curi et al. 1970a, Curi et al. 1970b, Fazuoli and Lordello 1978).

Hybrids resulting from the cross of *C. arabica* x *C. canephora* (Icatu, Sarchimor and Catimor, among others) have been intensely studied including their reaction to the causal agent of coffee leaf rust (*Hemileia vastatrix*), since many plants are resistant to this fungus. In these hybrid populations resistant plants against *M. exigua*, *M. incognita* and *M. paranaensis* were observed, though segregating for these traits. Some Híbrido de Timor and Catimor plants are homozygous for *M. exigua* resistance and present desirable agronomical traits (Gonçalves et al. 1998, Malavolta et al. 1993). First studies of F_1 plants and F_2 populations derived from Híbrido de Timor, segregating for *M. exigua* resistance, indicated oligogenic determination of this trait (Bertrand et al. 2001).

Variety Icatu was obtained based on an interspecific hybridization of *C. canephora*, artificially duplicated with the *C. arabica* var. Bourbon Vermelho. The obtained F_1 population was selected and backcrossed thrice with var. Mundo Novo. The obtained population was evaluated for the trait nematode resistance (Fazuoli 1986, Fazuoli et al. 2002) and similar resistance levels to those in *C. canephora* were found (Silvarolla et al. 1998).

Based on the AFLP markers (Vos et al. 1995) used by Noir et al. (2003), the markers linked to *M. exigua* resistance (*Mex*-1) were evaluated in order to select consistent molecular markers to be used in assisted selection, thus contributing to coffee genetic breeding programs.

The objective of this study was to evaluate, through AFLP markers, the presence of the introgressed fragment associated to locus *Mex*-1 in different lines of *C. arabica* var. Icatu, to make the use of this variety in genetic coffee improvement programs possible and allowing the use of these markers as specific molecular markers to detect the presence of this locus in assisted selection.

MATERIAL AND METHODS

Twenty-one Icatu lines were selected and collected in Viçosa and Patrocínio, MG, aiming to verify the presence of the *M. exigua*-resistance-associated marker (Table 1). Three controls were used for comparison, two of which presented complete resistance to *M. exigua*, Iapar 59 and Híbrido de Timor, and one susceptible variety, Catuaí. For the DNA extraction, lyophilized leaves were used since they are easily storable for posterior studies. A specific DNA extraction protocol based on lyophilized leaves was developed for this purpose. Nine combinations of AFLP primers were used in this study.

Preparation of the extraction buffers

Two buffers were prepared. For the first, designated A or lysis buffer, 0.35 M Sorbitol; 0.10 M Tris-HCl and 5 mM EDTA were used. Buffer B, called extraction buffer, contained 2 M NaCl; 2% CTAB; 0.20 M Tris-HCl and 50 mM EDTA. Due to the viscosity of buffer B, it was necessary to maintain it under constant agitation and heating (40 °C) during the preparation phase and, after preparation, keep it cooled until using it. Buffer B was

Table 1. List of the analyzed plants for the presence or absence of markers linked to locus *Mex*-1

Number	Genotypes	Collection site
Control 1	Iapar 59	Londrina, PR
Control 2	Catuaí CH 2077-2-5-44	Viçosa, MG
Control 3	Hibrido de Timor 832/2	Viçosa, MG
1	Icatu 2901 VEP 273	Viçosa, MG
2	Icatu M 4782-13-79-9 EP 209	Viçosa, MG
3	Icatu H 4782-13-167-5 EP 209	Viçosa, MG
4	Icatu H 4782-7-785-11 EP 182	Viçosa, MG
5	Icatu 294 AEP 273	Viçosa, MG
6	Icatu 2899 AEP 273	Viçosa, MG
7	Icatu H 4782-10-198 EP 268	Viçosa, MG
8	Icatu H 4782-7-585 EP 209	Viçosa, MG
9	Icatu H 4782-10-397-8 EP 268	Viçosa, MG
10	Icatu H 4782-10-299 EP 182	Viçosa, MG
11	Icatu vermelho 4040-179	Patrocínio, MG
12	Icatu vermelho 4040-181	Patrocínio, MG
13	Icatu vermelho 4040-315	Patrocínio, MG
14	Icatu vermelho 4042-222	Patrocínio, MG
15	Icatu vermelho 4042-114	Patrocínio, MG
16	Icatu vermelho 4045-47	Patrocínio, MG
17	Icatu vermelho 4288-101	Patrocínio, MG
18	Icatu vermelho 2942	Patrocínio, MG
19	Icatu amarelo 2944	Patrocínio, MG
20	Icatu amarelo 3282	Patrocínio, MG
21	Icatu vermelho 4782	Patrocínio, MG

removed 30 min before the beginning of the extraction and stored at 65 °C until using it. Before the extraction, buffers A and B were mixed (42% (v/v) of the volume final of each one) with 16% (v/v) of the final volume of Sarcosil 5%; 1% (w/v) sodium bisulfite; 0.1% (w/v) active carbon and 2% (w/v) PVP-40.

DNA extraction

About 50 mg of lyophilized leaves were ground in a 2 mL Eppendorf tube. After grinding, 1 mL extraction solution was added and the tubes shaken vigorously for 5 min and immediately put in a 65 ºC water bath for 40 min. After this, the samples were centrifuged for 5 min at 13000 rpm and the supernatant was transferred to a new tube, to which 1 mL CIA was added (chloroform: isoamyl 24:1). The tubes were softly shaken for 10 min and centrifuged for 5 min at 12000 rpm. The supernatant was transferred to another tube and the same volume of frozen isopropanol added to it, where it was maintained at -20 ºC for 1 h.

After this period, the material was centrifuged at maximal speed for 5 min, the supernatant was discarded and the pellet washed with 70% ethanol. This step was repeated twice and after drying, the pellets were treated with 100 µL TE plus RNAse (10 mg μL^{-1}) for 30 min at 37 ºC and 65 ºC for 5 min. After this step, the DNA was purified with the addition of 100 µL TE, 100 µL water, 100 µL NaCl 5 M and 100 µL EDTA 0.5 M. The samples were homogenized and incubated on ice for 30 min and centrifuged for 5 min at maximum speed. The supernatant was transferred to another tube and the same volume of frozen isopropanol added. The following phases were the same as described above. After drying, the pellet was diluted in 60 µL TE. The DNA quality and quantity were tested in 1% agarose gel in 0.5X TAE buffer, stained with ethidium bromide and visualized under UV light.

AFLP

The genetic map proposed by Noir et al. (2003) was taken as base for the selection of the markers used in this study. Markers Exi-1, Exi-2, Exi-3, Exi-4, Exi-5, Exi-7, Exi-10, Exi-11, and Exi-13 were selected. The combination of each primer as well as the fragment sizes are described in Table 2. The Gibco® AFLP Kit was used according to the manufacturer's instructions. Other primer combinations, not included in the kit, were used (Table 2).

The steps of digestion, adapter ligation, preamplification and amplification were realized according to kit instructions, with some modifications regarding the digestion and the ligation control to obtain the same final concentration in the samples. The amplification conditions used for the primer combinations in this study were similar to those proposed by Noir et al (2003). After the amplification step, 8 µl of formamide blue (98% formamide; 10 mM EDTA; bromophenol blue and xylene cyanol) were added to each tube. These samples were denatured for 5 min at 95 °C and immediately ice-cooled to avoid DNA renaturation.

On a 6% polyacrylamide vertical gel, 6 ml of each reaction product and 4 ml of molecular weight marker (10

Table 2. Relation of the nine primer combinations in conection with the sequence of bases of the final extremity of the *Eco* and *Mse* primers used in the amplification step and the expected size of each fragment

Combination code	Sequence of *Eco/Mse* extremity primers	Expected fragment size (pb)
Exi-1	E-AGC/M-CAA	201
Exi-2	E-AGG/M-CTT	141
Exi-3	E-CAT/M-CTT	109
Exi-4	E-CAT/M-AAC	124
Exi-5	E-ACG/M-ACC	247
Exi-7	E-AGC/M-ACA	77
Exi-10	E-ACT/M-AAC	110
Exi-11	E-CAC/M-CTA	194
Exi-13	E-CAC/M-CAT	114

Adapted from Noir et al. (2003)

or 100 pb) was applied to a 48-well gel. However, before the sample application, a pre-electrophoresis was run for 40 min at 70 W in a Gibco Life Technologies™ model S-2001 apparatus. After the application the samples were subjected to electrophoresis for about 2 h at 70 W. In the end of the electrophoresis the acrylamide gel was stained with silver nitrate (Gibcoâ Kit manufacturer's instructions) to visualize the obtained bands.

RESULTS AND DISCUSSION

The protocol used for DNA extraction based on lyophilized leaves was developed specifically for this study. Besides coffee leaves it was also tested for other crops (cacao, soybean, pepper). The other protocols tested for DNA extraction based on lyophilized coffee leaves presented a low yield and high oxidation level, due to the high level of phenolic compounds present in coffee leaves. In view of this problem, the protocol described in this study proved reliable, since the extraction obtained an optimum yield with little material and a low level of DNA oxidation.

Four of the nine primer combinations (Exi-4, Exi-5, Exi-7 and Exi-10) did not follow the amplification pattern for the presence of locus *Mex*-1. The other combinations, Exi-1, Exi-2, Exi-3, Exi-11 and Exi-13, detected the presence of the introgressed fragment associated to *M. exigua* resistance.

The five AFLP selected markers showed the presence of the resistance-associated molecular markers in the resistant controls (Iapar 59 and Híbrido de Timor) and in the 21 analyzed Icatu accessions. As expected, they did not present the resistance marker in the susceptible control since this accession does not present the introgressed fragment of *C. canephora* containing the dominant allele of *Mex*-1 locus.

According to Noir et al. (2003), AFLP markers associated to *Mex*-1, with exception of Exi-10, were detected in *C. canephora* accessions, but were not found in other *C. arabica* cultivars, thus confirming the origin of the *M. exigua* resistance gene from *C. canephora*, introgressed in *C. arabica*. Consequently, not all plants that present this introgression will be *M. exigua* resistant, demonstrating that the introgressed fragment may not contain the *Mex*-1 resistance gene.

Using AFLP marker Exi-2, the presence of the introgressed fragment was confirmed. This marker, according to Noir et al. (2003), is at a distance of about 4.6 cM from the *Mex*-1 locus. AFLP marker Exi-2 was isolated from the gel and converted into SCAR marker Exi-2 (data not shown); it was however not possible to obtain the same amplification profile for AFLP marker Exi-2, which was therefore discarded. This fact might have been a consequence of poor sequencing quality or problems associated to the isolation of the band from the acrylamide gel.

AFLP marker Exi-3 amplified a fragment of 109 pb and was 2.3 cM from the analyzed locus. AFLP detected the association of this marker with locus *Mex*-1. When this AFLP marker was used as probe to analyze high density filters of a BAC (bacterial artificial chromosome) coffee library (data not shown), it was also associated to *Mex*-1. Its performance was not that of a repetitive marker, which makes its use possible. It was however not possible to convert this AFLP into a SCAR marker since the molecular marker associated to resistance was very close to another molecular marker not associated to *Mex*-1, so a correct isolation of the band from the acrylamide gel was not possible.

The AFLP marker Exi-11, theoretically, target the *Mex*-1 locus and amplifies a fragment of 194 pb. The presence of this locus was detected in all analyzed Icatu accessions. The marker was however not found in the control Híbrido de Timor. This accession may or not present the amplification site for this AFLP primer, in spite of being

M. exigua-resistant. Or a genetic recombination may have occurred at the local associated to the AFLP marker Exi-11, which would justify the loss of the amplification site of this marker, while maintaining the resistance trait of the plant. Híbrido de Timor, when amplified with the other AFLP primers, presented the band associated to locus *Mex*-1. As the other molecular markers used to amplify the *Mex*-1 region are more distant from the gene, the recombination might have occurred in only one specific region.

The AFLP markers Exi-1 (Figure 1) and Exi-13 amplified fragments of 201 pb (Exi-1) and 114 pb (Exi-13) at a distance of 2.4 cM and 1.2 cM, respectively. Marker Exi-13 is closer to locus *Mex*-1 than Exi-2. Both presented the *Mex*-1-associated band. For AFLP marker Exi-1, the *Mex*-1-associated band is very close to another band, which is not associated to the resistance marker, so the isolation of this band and its use as SCAR marker was not possible (the same problem as described for marker AFLP Exi-3) (Figure 1).

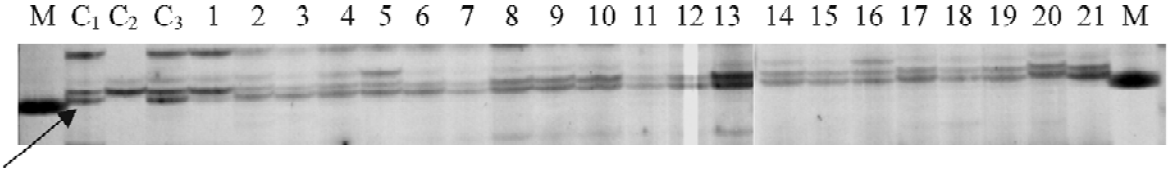

Figure 1. Polyacrylamide gel with the AFLP Exi-1 (band of 201pb) marker. The arrow indicates the presence of the introgressed fragment associated to locus *Mex*-1. M: Molecular weight marker. Controls- C_1: Iapar 59, C_2: Catuaí and C_3: Hibrido de Timor. 1 to 21: Icatu lines

When the AFLP markers Exi-11 and Exi-13 were used as probes for hybridization of BAC high density filters, they behaved as highly repetitive probes (data not shown). The use of these molecular markers for the physical mapping of the *Mex*-1 region became difficult, since these molecular markers are the closest to the resistance gene. So, based on the available markers for the *Mex*-1 locus mapping, it was observed that the AFLP markers Exi-1, Exi-3, Exi-11, and Exi-13 were able to detect polymorphism associated to *M. exigua* resistance. The amplification of these markers was repeated and the same results were found, demonstrating the reliability of these molecular markers.

The use of these molecular markers in coffee improvement programs will allow breeders to detect nematode-resistant plants more quickly and effectively, so only coffee trees of interest will be planted on the field in continuation of the improvement, preventing the loss of funds and time, besides diminishing the area required to evaluate the experiments. Therefore, whenever one needs to know whether a plant has the *Mex*-1 resistance gene or not, it is sufficient to use one of the 4 AFLP markers (Exi-1, Exi-3, Exi-11 and Exi-13). The presence of the band will indicate the presence of the *Mex*-1 gene. In turn, the lack of this band can be interpreted as absence of the resistance gene in the plant.

CONCLUSIONS

The DNA extraction protocol based on lyophilized leaves described in this study proved effective, providing a very good yield and low levels of DNA oxidation, and it can be used in other crops as well. The selected AFLP markers (Exi-1, 2, 3, 11 and 13) used to identify the introgression of the *Mex*-1-associated fragment proved useful and reliable since they were reproducible. Of the nine primer combinations we used, five were associated to the presence of locus *Mex*-1 which confers resistance to *M. exigua*. The introgression of this locus can have the dominant allele or not, thus conditioning the nematode resistance of the plant. The identification of molecular markers linked to the *M. exigua* resistance locus represents a turning point by beginning to support backcrosses in breeding programs and detecting resistance earlier, in the seed stage, allowing breeders to spare time in improvement programs as well as reduce the costs and area required for the experiments, while it gives coffee producers quicker accession to root-knot nematode (*M. exigua*) resistant plants.

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Análise de marcadores AFLP associados ao *locus* de resistência *Mex*-1 em progênies de Icatu

RESUMO - *O nematóide de galhas,* Meloidogyne exigua*, parasita o cafeeiro (*Coffea *sp.) e sua erradicação das regiões infestadas é praticamente impossível. A ampla disseminação deste nematóide nos cafezais do sul de Minas Gerais tem causado grandes prejuízos aos cafeicultores desta região. Estudos anteriores mostraram que a resistência a* M. exigua *é controlada por um gene dominante de herança simples, chamado* Mex-*1, presente em* C. canephora*. Linhagens de café arábica resistentes têm sido desenvolvidas em programas de melhoramento genético por meio da introgressão deste gene. Para confirmar esta introgressão, 21 linhagens de Icatu foram analisadas e comparadas a outros cultivares, dois resistentes, Iapar 59 e Híbrido de Timor e um susceptível, Catuaí. Dentre os marcadores AFLP utilizados, cinco marcadores confirmaram a presença do fragmento introgredido associado à resistência a* Mex-*1, mostrando que esta marca pode ser utilizada em programas de seleção assistida.*

Palavras-chave: *Coffea arabica*, *Meloidogyne exigua*, nematóide, resistência, AFLP.

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