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Identification of Infectious Agents in Onychomycoses by PCR-Terminal Restriction Fragment Length Polymorphism

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A fast and reliable assay for the identification of dermatophyte fungi and nondermatophyte fungi (NDF) in onychomycosis is essential, since NDF are especially difficult to cure using standard treatment. Diagnosis is usually based on both direct microscopic examination of nail scrapings and macroscopic and microscopic identification of the infectious fungus in culture assays. In the last decade, PCR assays have been developed for the direct detection of fungi in nail samples. In this study, we describe a PCR-terminal restriction fragment length polymorphism (TRFLP) assay to directly and routinely identify the infecting fungi in nails. Fungal DNA was easily extracted using a commercial kit after dissolving nail fragments in an Na_2S solution. *Trichophyton* spp., as well as 12 NDF, could be unambiguously identified by the specific restriction fragment size of 5′-end-labeled amplified 28S DNA. This assay enables the distinction of different fungal infectious agents and their identification in mixed infections. Infectious agents could be identified in 74% (162/219) of cases in which the culture results were negative. The PCR-TRFLP assay described here is simple and reliable. Furthermore, it has the possibility to be automated and thus routinely applied to the rapid diagnosis of a large number of clinical specimens in dermatology laboratories.

Onychomycosis is the most frequent nail disease that affects all ages and populations (10). Diagnosis is currently based on both direct microscopic examination of nail scrapings and macroscopic and microscopic identification of the infectious fungus in culture assay. At present, direct microscopic examination using fluorescence techniques is by far the most sensitive technique for detecting rare hyphae and spores in dermatological samples but does not provide genus or species identification of the infecting fungus. Results from culture assays are often difficult to interpret, as various nondermatophyte filamentous fungi (NDF) are often isolated from abnormal nails. Only recurrent isolations of the same NDF indicate its involvement as an infectious agent in onychomycosis (19, 38). The frequency of positive dermatophyte cultures from nail samples (*Trichophyton rubrum* and, to a lesser extent, *Trichophyton interdigitale*) was found to be only approximately 30% when direct nail mycological examination showed fungal elements (5, 32). The failure of fungi to grow in cultures is another complication in onychomycosis diagnosis. Culture assays were found to remain sterile in roughly 40% of cases even when direct mycological examinations were positive. Negative culture assays may be the consequence of previous antifungal therapy. Correct identification of the infectious agent in nail infections is essential, as *Fusarium* spp. and nondermatophyte molds have been shown to be resistant to systemic terbinafine and azole treatments (4).

In the last few years, many PCR assays have been developed for the direct detection of fungi in nail samples using specific-region primers (21). However, these assays mainly focused on the identification of dermatophytes or the discrimination of *Scytalidium* spp. from dermatophytes in nail infections (1–3, 6–8, 15, 17, 20, 23, 25, 27–29, 36, 39). We recently used a PCR-sequencing/restriction fragment length polymorphism (RFLP) assay to identify dermatophytes, as well as *Fusarium* spp. and other less frequently isolated NDF in onychomycosis (5, 32). Identification of fungi in nails using PCR methods provides a significant improvement over results obtained by culture: (i) NDF can be identified with certainty as the infectious agents of onychomycosis and can be discriminated from dermatophytes, as well as from transient contaminants; (ii) it is possible to identify the infectious agent when direct nail mycological examination showed fungal elements but negative results were obtained from fungal culture; and (iii) identification of the infectious agent can be obtained in 24 h with PCR-RFLP, whereas results from fungal culture can take as long as 1 to 3 weeks (5). Although simple, reliable, and sensitive, all proposed PCR methods to identify dermatophytes and NDF in onychomycosis are relatively time-consuming and not ideal for routine laboratory studies.

Terminal restriction fragment length polymorphism (TRFLP) is a DNA-fingerprinting technique used to investigate the compositions of microbial communities in different ecological systems, such as soil and water (13, 16, 26, 40). TRFLP assays have also been previously performed for bioremediation studies (9, 18, 24). In medicine, they have been used to characterize the oral bacterial flora in saliva from healthy subjects and patients with periodontitis (34). We applied this technique to fungi in nails in order to develop a fast and reliable assay to identify dermatophytes and NDF that can be routinely utilized on a large number of samples.

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MATERIALS AND METHODS

Nail samples. Nail samples were obtained from patients examined for suspected onychomycosis at the Department of Dermatology, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. A total of 679 samples from abnormal nails were analyzed in the present work. In 624 samples, fungal elements were observed *in situ* by direct mycological examination (positive samples). In 55 samples, fungal elements were not observed (negative samples). The clinical diagnosis of onychomycosis was based on a positive direct mycological examination in an abnormal nail. Twenty samples from healthy patients were also tested as negative controls.

Clinical-sample processing. Routinely, one portion of each clinical sample was examined in a dissolving solution using a fluorochrome (4, 5, 30). The solution was prepared by dissolving 1 g of sodium sulfide (Na_2S) (Sigma, St. Louis, MO) in 7.5 ml distilled water and subsequently adding 2.5 ml ethanol. Thereafter, 20 μl of a 1% aqueous solution of Tinopal UNPA-GX (fluorescent brightener 28; Sigma, St. Louis, MO) was added to this mixture. The sample preparations were examined using a Zeiss Axioskop fluorescence microscope with excitation between 400 and 440 nm (Zeiss, Thornwood, NY). In parallel, another portion of each sample was divided into two test tubes. The first tube contained Sabouraud's agar medium with chloramphenicol (50 μg/ml), and the second tube contained Sabouraud's agar medium with chloramphenicol plus actidione (400 μg/ml) (Bio-Rad, Hercules, CA) (5). The cultures were incubated at 30°C. Growing fungi were identified after 10 to 14 days of growth by macroscopic and microscopic examination, as previously described (12). A third portion of the samples was finally stored at room temperature in a dry box for further DNA extraction, PCR, and TRFLP analysis.

Fungal-DNA extraction. Fungal DNA was extracted from nail samples and fresh fungal cultures on Sabouraud's agar medium using the DNeasy Plant Minikit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Nail fragments (20 to 100 mg) were incubated overnight in 500 μl of sodium sulfide dissolving solution (10% [wt/vol] Na_2S [Sigma], 25% [vol/vol] ethanol) (see above) without fluorochrome. After centrifugation at 8,000 × *g* for 2 min, the sample precipitate containing fungal elements was washed twice with distilled water (5). Approximately 1 cm^2 of growing mycelium was used. The extracted DNA was stored at −20°C for subsequent repetition of TRFLP analyses.

Fungal 28S rDNA TRFLP assay (Fig. 1). 28S ribosomal DNA (rDNA) was amplified by PCR using ReadyMix *Taq* PCR Mix with $MgCl_2$ (Sigma) coupled with large-subunit forward fluorescently labeled primer LSU1 (5′-GATAGCGMACAAGTAGAGTG-3′) and reverse primer LSU2 (5′-GTCCGTGTTTCAAGACGGG-3′) (33). LSU1 primer was fluorescently labeled at the 5′ terminus with either Red-ATTO565 or Yellow-ATTO550 (Microsynth AG, Balgach, Switzerland). Red-ATTO565 was utilized to label amplicons used to prepare the reference ladder (see below). Yellow-ATTO550 was utilized to label amplicons from clinical strains or clinical samples. Extracted fungal DNA (5 μl), 1 μM (each) forward and reverse primers, and 25 μl of DNA polymerase reaction mixture were mixed with nuclease-free water to give a total reaction volume of 50 μl. The reaction mixture was incubated for 1 min at 94°C; subjected to 30 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 0.5 min at 72°C; and finally incubated for 10 min at 72°C on an ABI 2720 thermocycler (Applied Biosystems, Inc., Carlsbad, CA).

Restriction enzyme digestions were performed at 37°C for 60 min. Twenty microliters of PCR product; 1 μl of AvaI, 1 μl of AvaII, and 1 μl of StuI restriction endonucleases (New England BioLabs, Ipswich, MA); and 5 μl of 10× reaction buffer (NEBuffer 4) were mixed with deionized water to give a total reaction volume of 50 μl. Restriction fragments were subsequently purified using a High Pure PCR Purification kit (Roche Diagnostics, Basel, Switzerland).

Concentrations of PCR products from nail samples were estimated on 0.8% (wt/vol) agarose gels with a known amount of DNA Molecular Weight Marker XIV (Roche) and ranged from no detection to 150 ng/μl. After purification of digested PCR products, the DNA concentration was measured for 50 samples with a spectrophotometer (BioPhotometer Plus; Vaudaux-Eppendorf, Basel, Switzerland). The DNA concentration varied from 2 to 60 ng/μl, and different concentrations were used to test the limits of detection by a DNA analyzer.

Trichophyton spp. and NDF infecting nail samples were identified by the specific restriction fragment size of 5′-end-labeled amplified 28S rDNA. TRFLP analysis was performed in a MicroAmp Optical 96-well reaction plate (Applied Biosystems). GeneScan-LIZ[500] size standard (0.3 μl; Applied Biosystems) and 11.7 μl of Hidi formamide (Applied Biosystems) was added to 1.5 μl of the purified PCR product and 1.5 μl of the reference ladder (see "Preparation of a reference ladder for TRFLP assay" below). At this step, the manufacturer of the DNA analyzer suggests a DNA concentration ranging between 5 and 20 ng. The fluorescently labeled terminal restriction fragments were separated and detected on a 3730 DNA Analyzer using POP-7 polymer, the G5 dye set, and a Genemapper_G5_50_POP7 run module (Applied Biosystems) at the Centre intégratif de Génomique (CIG), Génopode-UNIL, Lausanne, Switzerland (Fig. 1). Data collected with the sequencer were then uploaded in GeneMapper software v4.0 (Applied Biosystems) for analysis.

The enzymes for RFLP and TRFLP were chosen by bioinformatics analysis. Theoretical digestions and restriction fragment length polymorphisms were analyzed using ApE-A plasmid Editor software (M. W. Davis). In a previous study, we showed that *Trichophyton* spp. (*T. rubrum* and *T. interdigitale*), *Fusarium* spp. (*Fusarium oxysporum* and *Fusarium solani*), and other NDF (*Aspergillus* spp. [*Aspergillus versicolor* and *Aspergillus flavus*], *Acremonium* spp. [*Acremonium alternatum* and *Acremonium strictum*], *Candida* spp. [*Candida albicans* and *Candida parapsilosis*], *Scopulariopsis brevicaulis*, and *Penicillium citrinum*) were infectious agents in onychomycoses (5). Bioinformatics analysis allowed the discrimination of these fungi by RFLP using combined AvaI, AvaII, and StuI digestions (Table 1).

Preparation of a reference ladder for TRFLP assay. One isolate representing each of 12 nail-infectious species was used to prepare a reference ladder for the TRFLP assay (Fig. 2 and 3). 28S rDNA was amplified under standard conditions using primer LSU1 fluorescently labeled at the 5′ terminus with Red-ATTO565 and primer LSU2. Digestion of the PCR products was then performed using mixed AvaI, AvaII, and StuI. The restriction products were purified using filter tubes as described in Materials and Methods. The purified digested products were visualized on agarose gels stained with ethidium bromide (Fig. 2). Comparable amounts of fluorescently labeled terminal restriction products from each fungus were mixed to generate a ready-to-use reference ladder (final concentration, 6 ng/μl). A clear peak separation for either the species or the genus of the incriminated fungi was observed using DNA Analyzer and GeneMapper software (Fig. 3). *T. rubrum* and *T. interdigitale* were not distinguished from each other, nor were *C. parapsilosis* and *C. albicans*.

Different concentrations of digested DNA ranging from 2.5 to 30 ng/μl were tested to find the one giving the clearest result by TRFLP analysis. Optimal results were obtained using DNA concentrations ranging between 5 and 15 ng/μl. Below 5 ng/μl, peaks were not discernible, and above 15 ng/μl, they were too intense, leading to a high background and difficulty in visualizing the ladder.

Agarose gel RFLP analysis. An RFLP analysis was performed as a control for the sizes of the PCR products obtained from reference strains. PCR products were loaded onto 1.5% (wt/vol) agarose gels (Tris-borate-EDTA [TBE] buffer) and stained with ethidium bromide. A DNA PCR Low Ladder Marker Set (20- to 100-bp ladder; Sigma) was used. After running for 1.5 h at 6 V/cm, the DNA fragments were visualized with UV radiation (300 nm) and recorded photographically.

Species identification by DNA sequencing. DNA sequence analysis of the amplified 28S rDNA was used for species identification of 63 culture isolates and 42 nail samples where the infectious species remained unidentified by TRFLP. DNA sequencing was performed by Microsynth AG (Balgach, Switzerland) on an FLX Genome Sequencer (454 Sequencing;

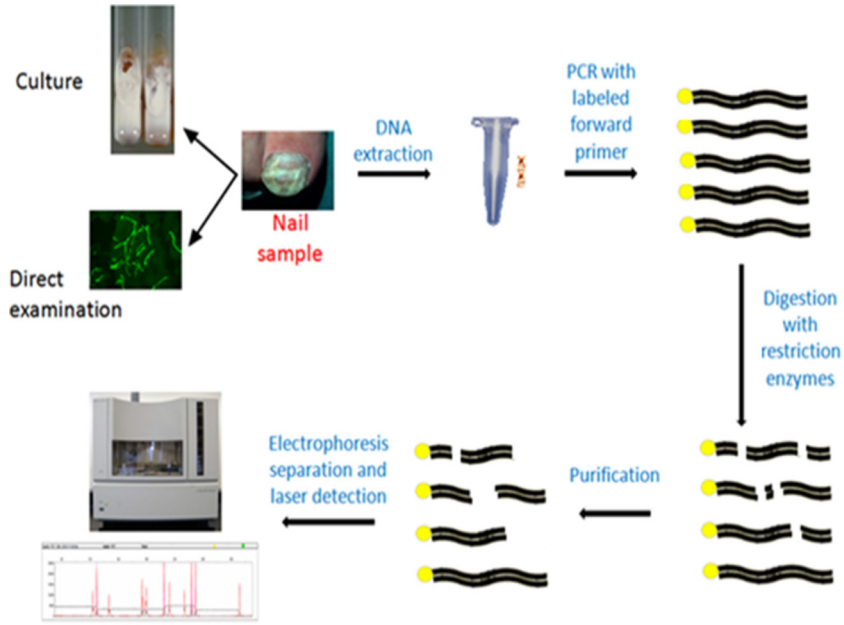

FIG 1 Overview of the PCR-TRFLP assay developed for fungal identification in onychomycosis. First, fungal DNA was extracted from nail samples. Then, 28S rDNA was amplified using a labeled forward primer. A single-step digestion of PCR amplicons with AvaI, AvaII, and StuI was completed. The digested PCR products were purified, and finally, the purified, digested, and labeled PCR products were separated using a DNA analyzer, and output data were analyzed with GeneMapper.

Roche) with part of the PCR DNA used for TRFLP analysis. The sequences were aligned with Multalin (10a; http://multalin.toulouse.inra.fr/multalin/multalin.html) and compared by BLAST on the NCBI database.

RESULTS

Principle of the developed TRFLP assay. An overview of the developed TRFLP assay is shown in Fig. 1. Total DNA was first extracted from nail samples, and the 28S rDNA was amplified using primer LSU1 fluorescently labeled with Yellow-ATTO550 and reverse primer LSU2. The PCR product was subsequently subjected to restriction enzyme digestion and purified. This step generated one fluorescently labeled fragment (the fragment retaining the 5′

TABLE 1 Fungal 28S rDNA sequences and RFLP used for preparation of the TRFLP reference ladder

Species	GenBank accession no.	28S PCR size (bp)[a]	RFLP fragment size (bp)[b]
T. interdigitale	AF378738	313	**98** + 49 + 55 + 111
T. rubrum	AF378734	314	**98** + 50 + 166
A. versicolor	AY235001	312	**104** + 36 + 102 + 70
C. albicans	AY233747	313	**121** + 192
C. parapsilosis	AY497686	311	**121** + 190
F. oxysporum	AF060383	311	**167** + 144
P. citrinum	AF033422	312	**179** + 22 + 41 + 70
A. alternatum	U57349	308	**200** + 46 + 62
Alternaria sp.	AY234951	313	**209** + 41 + 63
S. brevicaulis	AF378737	308	**231** + 14 + 63
A. flavus	AY216669	312	**242** + 70
A. strictum	AY138482	308	**246** + 62
F. solani	AY097316	311	**311**

[a] LSU1 and LSU2 primers were used.
[b] Restriction fragment size after digestion by a mixture of AvaI, AvaII, and StuI. Labeled terminal fragment sizes are in boldface.

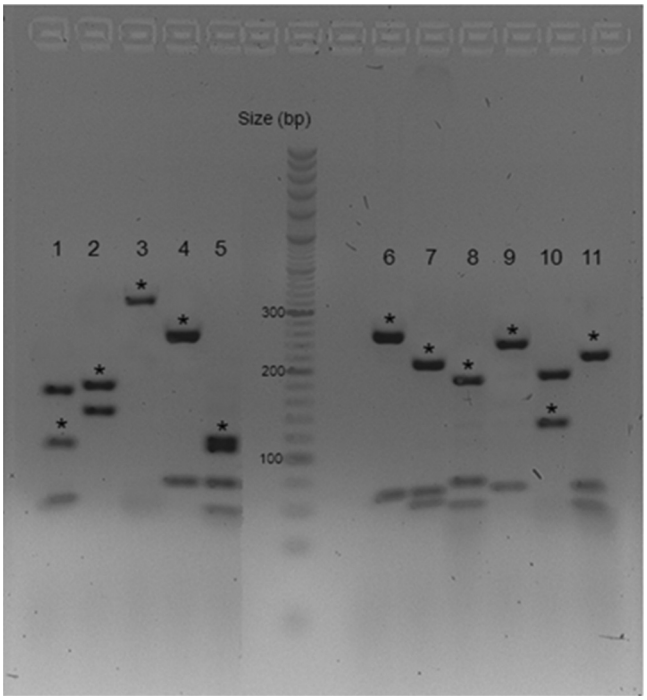

FIG 2 RFLP profiles of 28S ribosomal DNA PCR products used to prepare the TRFLP reference ladder. DNA was loaded onto a 1.5% agarose gel and stained with ethidium bromide. The PCR low ladder set (Sigma) was used as a molecular size marker. The different profiles were obtained from one strain representing each of the common infectious fungi in onychomycosis. Lane 1, *T. rubrum*; lane 2, *F. oxysporum*; lane 3, *F. solani*; lane 4, *A. flavus*; lane 5, *A. versicolor*; lane 6, *A. strictum*; lane 7, *A. alternatum*; lane 8, *P. citrinum*; lane 9, *S. brevicaulis*; lane 10, *C. parapsilosis*; lane 11, *Alternaria* spp. The 28S ribosomal DNA sequences and fragment sizes obtained by AvaI, AvaII, and StuI digestion are listed in Table 1. The fragments that are red labeled in the reference ladder are indicated by asterisks.

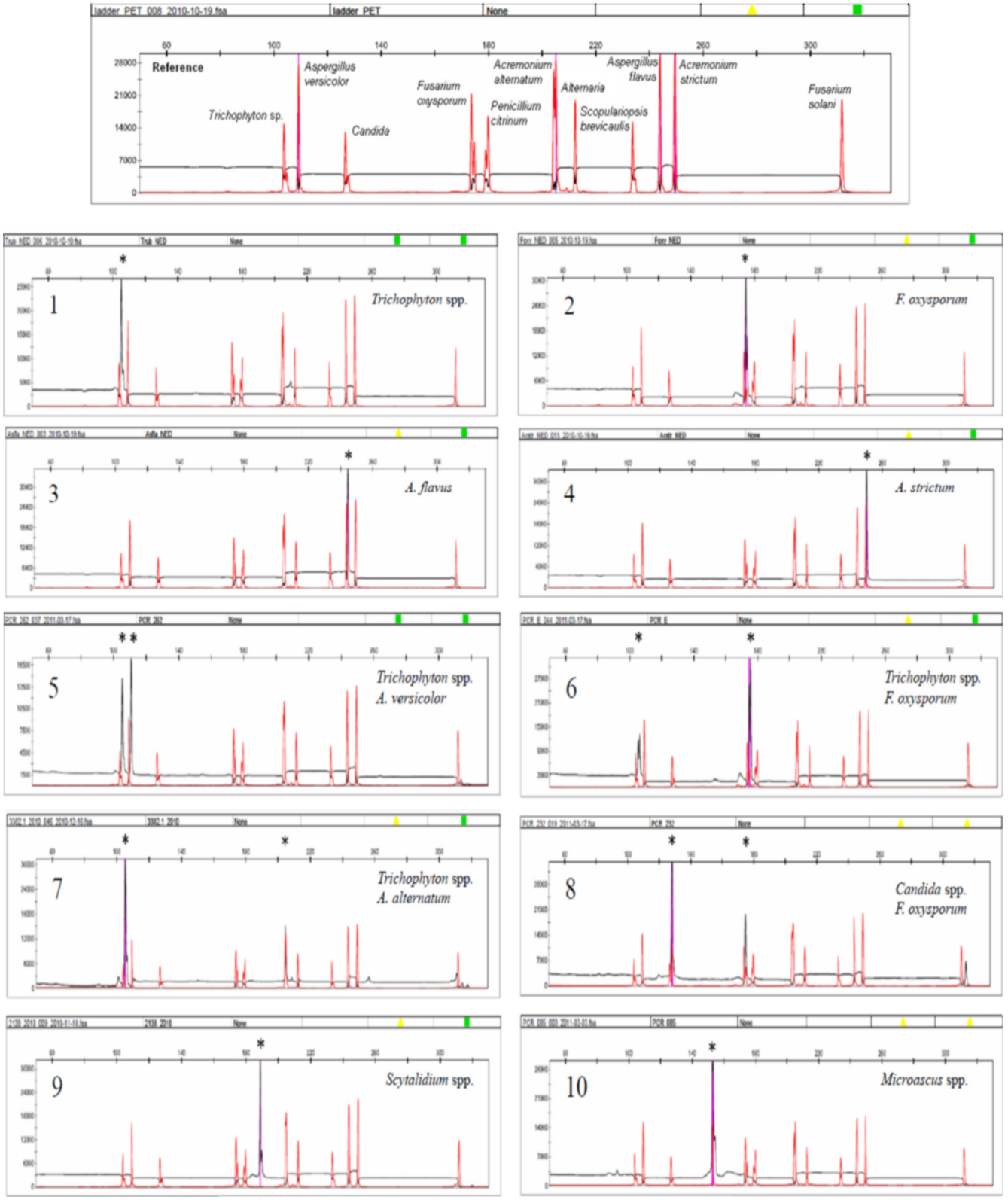

FIG 3 Identification of nail-infectious fungi in onychomycosis (panels 1 to 10) by TRFLP analysis. Fragments of the reference ladder were labeled with Red-ATTO565 (red). Fragments used to discriminate infectious fungi in onychomycosis by TRFLP analysis were labeled with Yellow-ATTO550 (black). Eleven fungi were discriminated: *Trichophyton* spp., *A. versicolor*, *Candida* spp., *F. oxysporum*, *P. citrinum*, *A. alternatum*, *Alternaria* spp., *S. brevicaulis*, *A. flavus*, *A. strictum*, and *F. solani*. Mixed infections are highlighted by multiple peaks. Panels 9 and 10 are examples where the detected peak did not correspond to any of the peaks in the reference ladder. *Scytalidium* spp. and *Microascus* spp. were identified by sequencing of the amplicons.

label from the original primer) and other, nonlabeled fragments. The digested products were then separated by capillary electrophoresis and analyzed by laser detection (3730 DNA Analyzer; Applied Biosystems), together with a mixture of Red-ATTO565 fluorescently labeled terminal restriction products generated from a set of different fungi used as a reference (reference ladder). These fungi were those known to be possible infectious agents in onychomycosis. The output of the sequencer was a series of yellow peaks of various sizes and heights that represented the profile relative to the nail sample and a series of red peaks of comparable sizes and heights that represented the profile of the reference ladder. Nail-infectious fungi were identified by the superimposition of yellow peaks from the sample on red peaks from the ladder.

Validation of the reference ladder with referenced strains. The reference ladder was first tested to identify various fungal strains isolated from infected nails. Sixty-three isolates of 2 dermatophyte and 11 nondermatophyte species (*Trichophyton* spp. [*T. rubrum* and *T. interdigitale*], 14; *F. oxysporum*, 7; *F. solani*, 4; *A. versicolor*, 7; *A. flavus*, 4; *A. alternatum*, 3; *A. strictum*, 4; *Candida* spp. [*C. parapsilosis* and *C. albicans*], 6; *Alternaria* spp., 5; *P. citrinum*, 4; and *S. brevicaulis*, 5) isolated from nail samples were used. The isolates were identified by superimposition between peaks from the reference ladder (red peaks) and the strain sample (black peaks) (Fig. 3). The results agreed with the species identification obtained by sequencing of amplified 28S rDNA for all 63 isolates tested and therefore validated the use of the reference ladder to further identify infectious fungi in nail samples (see Table S1 in the supplemental material).

Identification of fungi in onychomycoses by terminal restriction fragment length polymorphism analysis. Using the designed TRFLP ladder, infectious fungi were identified in 624 nail samples that showed fungal elements by direct mycological examination (positive samples), and the results were compared to those of culture assays (Table 2 and Fig. 3). *Trichophyton* spp. were identified as the single infecting fungal agent in 71 of 81 cases (88%) where either *T. rubrum* or *T. interdigitale* grew in cultures (Table 2, boldface). Likewise, *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp. and *Candida* spp. were revealed in 76% (38/50), 39% (11/28), 80% (4/5), 50% (5/10), 14% (3/22), and 59% (26/44) of cases, respectively, when these NDFs grew as single species in culture assays (Table 2, boldface). In some cases where a single fungus was recovered in culture, TRFLP results demonstrated the presence of 2, 3, or 4 fungal DNAs, one of which matched the agent recognized in the culture (mixed infections 1 to 20 in Table 2). The identified species are listed in Table S2 in the supplemental material.

Most often, a single infectious agent was found by TRFLP. However, in several cases, one agent was recovered in culture and a different agent was detected by TRFLP. For instance, *Trichophyton* spp. were detected in 4, 7, 1, 2, 12, and 4 cases when *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp., and *Candida* spp., respectively, grew as a single fungus in culture (Table 2, row 1, shaded cells). When *Alternaria* spp. were isolated in culture assays (19 cases), *Trichophyton* spp. were identified as the infectious agent in 15 cases (Table 2; see Table S2 in the supplemental material). *Alternaria* spp. were identified only once, but in a mixed infection with *Trichophyton* spp.

TRFLP was used to identify the infectious fungi in nails when other molds (i.e., species different from those used for the design of the ladder) grew in culture (64 cases) and when culture assays remained sterile (218 cases) (Table 2). The analysis results revealed a prevalence of *Trichophyton* spp. with frequencies of 48% (31/64) and 55% (120/218). One NDF among *Fusarium* spp., *Acremonium* spp., *Aspergillus* spp., *S. brevicaulis*, *Penicillium* spp., *Candida* spp., and *Alternaria* spp. was clearly identified in 19% (12/64) and in 16% (34/218) of these cases (Table 2). Altogether, 17 mixed infections with the aforementioned fungi were detected (Table 2, mixed infections 22 to 38). In total, infectious agents could be identified in 74% (162/218) of the cases where negative results were obtained by means of cultures.

Infectious fungi could not be identified in 74 of 624 cases (12%); either TRFLP results were not interpretable due to too much background noise or no peak was detected because of a failure in the PCR amplification. In these cases, retrospective investigations revealed that either direct mycological examination showed a small quantity of fungal elements or the assay had been performed using a small amount of nail sample.

Mixed infections. Two examples of TRFLP analysis results revealing two peaks and attesting to mixed infections are shown in Fig. 3. When either *Trichophyton* spp., *Fusarium* spp., *Aspergillus* spp., *Acremonium* spp., *Scopulariopsis* spp., *Penicillium* spp., or *Candida* spp. grew as a single fungus in culture (81, 50, 28, 5, 10, 22, and 44 cases, respectively [Table 2]), 20 cases of mixed infections (sorted mixed infections 1 to 20) were detected, representing 8% of the total of 240 cases. The ratio of mixed infections detected by TRFLP analyses was higher (16%) when two or more species grew in culture (14 cases among 83 nail samples) (Table 2; see Table S3 in the supplemental material). Only one species was identified by TRFLP analysis in 73% of these cases (61/83) using the reference ladder generated in this study.

After a 6-month period, TRFLP analysis was repeated for the cases where the agent identified by TRFLP was different from that identified in culture (Table 2, shaded cells) and the 52 cases of recorded mixed infections. We used the same stocks of DNA isolated from nail specimens, which were conserved at −20°C. The results were confirmed, with the exception of 6 cases of mixed infections, where one signal was lost. The reproducibility of the analyses permitted us to rule out exogenous contamination in most cases. Discrepancies in the 6 cases of mixed infections may also be explained by long-term conservation of DNA.

Identification of other infectious fungi in onychomycoses and completion of markers in the reference ladder. Further investigation was performed using DNA extracted from 42 samples where the infectious species remained unidentified, as the detected peak in TRFLP analysis did not correspond to any of the peaks in the reference ladder (Table 2 and Fig. 3; see Table S3 in the supplemental material). Twenty-four of these 42 samples showed a single species, and 18 samples were from the 52 samples in which mixed infections were detected. Sequencing of amplified 28S rDNA allowed identification of *Scytalidium* spp. and *Microascus* spp. (teleomorphs of *Scopulariopsis* spp. non-*brevicaulis*), as the single infectious agent in four and two onychomycoses, respectively. Although *Scytalidium* spp. are commonly reported as etiological agents in onychomycoses, this was the first time the fungus had ever been reported as an infectious agent in Switzerland. On the other hand, *Microascus cirrosus* had previously been reported as an infectious agent in onychomycoses (14). A unique identification was obtained in 12 cases (Table 3). The species could not be identified by sequencing in 24 cases. In 5 cases, the amount of DNA was too small. In 19 cases, the sequencing trace files were not readable because of superimposition of sequences in-

TABLE 2 PCR-TRFLP identification of fungi in onychomycosis in comparison to culture results[a]

PCR-TRFLP identification result	Culture identification result (no.)											
	Trichophyton spp.	*Fusarium* spp.	*Aspergillus* spp.	*Acremonium* spp.	*Scopulariopsis brevicaulis*	*Penicillium citrinum*	*Candida albicans* or *C. parapsilosis*[b]	*Alternaria* or *Curvularia*	Other filamentous fungi[c]	Sterile	Mixed culture	Total
Trichophyton spp.	**71**	4	7	1	2	12	4	15	31	120	44	311
F. oxysporum		**31**	1				1			8	4	45
F. solani		**7**				1			2	5	2	17
A. versicolor		1	**9**		1		1		3	8	1	24
A. flavus			**2**							4		6
A. alternatum			2	**2**		1		1	2	2	1	11
A. strictum				**2**					1	1	1	5
S. brevicaulis			1		**5**							6
P. citrinum			1			**3**						4
Candida spp.							**26**		3	5	7	41
Alternaria or *Curvularia*						1		**0**	1	1	1	4
Mixed infection with species identified in cultures[d]	5 (1–5)	4 (6–9)	2 (10, 11)	0	1 (12)	1 (13)	7 (14–20)	1 (21)	9 (22–30)	8 (31–38)	14 (39–52)	52
Fungi not in the TRFLP ladder[e]	1	2	1			1	1		5	9	4	24
Negative result[f]	4	1	2		1	2	4	2	7	**47**	4	74
Total	81	50	28	5	10	22	44	19	64	218	83	**624**
Result identity (%)[g]	93.83	84.00	46.43	80.00	60.00	18.18	75.00	5.26	81.25	78.44		

[a] All specimens were from abnormal nails that were positive by direct mycological examination. The shaded cells represent discrepancies between the culture result and the PCR-TRFLP result (the agent identified in culture was not that detected by TRFLP analysis). Boldface indicates identification of single infecting species in culture assays.

[b] All the cases where *Candida* spp. were detected were nail infections without paronychias.

[c] A fungus not in the ladder was cultured.

[d] TRFLP detected both the agent identified in culture and another agent. Identified species are listed in Table S2 in the supplemental material. The ID numbers of the sorted mixed infections are in parentheses.

[e] Fungi further identified by 28S rDNA sequencing are listed in Table 3.

[f] No peak was detected by TRFLP analysis.

[g] Percentages of the cases where the fungus identified by TRFLP (as a single agent or in mixed infections) corresponded to the fungus identified in culture.

TABLE 3 Infectious fungi not identified by TRFLP analysis using the prepared reference ladder[a]

Culture[a]	TRFLP identification[b]	Peak size (bp)	Identification by 28S rDNA sequencing	Corresponding GenBank accession no.
T. rubrum	*Trichophyton* spp. + *Candida* spp. + *A. alternatum* + ND	115.67	Superimposed sequences on sequencing	
T. interdigitale + *Aspergillus* spp. + molds	*Trichophyton* spp. + ND	146.28	Superimposed sequences on sequencing	
Sterile	ND	146.38	*Eurotium amstellodami*	AY213699
Alternaria spp. + *Fusarium* spp.	*A. versicolor* + ND	146.44	Superimposed sequences on sequencing	
Sterile	ND	152.89	*Microascus desmosporus* (= *cirrosus*)	AF400861
Molds	ND	153.15	*Microascus cinereus*	AF400859
Molds	ND	184.64	*Macrovalsaria megalospora*	FJ215701
Molds	ND	184.68	*Massarina corticola*	FJ795448
Fusarium spp.	ND	185.33	Too little DNA for sequencing	
Sterile	ND	186.18	*Pleurophoma pleurospora*	EU754200
Molds + *P. citrinum*	*Trichophyton* spp. + ND	186.57	Superimposed sequences on sequencing	
T. soudanense	ND	188.4	*Cladosporium* spp.	HQ026794
Candida spp.	ND	188.44	Too little DNA for sequencing	
Molds	ND	188.84	*Scytalidium* spp.	EF585552
P. citrinum	ND	188.84	*Scytalidium* spp.	DQ377925
Molds + *P. citrinum*	ND	188.86	*Scytalidium* spp.	DQ377925
Molds	ND	188.88	*Scytalidium* spp.	DQ377925
Trichosporon + Sterile	*Trichophyton* spp. + ND	203.69	Superimposed sequences on sequencing	
Candida spp.	*Candida* spp. + ND	207.5	Superimposed sequences on sequencing	
Fusarium spp.	ND	208.65	*Epicoccum nigrum*	HQ691437
Aspergillus spp.	*Candida* spp. + ND	208.97	Superimposed sequences on sequencing	
Sterile	ND	247.33	*Pseudallescheria boydii* or *Scedosporium prolificans*	AB363763 or AF027679
P. citrinum	ND	253.28	*Cochliobolus* spp.	AF163979
Sterile	ND	253.47	*Xenostigmina zilleri*	FJ839676
Sterile	ND	263.77	Too little DNA for sequencing	
Sterile	*Candida* spp. + ND	263.83	Superimposed sequences on sequencing	
Candida spp. + *Fusarium* spp.	*Trichophyton* spp. + *Candida* spp. + ND	263.84	Superimposed sequences on sequencing	
T. rubrum	*Trichophyton* spp. + ND	287.74	Superimposed sequences on sequencing	
Sterile	ND	292.38	Too little DNA for sequencing	
Candida spp.	*A. versicolor* + *Candida* spp. + ND	293.27	Superimposed sequences on sequencing	
Sterile	ND	313.95	*Albonectria rigidiuscula*	HM042412
Sterile	ND	314.01	Too little DNA for sequencing	
Scedosporium spp.	ND	314.21	*Arthroderma multifidum*	AB359438
Geotrichum spp.	*Trichophyton* spp. + ND	314.22	Superimposed sequences on sequencing	
Molds	*Alternaria* spp./*Curvularia* spp. + ND	314.24	Superimposed sequences on sequencing	
Molds	*A. alternatum* + ND	314.86	*Candida guilliermondii*	HM771258
Mucor spp.	*Mucor* spp.?	316.23	No correct identification by sequencing	
Molds	*Trichophyton* spp. + ND	317.38	Superimposed sequences on sequencing	
Candida spp.	*Candida* spp. + ND1 + ND2	150 + 292.23	Superimposed sequences on sequencing	
Molds	*Trichophyton* spp. + *Alternaria* spp./*Curvularia* spp. + ND	188.3 + 255.2	Superimposed sequences on sequencing	
Candida spp. + *P. citrinum*	ND	188.52 + 314.63	Superimposed sequences on sequencing	
Molds + *Candida* spp.	*Candida* spp. + ND	188.74 + 223.23	Superimposed sequences on sequencing	

[a] Identification results were obtained by sequencing 28S rDNA amplicons. Sterile, negative result in culture.
[b] ND, other fungi.

dicative of mixed infections. As *Scytalidium* spp. and *Microascus* spp. were identified more than once and are known to be potential infectious agents in onychomycosis, the reference ladder was completed with labeled DNA from these fungi for their detection in future samples.

TRFLP analysis of nail samples showing negative results by direct examination. Fifty-five abnormal nail samples with negative direct examination results were analyzed by the method described here and were used as negative controls (data not shown). No peak was detected in 47 cases (85%). *Trichophyton* spp., *Candida* spp., and *Acremonium* spp. were identified in five, one, and two cases, respectively. Twenty nail scrapings from healthy patients were also tested, and all gave a negative result with the TRFLP assay.

DISCUSSION

The TRFLP technique was first used to study complex communities of bacteria by taking advantage of variations in their 16S rDNA. In the present study, a similar approach was adopted, and

TRFLP analysis was used to identify infectious fungi based on differences in their 28S rDNA amplicons. Other DNA sequences, such as that of the chitin synthase 1 gene or small ribosomal subunit 18S rRNA, were successfully used for fungal species delineation and identification (7, 27, 28). The polymorphism of the internal transcribed spacers (ITS) of ribosomal DNA regions (ITS1 and ITS2) flanking the DNA sequence composing the 5.8S rDNA is the most discriminating tool for distinguishing different fungi (1). ITS sequences have been used in previous studies for the identification of dermatophytes by RFLP (11, 41). However, the 320-bp 28S rDNA sequence was found to be more sensitive and suitable, as well as sufficient to routinely identify isolates from nails with high sensitivity, at least to the genus level. The TRFLP assay does not allow the identification of dermatophytes to the species level, but in practice, identifying the genus to which an infecting fungus belongs is what is most relevant for the dermatologist with respect to onychomycosis. The two anthropophilic species *T. rubrum* and *T. interdigitale* are the causes of 99% of tinea unguium (22, 31, 35, 37), and both species respond well to standard treatment with azoles and terbinafine, whereas special treatments may need to be prescribed for NDF onychomycosis (4). In other tinea infections, in particular tinea capitis, the treatment sometimes varies, depending on the species involved, and therefore, only in these cases would species determination be of any use.

The clinical sensitivity of the assay for identifying, at least to the genus level, infectious fungi in samples positive by direct mycological examination was 84% (526/624). The results of fungal identification obtained by the TRFLP assay described here are representative of the fungal community in the whole nail sample. In contrast, only a small part of the nail sample is seeded on an agar medium surface in culture assays. This may explain many of the discrepancies between culture and TRFLP analysis results. The reproducibility of results obtained for nail specimens in which mixed infections were detected allows one to rule out exogenous contamination.

TRFLP fungal identifications were performed using a 20- to 100-mg nail sample, provided that more than rare spores or/and filaments were detected by direct mycological examination. Failure of fungal identification occurred when the sample revealed a small quantity of fungal elements by direct mycological examination. During the development of the assay and preparation of the reference ladder, the lower limit of DNA that could be detected by the DNA analyzer (analytical sensitivity) for each species was found to be 7.5 ng.

No false positive was observed with nail scrapings from healthy individuals without suspected mycosis. In contrast, 10 positive samples were observed in a group of 55 abnormal nails that were negative by direct mycological examination (18%). However, in the samples where a fungal species can be identified, the diagnosis of a fungal infection should be confirmed with a second independent sampling. The detection of the same fungus by TRFLP should exclude accidental occurrence of NDF in the first nail sampling. In addition, new direct mycological examination may clarify a doubtful situation.

In this study, 174 (27%) of the 640 identified samples were NDF (not including mixed infections or undetermined species). This ratio is comparable to that revealed in a previous study using a PCR-RFLP assay (5). In the last decade, we observed an increasing prevalence of *Fusarium* spp. and *Acremonium* spp. in onychomycoses. The frequency of *Fusarium* isolates from nails is now reaching 15% of that of dermatophytes in our records from 2005 to 2010. As *Fusarium* spp. and NDF appear to be insensitive to standard systemic treatments with terbinafine and azoles, a reliable diagnosis of NDF from the laboratory allows the practitioner to tailor therapy as needed.

The PCR-TRFLP assay we describe here shows several improvements in comparison to PCR-RFLP assays using agarose gel electrophoresis.

First, the infectious agents can be precisely identified at least to the genus or the species level using a one-step digestion protocol. The results are easy to read and interpret by simply searching for peak superimposition.

Second, in the case of mixed infections, more than one infectious agent can be unambiguously identified. Different infectious fungi simultaneously appear as distinct peaks in a diagram (peak profile). Sequencing of 28S rDNA amplified from genomic DNA extracted from a mixture of 2 different fungi generates trace files that are not readable and are not suitable for fungal identification. Using conventional RFLP agarose gel methods, it is often difficult to interpret band profiles relating to more than one species in a nail sample. As mixed infections are detected in roughly 10% of onychomycoses (8% [52/624] in the present study), TRFLP analysis is ideally suited to this kind of analysis.

Third, PCR-TRFLP analysis allows the identification of new infectious agents. The reference ladder can be continuously upgraded with new species markers as they are discovered. From the results of this study, it could be updated for further analyses with markers for *Scytalidium* spp. and *Microascus* spp., which were detected in five and two cases, respectively. We are now able to identify 15 infectious agents that were found to be in more than 85% (530/624) of the samples at the genus or species level. Other fungi were detected in 3% of the cases. The names of these fungi (12 species identified only once [Table 3]) were recorded in our data bank for the possible inclusion of a corresponding new marker in the reference ladder if one of these fungi is repeatedly identified in the future.

The PCR-TRFLP assay described here is simple, reliable, and suitable for dermatology laboratories provided that enough nail material is collected for analysis. In practice, we are using TRFLP for fungal identification and not for the clinical diagnosis of an onychomycosis, which is based on a positive direct mycological examination in an abnormal nail. The cost for a complete TRFLP assay, including DNA extraction, PCR with labeled primer, digestion, purification, and loading on a DNA analyzer, is approximately €10. This is a higher price than for culture (€2) or RFLP assay (€8), but it is counterbalanced by the higher efficiency and sensitivity of the TRFLP method (32). Despite the higher cost, PCR methods are appropriate for routine onychomycosis diagnosis because of the high frequency of NDF and the commonly problematic interpretation of culture results. Automation of the technique can contribute to lowering the price and is under way using an automated DNA extraction system, PCR, digestion of PCR products, and their purification in 96-well microplates.

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REFERENCES

1. **Arca E, et al.** 2004. Polymerase chain reaction in the diagnosis of onychomycosis. Eur. J. Dermatol. **14**:52–55.
2. **Baek SC, Chae HJ, Houh D, Byun DG, Cho BK.** 1998. Detection and differentiation of causative fungi of onychomycosis using PCR amplification and restriction enzyme analysis. Int. J. Dermatol. **37**:682–686.
3. **Bagyalakshmi R, Senthilvelan B, Therese KL, Murugusundram S, Madhavan HN.** 2008. Application of a polymerase chain reaction (PCR) and PCR based restriction fragment length polymorphism for detection and identification of dermatophytes from dermatological specimens. Indian J. Dermatol. **53**:15–20.
4. **Baudraz-Rosselet F, Ruffieux C, Lurati M, Bontems O, Monod M.** 2010. Onychomycosis insensitive to systemic terbinafine and azole treatments reveals non-dermatophyte moulds as infectious agents. Dermatology **220**: 164–168.
5. **Bontems O, Hauser PM, Monod M.** 2009. Evaluation of a polymerase chain reaction-restriction fragment length polymorphism assay for dermatophyte and non-dermatophyte identification in onychomycosis. Br. J. Dermatol. **161**:791–796.
6. **Brasch J, Beck-Jendroschek V, Gläser R.** 2011. Fast and sensitive detection of *Trichophyton rubrum* in superficial *tinea* and onychomycosis by use of a direct polymerase chain reaction assay. Mycoses **54**:e313–317.
7. **Brillowska-Dabrowska A, Saunte DM, Arendrup MC.** 2007. Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum.* J. Clin. Microbiol. **45**:1200–1204.
8. **Brillowska-Dabrowska A, Nielsen SS, Nielsen HV, Arendrup MC.** 2010. Optimized 5-hour multiplex PCR test for the detection of *tinea unguium*: performance in a routine PCR laboratory. Med. Mycol. **48**:828–831.
9. **Burke DJ, Chan CR.** 2010. Effects of the invasive plant garlic mustard (*Alliaria petiolata*) on bacterial communities in a northern hardwood forest soil. Can. J. Microbiol. **56**:81–86.
10. **Chabasse D.** 2004. Epidémiologie et étiologie des onychomycoses, p 1–35. *In* Baran R and Piérard GE (ed), Onychomycoses. Masson, Paris, France.

10a. **Corpet F.** 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 10881–10890.

11. **De Baere T, Summerbell R, Theelen B, Boekhout T, Vaneechoutte M.** 2010. Evaluation of internal transcribed spacer 2-RFLP analysis for the identification of dermatophytes. J. Med. Microbiol. **59**:48–54.
12. **De Hoog GS, Guarro J, *Gen* Jé Figueras MJ.** 2000. Atlas of clinical fungi, 2nd ed. Centrallbureau voor Schimmelcultures, Utrecht, Netherlands.
13. **Derakshani M, Lukow T, Liesack W.** 2001. Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. Appl. Environ. Microbiol. **67**:623–631.
14. **De Vroey C, Lasagni A, Tosi E, Schroeder F, Song M.** 1992. Onychomycoses due to *Microascus cirrosus* (syn. *M. desmosporus*). Mycoses **35**:7–8.
15. **Ebihara M, Makimura K, Sato K, Abe S, Tsuboi R.** 2009. Molecular detection of dermatophytes and nondermatophytes in onychomycosis by nested polymerase chain reaction based on 28S ribosomal RNA gene sequences. Br. J. Dermatol. **161**:1038–1044.
16. **Eschenhagen M, Schuppler M, Röske I.** 2003. Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. Water Res. **37**:3224–3232.
17. **Garg J, et al.** 2007. Evaluation of pan-dermatophyte nested PCR in diagnosis of onychomycosis. J. Clin. Microbiol. **45**:3443–3445.
18. **Gough HL, Stahl DA.** 2011. Microbial community structures in anoxic freshwater lake sediment along a metal contamination gradient. ISME J. **5**:543–558.
19. **Gupta AK, Cooper EA, MacDonald P, Summerbell R.** 2001. Utility of inoculum counting (Walshe and English criteria) in the clinical diagnosis of onychomycosis caused by nondermatophytic filamentous fungi. J. Clin. Microbiol. **39**:2115–2121.
20. **Gupta AK, Zaman M, Singh J.** 2007. Fast and sensitive detection of *Trichophyton rubrum* DNA from the nail samples of patients with onychomycosis by a double-round polymerase chain reaction-based assay. Br. J. Dermatol. **157**:698–703.
21. **Hay RJ, Morris Jones R.** 2010. New molecular tools in diagnosis of superficial fungal infections. Clin. Dermatol. **28**:190–196.
22. **Järv H, Naaber P, Kaur S, Eisen M, Silm H.** 2004. Toenail onychomycosis in Estonia. Mycoses **47**:57–61.
23. **Kardjeva V, et al.** 2006. Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. J. Clin. Microbiol. **44**: 1419–1427.
24. **Karpouzas D, Singh BK.** 2010. Application of fingerprinting molecular methods in bioremediation studies. Methods Mol. Biol. **599**:69–88.
25. **Kondori N, Abrahamsson AL, Ataollahy N, Wenneras C.** 2010. Comparison of a new commercial test, dermatophyte-PCR kit, with conventional methods for rapid detection and identification of *Trichophyton rubrum* in nail specimens. Med. Mycol. **48**:1005–1008.
26. **Lee H-K, Kim H-R, Mengoni A, Lee DH.** 2008. Modified T-RFLP methods for taxonomic interpretation of T-RF. J. Microbiol. Biotechnol. **18**:624–630.
27. **Litz CE, Cavagnolo RZ.** 2010. Polymerase chain reaction in the diagnosis of onychomycosis: a large, single-institute study. Br. J. Dermatol. **163**: 511–514.
28. **Machouart-Dubach M, et al.** 2001. Rapid discrimination among dermatophytes, Scytalidium spp., and other fungi with a PCR-restriction fragment length polymorphism ribotyping method. J. Clin. Microbiol. **39**:685–690.
29. **Menotti J, et al.** 2004. Polymerase chain reaction for diagnosis of dermatophyte and *Scytalidium* spp. onychomycosis. Br. J. Dermatol. **151**: 518–519.
30. **Monod M, Baudraz-Rosselet F, Ramelet AA, Frenk E.** 1989. Direct mycological examination in dermatology: a comparison of different methods. Dermatologica **179**:183–186.
31. **Monod M, et al.** 2002. Survey of dermatophyte infections in the Lausanne area (Switzerland). Dermatology **205**:201–203.
32. **Monod M, et al.** 2006. Fast and reliable PCR/sequencing/RFLP assay for identification of fungi in onychomycoses. J. Med. Microbiol. **55**:1211–1216.
33. **Ninet B, et al.** 2003. Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. J. Clin. Microbiol. **41**:826–830.
34. **Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y.** 2003. Application of terminal RFLP analysis to characterize oral bacteria flora in saliva of healthy subjects and patients with periodontitis. J. Med. Microbiol. **52**:79–89.
35. **Saunte DM, et al.** 2008. Laboratory-based survey of dermatophyte infections in Denmark over a 10-year-period. Acta Derm. Venereol. **88**:614–616.
36. **Savin C, et al.** 2007. Multicenter evaluation of a commercial PCR-enzyme-linked immunosorbent assay diagnostic kit (Onychodiag) for diagnosis of dermatophytic onychomycosis. J. Clin. Microbiol. **45**:1205–1210.
37. **Simonnet C, Berger F, Gantier J-C.** 2011. Epidemiology of superficial fungal diseases in French Guiana: a three-year retrospective analysis. Med. Mycol. **49**:608–611.
38. **Summerbell RC, Cooper E, Bunn U, Jamieson F, Gupta AK.** 2005. Onychomycosis: a critical study of techniques and criteria for conforming the etiologic significance of nondermatophytes. Med. Mycol. **43**:39–59.
39. **Turin L, Riva F, Galbiati G, Cainelli T.** 2000. Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. Eur. J. Clin. Invest. **30**: 511–518.
40. **Vinten AJ, et al.** 2011. Comparison of microbial community assays for the assessment of stream biofilm ecology. J. Microbiol. Methods **85**:190–198.
41. **Yang G, Zhang M, Li W.** 2008. Direct species identification of common pathogenic dermatophyte fungi in clinical specimens by semi-nested PCR and restriction fragment length polymorphism. Mycopathologia **166**: 203–208.