Short Communication

Terminal repeat retrotransposons as DNA markers in fungi

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In this study, we demonstrate that CIIRAP primers designed using the transposable element *RetroCl1* sequence from *Colletotrichum lindemuthianum* can be used to generate an efficient IRAP (inter-retrotransposon amplified polymorphism) molecular marker to study intra- and inter-species diversity in fungi. It has been previously demonstrated that primers generated from this TRIM-like element can be used in the *Colletotrichum* species. We now prove that the *RetroCl1* sequence can also be used to analyze diversity in different fungi. IRAP profiles were successfully generated for 27 fungi species from 11 different orders, and intra-species genetic variability was detected in six species. The CIIRAP primers facilitate the use of the IRAP technique for a variety of fungi without prior knowledge of the genome.

Keywords: IRAP / Molecular marker / Retrotransposon / TRIM

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Introduction

DNA-based molecular markers are used to study genetic variability and diversity as well as for linkage map construction. A large fraction of repetitive sequences in fungal genomes is predominantly composed of transposable elements (TEs) [1, 2]; thus, they may be useful as molecular markers.

TEs can be divided into two classes, which are differentiated by the presence or absence of an RNA intermediary. For the class II TEs, the excision process is direct and is followed by integration. For class I, reverse transcriptase catalyzes DNA synthesis based on a copy of the retrotransposon RNA, which can then be inserted into a target site [3]. Retrotransposons have two primary subclasses, long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons (LINEs, long interspersed nuclear elements, and SINEs, short interspersed nuclear elements), which are distinguished by the respective presence or absence of LTRs at the end. Furthermore, groups of non-autonomous TEs lack one or more of the genes essential for transposition, including miniature

inverted-repeat terminal elements (MITEs) for class II, SINEs for non-LTR retrotransposons, and terminal-repeat retrotransposon in miniature (TRIM) retrotransposons and large retrotransposon derivatives (LARDs) for LTR retrotransposons [4].

Retrotransposons are advantageous molecular markers because of their replication structure and strategy. They contain long, defined, conserved sequences that can be used as specific markers for primer design. In addition, the replication activity of retrotransposons produces a high degree of polymorphism in the genome. The insertions can be detected and used for phylogenetic analysis. In this context, fingerprint techniques used for genetic diversity studies have been based on retrotransposons, such as sequence-specific amplified polymorphism (S-SAP) [5], inter-retrotransposon amplified polymorphism (IRAP) [6], retrotransposon-microsatellite amplified polymorphism (REMAP) [6], and retrotransposon-based insertion polymorphism (RBIP) [7].

Among these techniques, IRAP has been widely used primarily due to its simplicity [4]. The IRAP technique can examine polymorphisms in regions where retrotransposons are inserted. In this technique, primers are designed for conserved retrotransposon regions, such as LTRs [6]. Recently, it was demonstrated that TRIMs are ubiquitous in many species and may be useful for studying diversity among correlated species [8, 9]. In particular, Santos *et al.* [10] characterized a TRIM-like element, *RetroCl1*, and

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demonstrated through IRAP that this element could be used to analyze genetic variability in different species from the genus *Colletotrichum*. Herein, we examine the utility of IRAP markers based on primers generated from *RetroCl1* as a molecular marker for different fungi species.

Materials and methods

Species and DNA extraction

The isolates used belong to the mycological collection in the Molecular Genetics of Microorganisms Laboratory of the Universidade Federal de Viçosa (Table 1), and they were cultivated on a potato dextrose agar (PDA) medium.

The total DNA was extracted using the Ultra Clean™ Microbial DNA Isolation extraction kit from MOBIO Laboratories, Inc. Twenty-seven fungi species were used, and six species were selected for intra-specific polymorphisms analysis.

IRAP–PCR amplification and primers

CIIRAP1 (5'/CGTACGGAACACGCTACAGA3') and CIIRAP4 (5'/CTTTTGACGAGGCCATGC 3') primer combinations were used, which have been previously described by Santos *et al.* [10]. In the above combination, the primers are in the same sense of amplification. A new primer, CIIRAP2 (5'/AATAACGTCTCGGCCTTCAG3'), was designed based on the direct terminal repeats (DTRs) in *RetroCl1* (access no. JF313218). This primer was used in

Table 1. Isolates used in the IRAP analysis.

Identification in the collection	Species	Phylum	Class	Order
09	<i>Moniliophthora perniciosa</i>	Basidiomycota	Agaricomycetes	Agaricales
17	<i>Moniliophthora perniciosa</i>	Basidiomycota	Agaricomycetes	Agaricales
08	<i>Moniliophthora perniciosa</i>	Basidiomycota	Agaricomycetes	Agaricales
CMT28	<i>Cryptococcus zeae</i>	Basidiomycota	Tremellomycetes	Tremellales
C18	<i>Sporobolomyces oryicola</i>	Basidiomycota	Pucciniomycetes	Erythrobasidiales
C8	<i>Rhodotorula sp.</i>	Basidiomycota	Urediniomycetes	Sporidiales
	<i>Stemphylium solani</i>	Ascomycota	Dothideomycetes	Pleosporales
CMON10	<i>Microsphaeropsis arundinis</i>	Ascomycota	Dothideomycetes	Pleosporales
CMT35	<i>Leptosphaerulina chartarum</i>	Ascomycota	Dothideomycetes	Pleosporales
CMT47	<i>Cochliobolus kusanoi</i>	Ascomycota	Dothideomycetes	Pleosporales
M51	<i>Ampelomyces sp.</i>	Ascomycota	Dothideomycetes	Pleosporales
MAP15C	<i>Epicoccum nigrum</i>	Ascomycota	Dothideomycetes	Pleosporales
C15	<i>Cladosporium pini-ponderosae</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT1	<i>Cladosporium tenuissimum</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT48	<i>Cladosporium tenuissimum</i>	Ascomycota	Dothideomycetes	Capnodiales
CMON40	<i>Cladosporium tenuissimum</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT52	<i>Cladosporium tenuissimum</i>	Ascomycota	Dothideomycetes	Capnodiales
CAP18C	<i>Cercospora zebrinae</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT27	<i>Cercospora kikuchii</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT54	<i>Cercospora kikuchii</i>	Ascomycota	Dothideomycetes	Capnodiales
CMT63	<i>Cercospora kikuchii</i>	Ascomycota	Dothideomycetes	Capnodiales
CMON3	<i>Cercospora sp.</i>	Ascomycota	Dothideomycetes	Capnodiales
CMON18	<i>Penicillium brevicompactum</i>	Ascomycota	Eurotiomycetes	Eurotiales
CMT13	<i>Cladophialophora sp.</i>	Ascomycota	Eurotiomycetes	Chaetothyriales
M2P16F	<i>Fusarium proliferatum</i>	Ascomycota	Sordariomycetes	Hypocreales
MBP21D	<i>Fusarium equiseti</i>	Ascomycota	Sordariomycetes	Hypocreales
CMT37	<i>Fusarium equiseti</i>	Ascomycota	Sordariomycetes	Hypocreales
C3P34D	<i>Fusarium equiseti</i>	Ascomycota	Sordariomycetes	Hypocreales
C13.1	<i>Myrothecium inundatum</i>	Ascomycota	Sordariomycetes	Hypocreales
M45	<i>Annulohyphoxylon stygium</i>	Ascomycota	Sordariomycetes	Xylariales
C28	<i>Xylaria berteri</i>	Ascomycota	Sordariomycetes	Xylariales
CMT29	<i>Anthostomella sp.</i>	Ascomycota	Sordariomycetes	Xylariales
M30	<i>Hypoxylon sp.</i>	Ascomycota	Sordariomycetes	Xylariales
M39	<i>Diaporthe phaseolorum</i>	Ascomycota	Sordariomycetes	Diaporthales
C6	<i>Diaporthe helianthi</i>	Ascomycota	Sordariomycetes	Diaporthales
C29	<i>Diaporthe helianthi</i>	Ascomycota	Sordariomycetes	Diaporthales
C62	<i>Diaporthe helianthi</i>	Ascomycota	Sordariomycetes	Diaporthales
CMT33	<i>Phomopsis longicolla</i>	Ascomycota	Sordariomycetes	Diaporthales
CMT38	<i>Phomopsis longicolla</i>	Ascomycota	Sordariomycetes	Diaporthales
CMON20	<i>Phomopsis longicolla</i>	Ascomycota	Sordariomycetes	Diaporthales

conjunction with CIIRAP4; in this combination, the primers are in opposite sense of amplification.

The IRAP reactions were performed in a reaction volume of 25 μ l containing 1 $\times$ Thermophilic DNA Polymerase Buffer (Promega), 2.0 μ mol L⁻¹ MgCl₂ (Promega), 100 μ mol L⁻¹ of each dNTP, 0.2 μ mol L⁻¹ of each primer, 40 ng of DNA and 1 unit of Taq DNA polymerase (Promega). PCRs were performed in a PTC-100 thermal cycler (MJ Research) programmed to undergo an initial denaturation step of 2 min at 94 °C, six cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C. Twenty-four cycles were added to these six initial cycles, with 30 s added to the extension time (at 72 °C) every six cycles. The final extension step was 10 min at 72 °C. Differences in the amplicon patterns among the isolates were visually evaluated using a 1.5% agarose gel. The reproducibility of

the DNA band profile was tested by repeating the PCR with each of the selected primers.

Results and discussion

Many TEs are ubiquitous and have been used as molecular markers to analyze genetic diversity [10, 11], classify banana cultivars [12], detect similarity among rice cultivars [13], and fingerprint different mushroom species [14]. In the large majority of cases, whether TEs are used as molecular markers depends on prior knowledge of the genome or the TE sequences.

Herein, we demonstrate that the IRAP technique can be used with primers designed for a TRIM-like element, *RetroCl1* from *Colletotrichum lindemuthianum* [10], in

Figure 1. IRAP markers generated with the CIIRAPs primers. The band profile generated with the primer combinations CIIRAP2 and CIIRAP4 (A) as well as CIIRAP1 and CIIRAP4 (B). The species and respective identifications in the mycological collection are represented by numbers from 1 to 22. 1, *P. brevicompactum* (CMON 18); 2, *C. zeae* (CMT28); 3, *S. oryzoicola* (C18); 4, *Rhodotorula* sp. (C8); 5, *E. nigrum* (MAP15C); 6, *Ampelomyces* sp. (M51); 7, *C. kusanoi* (CMT47); 8, *L. chartarum* (CMT 35); 9, *M. arundinis* (CMON 10); 10, *S. solani* (C30); 11, *F. proliferatum* (M2P16F); 12, *M. inundatum* (C13.1); 13, *C. pini-ponderosae* (C15); 14, *C. tenuissimum* (CMON40); 15, *C. zebrinae* (CAP18C); 16, *Cercospora* sp. (CMON3); 17, *A. stygium* (M45); 18, *X. berteri* (C28); 19, *Anthostomella* sp. (CMT29); 20, *Hypoxyton* sp. (M30); 21, *D. phaseolorum* (M39); and 22, *Cladophialophora* sp. (CMT 13). M represents the 1 kb DNA Ladder molecular marker and the respective molecular weights in kilo bases (kb).

different species of fungi. 27 species were evaluated using RetroCI1 primers to generate polymorphic markers using the IRAP technique. Six isolates representative of the class Basidiomycota and 21 isolates representative of the class Ascomycota were analyzed, which correspond to 11 orders. In the repeated experiments performed for each primer set, there were no changes in the band profiles obtained for each isolate. This technique generated amplicons that varied from approximately 4 kb in *Cladophialophora* sp. to <250 bp in different fungi such as *Microsphaeropsis arundinis* (Fig. 1). The number of amplicons generated varied in accordance with the species and combination of primers used. Therefore, the primers designed for RetroCI1 could amplify DNA sequences from different fungi species, which demonstrates their potential for discriminating different fungi species.

Six fungi species were used to determine the capacity of this technique for intra-specific polymorphism detection. The combinations of primers analyzed herein facilitated polymorphism detection even with a small number of isolates (three) from the same species (Fig. 2). In certain species such as *Cercospora kikuchii*, many amplicons were detected using both combinations of primers (Fig. 2). However, in *Fusarium equiseti*, the CIIRAP2 and CIIRAP4 combination generated more polymorphic bands. In addition to primer selection, the efficiency for polymorphism detection may vary with TE orientation in the genome. Nearby TEs with different orientations (head to head, tail to tail, head to tail) may be detected.

The CIIRAPs primers were efficient in detecting inter- and intra-species polymorphisms. In addition to the IRAP technique, the RetroCI1 primers can be used with primers for different microsatellite regions in the REMAP technique. For the REMAP technique, the PCR amplification product contains sequences between TEs and microsatellites, which are also widely dispersed in the genome. Thus, it is expected that REMAP will generate more polymorphic markers than IRAP [6]. These techniques may allow for exclusive amplification of products from certain species or isolates, which facilitates the use of these markers to generate primers for sequence characterized amplified region- (SCAR) based molecular markers.

IRAP and REMAP are advantageous relative to other techniques used in population analysis because they are versatile, they use combinations of different primers that anneal to conserved regions in retrotransposons (IRAP) or microsatellites (REMAP), they are highly reproducible because they use specific primers and involve a simple PCR process, they are inexpensive, they require little work, and they generate a large number of polymorphic markers.

Figure 2. IRAP markers generated using CIIRAP primers. The band profile generated from the primer combinations CIIRAP2 and CIIRAP4 (A) as well as CIIRAP1 and CIIRAP4 (B). The species and respective identifications in the mycological collection are represented by numbers from 1 to 6 and by letters from “a” to “c” to identify different isolates of the same species. 1a, *D. helianthi* (C6); 1b, *D. helianthi* (C29); 1c, *D. helianthi* (C62); 2a, *F. equiseti* (MBP21D); 2b, *F. equiseti* (CMT37); 2c, *F. equiseti* (C3P34D); 3a, *M. pernicioso* (09); 3b, *M. pernicioso* (17); 3c, *M. pernicioso* (08); 4a, *C. kikuchii* (CMT27); 4b, *C. kikuchii* (CMT54); 4c, *C. kikuchii* (CMT63); 5a, *C. tenuissimum* (CMT1); 5b, *C. tenuissimum* (CMT48); 5c, *C. tenuissimum* (CMT52); 6a, *P. longicolla* (CMT33); 6b, *P. longicolla* (CMT38); and 6c, *P. longicolla* (CMON20). M represents the 1 kb DNA Ladder molecular marker and the respective molecular weights in kilo bases (kb).

In conclusion, the IRAP technique based on the CIIRAP primers constructed for the TRIM-like element RetroCI1 has high potential for use in studying inter- and intra-species diversity and variability in fungi without knowing the TEs in the genome.

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