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Autoscreening of Restriction Endonucleases for PCR-Restriction Fragment Length Polymorphism Identification of Fungal Species, with *Pleurotus* spp. as an Example▽†

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A molecular method based on PCR-restriction fragment length polymorphism (RFLP) analysis of internal transcribed spacer (ITS) ribosomal DNA sequences was designed to rapidly identify fungal species, with members of the genus *Pleurotus* as an example. Based on the results of phylogenetic analysis of ITS sequences from *Pleurotus*, a PCR-RFLP endonuclease autoscreening (PRE Auto) program was developed to screen restriction endonucleases for discriminating multiple sequences from different species. The PRE Auto program analyzes the endonuclease recognition sites and calculates the sizes of the fragments in the sequences that are imported into the program in groups according to species recognition. Every restriction endonuclease is scored through the calculation of the average coefficient for the sequence groups and the average coefficient for the sequences within a group, and then virtual electrophoresis maps for the selected restriction enzymes, based on the results of the scoring system, are displayed for the rapid determination of the candidate endonucleases. A total of 85 haplotypes representing 151 ITS sequences were used for the analysis, and 2,992 restriction endonucleases were screened to find the candidates for the identification of species. This method was verified by an experiment with 28 samples representing 12 species of *Pleurotus*. The results of the digestion by the restriction enzymes showed the same patterns of DNA fragments anticipated by the PRE Auto program, apart from those for four misidentified samples. ITS sequences from 14 samples (of which nine sequences were obtained in this study), including four originally misidentified samples, confirmed the species identities revealed by the PCR-RFLP analysis. The method developed here can be used for the identification of species of other living microorganisms.

The PCR-restriction fragment length polymorphism (RFLP) technique, consisting of PCR amplification of target DNA fragments and subsequent digestion of the PCR products with restriction endonucleases to obtain band patterns, has been used extensively for molecular identification of living organisms, especially microorganisms (25, 37). The selection of suitable endonucleases is the key step of the technique. Endonucleases have been selected through extensive experimentation with restriction enzyme cleavage to distinguish different species of bacteria (31, 34), yeasts (9, 30), filamentous fungi (21–23), and other organisms (4, 5) in the recent years. As more DNA sequences are becoming readily available, the selection of endonucleases can be based on sequence analyses through the examination of recognition sites of restriction enzymes from the alignment of sequences, e.g., from bacteria (10), fungi (7, 41), and other living organisms (1, 48).

In recent years, various computer programs have been used for the selection of these restriction endonucleases. Programs have been designed to search for the recognition sites of endonucleases, to locate the positions of these sites in sequences, and to calculate the sizes of fragments after digestion. Some of the software packages, e.g., Gene Runner version 3.05 (24), Genetyx version 6.1 (26), MapDraw of DNASTAR version 3.14 (16, 32), NEBcutter version 2.0 (35), Webcutter version 2.0 (40), BioEdit (45), DNA Club (47), the Infobiogen restriction program (29), and REBsites (14), have been adopted to assist in the selection of endonucleases in the PCR-RFLP experimental design. However, these programs perform restriction analysis only for individual sequences. Although the SNP Cutter software (51) can analyze the data from multiple sequences, the subsequent comparison of banding profiles of different sequences after digestion is done manually. Therefore, employing these programs is time-consuming, tedious, and often error prone, especially when a large number of taxa with different sequences are involved. There is a need to develop a strategy to select the endonucleases rapidly through the restriction analysis of a large number of sequences.

The purpose of this work was to develop a method of rapid selection of restriction endonucleases for PCR-RFLP identification of fungal species with the aid of a computer program based on the analysis of a considerably large number of DNA sequences. Species of *Pleurotus* (Fr.) P. Kumm., a fungal genus, are used as examples owing to the reasonable number of species in the genus and the considerable molecular data on internal transcribed spacer (ITS) sequences.

Species of *Pleurotus*, commercially called oyster mushrooms,

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are a group of the most important edible fungi, accounting for 25% of the total world production of commercially cultivated mushrooms (17). In addition, *Pleurotus* species are also used as a tool for environmental control in the biotransformation-biodegradation of industrial effluents owing to their unique ligninolytic system (6). Further, some species of *Pleurotus* are known as predaceous fungi (39) and have the potential for controlling nematode diseases of agricultural crops. Traditionally, species-level identification of *Pleurotus* has been based mainly on morphology and compatibility relationships (3, 18, 49). However, many difficulties have been associated with the identification of species of this genus, especially those of commercially cultivated strains, due to their similar morphological characteristics and morphological plasticity in cultivation (19). The long period from the inoculation of spawn to the production of fruiting bodies of these fungi also delays any positive determination (33). Various molecular methods, such as DNA sequencing and RFLP and PCR-RFLP techniques, have been used to identify *Pleurotus* species. The application of analyses of sequences of ITS regions and large subunits (LSU) of nuclear ribosomal DNA (rDNA) to the determination of *Pleurotus* species (42, 43, 50) is very powerful at the species level, but these analyses are often used for particular research and have not been used for routine or rapid identification. RFLP and PCR-RFLP methods have also been applied to the study of molecular systematics and the genotyping of *Pleurotus* (2, 12). In the two latter studies (2, 12), however, the numbers of restriction enzymes screened (four and seven, respectively) and species identified (5 and 10, respectively) were limited because the selection of restriction endonucleases was based on results from extensive experiments. It is necessary to establish a rapid, accurate, and simple molecular method for the identification of *Pleurotus* species, based on the analysis of a large number of sequences and the selection of restriction enzymes from thousands of candidate endonucleases.

There are about 20 *Pleurotus* species recognized worldwide (15). Recent molecular phylogenetic analysis of *Pleurotus* provides a useful framework for understanding species concepts and taxonomy. Relationships between species and species determination have also been studied, and the results of systematic analyses of LSU and ITS sequences in nuclear rDNA (42, 43, 50) and of small-subunit sequences in mitochondrial rDNA (8) show that ITS sequences are an ideal marker for species identification. A large number of ITS sequences from this genus have been accumulated, and a molecular identification method using the PCR-RFLP technique to distinguish *Pleurotus* species can be developed through the DNA sequence analyses.

MATERIALS AND METHODS

Fungal material. A total of 17 living strains and 11 dried specimens were used for this study and are listed in Table 1. The strains were stored at 4°C on potato dextrose agar medium and subcultivated at 25°C in liquid potato dextrose medium for 10 days to collect the mycelia for extracting DNA. Strains and specimens of fungal material were designated according to the acquisition sources, as indicated in Table 1.

DNA isolation, PCR amplification, and sequencing. Genomic DNA was isolated from well-preserved herbarium specimens and fresh fungal cultures by using the modified cetyltrimethylammonium bromide method as described by Li and Yao (20). The ITS region of rDNA was amplified using the primers ITS4 and ITS5 (46). PCR amplification was carried out in a 25-μl reaction volume containing 12.5 μl of 2× reaction mix (200 M deoxynucleoside triphosphates, 4.0 mM $MgCl_2$, 2.5 U of *Taq* DNA polymerase), 50 pmol of each primer, and 2 μl of template DNA. The thermal cycling conditions consisted of an initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis in a 1.0% (wt/vol) agarose gel in 10× Tris-borate-EDTA buffer and were subsequently visualized by UV illumination after ethidium bromide staining.

PCR products were purified using cleanup plates (Millipore Corporation). Sequencing was performed by the cyclic reaction termination method on an ABI Prism 3100 genetic analyzer (Applera Corporation), and data were collected on a Dell computer with the ABI Prism DNA sequencing analysis software, version 3.7. Each fragment was sequenced in both directions for confirmation, and the sequences of the two strands were assembled with ABI Prism SeqScape software, version 1.1.

Sequence analysis. The retrieval tool from the National Centre of Biotechnology Information was used to search ITS sequences by using the phrase "*Pleurotus* internal transcribed spacer 1 internal transcribed spacer 2" as keywords. All available ITS sequences from GenBank were aligned using Clustal X 1.81 (38) and then further manually adjusted using BioEdit 5.0.6 (11) to reduce some obvious mismatching of sequences created by computer alignment. Sequences with regions of consecutive ambiguous bases or with potentially confounding ambiguous bases, i.e., unresolved bases or ambiguous bases in a position that may affect a restriction site, were excluded. Only sequences from material identified to the species level were used, and pairs of identical sequences were represented by one haplotype. A total of 259 ITS sequences were retrieved, and 85 haplotypes representing 151 sequences met all of the above criteria of selection for study. These sequences were used for phylogenetic analysis and for the selection of restriction enzymes (Table 2). Phylogenetic analyses were performed by PAUP 4.0b10 for Macintosh (36) by using ITS sequences from *Hohenbuehelia grisea* as the outgroup. A parsimony analysis was performed using a heuristic search, with the random addition of sequences with 1,000 replicates, tree bisection-reconnection as the branch-swapping algorithm, one tree held at each step during stepwise addition, and the MULTREES option off. Gaps were treated as missing data. Bootstrap values were calculated from 1,000 replicates.

Architectural structure of PRE Auto program. The PCR-RFLP endonuclease autoscreening (PRE Auto) program was written using Borland Delphi 6.0. A schematic diagram of the architectural framework of the program is shown in Fig. 1. The program consists of four main modular components, i.e., sequence grouping, restriction enzyme analysis, scoring algorithm, and electrophoresis map output. A restriction endonuclease database including listings for 2,992 enzymes was downloaded from REBASE, the restriction enzyme database of New England Biolabs, Inc. (http://rebase.neb.com). Endonucleases listed in the database can also be added to the PRE Auto program manually. In the sequence grouping module, the user can divide the sequences into different groups according to the results of phylogenetic analysis. The sequence data can be imported into the program in FASTA format. In the restriction enzyme analysis module, the PRE Auto program searches for recognition sites, calculates the restriction fragment sizes for different sequences, and transforms the data into a (0,1) matrix. In the scoring algorithm module, the program computes the Dice coefficient $[2a/(2a + b + c)]$ (27) through the (0,1) matrix for the sequences, and every restriction enzyme is scored through the calculation of the average coefficient for the groups and the average coefficient for the sequences within a group. In the electrophoresis map output module, a virtual electrophoresis map of fragments after digestion with different enzymes can be displayed.

Selection of restriction endonucleases and PCR-RFLP procedure. Restriction enzymes were selected using the PRE Auto program developed in this study. The conservative partial 18S sequences from the annealing position of the primer ITS5 and partial 28S sequences from the position of ITS4 were added to both ends of the ITS sequences, if they were incomplete at the ends when downloaded from GenBank, to ensure the accuracy of the prediction of fragment sizes after digestion with restriction endonucleases. Data for all the 85 ITS sequence haplotypes were input into the PRE Auto program as FASTA files.

The screening for a single enzyme was followed by a further search for a combination of two enzymes, because all 12 species could not be distinguished by any one enzyme. Candidate restriction enzymes were determined based on two parameters, the scoring and the degree of cleavage site coverage, after the autoscreening of restriction enzymes was implemented. The predicted band patterns after digestion by candidate enzymes having a coefficient above 0.1 and a cleavage site coverage of 100% for a single enzyme and 50% for a combination of two enzymes were examined through the electrophoresis map output from the PRE Auto program. The major selection criteria for enzymes were based on the minimum number of enzymes and the steps required to produce discriminating

TABLE 1. Fungal material used in this study

Species name	Sample[a]	Origin	GenBank accession no.[b] for ITS sequence	Reidentification of species[c]
P. abalonus Han, Chen, et Cheng	MG 005	China		
	CGMCC 5.409	China		
P. calyptratus (Lindblad) Saccardo	HMAS 63355	China	AY562495	
	HMAS 77117	China		
P. cornucopiae (Paulet) Rolland	ATCC 38547	Germany		
	HMAS 76520	China	**EF514242**	*P. ostreatus*
	MG 504	China		
P. cystidiosus Mill	ATCC 28597	United States	**EF514244**	
	CGMCC 5.467	China	AY540320	*P. abalonus*
	CGMCC 5.494	China	AY540321	*P. abalonus*
P. djamor (Rumphius ex Fries) Boedijn	CGMCC 5.600	China		
	CGMCC 5.407	China		
P. dryinus (Persoon) Kummer	F 14011	Finland		
	CBS 44977	Former Czechoslovakia	**EF514249**	
	HKAS 17450	China	**EF514243**	*P. pulmonarius*
P. eryngii (Lanzi) Saccardo	MG 497	China		
	HMAS 25978	China	**EF514246**	
P. nebrodensis (Inzenga) Quélet	MG 500	China		
	HMAS 86357	China	**EF514245**	
P. pulmonarius (Fries) Quélet	MG 502	China		
	HMAS 76672	China		
	HMAS 72869	China		
P. ostreatus (Jacquin: Fries) Kummer	HMAS 66080	China	**EF514248**	
	CGMCC 5.37	China	**EF514247**	
	CGMCC 5.344	China	AY540325	
P. tuber-regium (Rumphius ex Fries) Singer	MG 506	China		
	HMAS 84647	China	**EF514250**	
P. smithii Guzmán	IE 74	Mexico	AY315779	

[a] The prefixes in the sample designations indicate the sources, as follows: ATCC, American Type Culture Collection, Rockville, MD; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CGMCC, China General Microbiological Culture Collection Center, Beijing, China; F, National Museum of Natural Science, Taiwan, China; HKAS, Herbarium, Kunming Institute of Botany, Academia Sinica, Kunming, China; HMAS, Mycological Herbarium, Institute of Microbiology, Academia Sinica, Beijing, China; IE, Instituto de Ecologia, Xalapa, Mexico; and MG, Macrofungi Group, Chinese Academy of Science, China.

[b] GenBank accession numbers in bold represent sequences obtained in this study.

[c] For samples for which no reidentification is listed, the original determination was confirmed.

band patterns. In addition, enzymes producing too many small fragments (<50 bp) or showing intraspecific polymorphism were avoided. The availability and the cost of enzymes were also taken into account. Rare enzymes difficult to procure were also avoided, and common and low-cost enzymes were selected when multiple candidates with the same recognition site were available.

Digestion reactions were performed for each of the selected restriction enzymes, i.e., HaeIII, AluI, and HpyCH4IV (all from New England Biolabs, Beverly, MA), in a total volume of 20 μl containing 2 μl of 10× reaction buffer, 0.5 μl of each restriction enzyme (10 U/μl), and 17.5 μl (for a single enzyme) or 17 μl (for a combination of two enzymes) of the PCR products of each sample. Digestions were incubated overnight at the optimal temperature (37°C). Digested products were separated by 3% agarose gel electrophoresis in 10× Tris-borate-EDTA buffer for 3 h at 80 V. The gels were stained with 0.1 g of ethidium bromide/liter and visualized with UV light. Sizes of restriction fragments were determined by comparison with a standard DNA molecular mass marker, a 100-bp DNA ladder (Beijing Yuanchen Bio Company, Beijing, China).

Experimental validation of PCR-RFLP species identification. A total of 28 samples representing 12 *Pleurotus* species were selected for PCR-RFLP identification and validation of the method (Table 1). ITS sequences from five of these samples had been sequenced previously and submitted to GenBank (accession no. AY562495, AY540320, AY540321, AY540325, and AY315779). Of the five sequenced samples, two (CGMCC 5.467 and CGMCC 5.494, identified as *P. cystidiosus*) were proven to be misidentified, as revealed by their ITS sequences (AY540320 and AY540321, deposited in GenBank under the name *P. cystidiosus*), and were used as negative controls, while the other three (HMAS 63355 [*P. calyptratus*], CGMCC 5.344 [*P. ostreatus*], and IE 74 [*P. smithii*]) were used as positive controls. Additional samples were randomly selected from materials available in our laboratory to make two to three samples for each species. After the PCR products from the selected samples were confirmed by electrophoresis, the PCR-RFLP procedure described above was performed for the molecular identification of the samples through the comparison of the actual band patterns after digestion by the tested restriction endonucleases with the patterns predicted by the PRE Auto program. The species identification of the samples was further confirmed by sequencing the PCR products from ITS sequences from nine samples representing six species of *Pleurotus*, in addition to the five sequenced samples used as controls (Table 1).

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers EF514242 to EF514250.

RESULTS

Phylogenetic analysis of ITS sequences from *Pleurotus* species. The phylogenetic analysis of a total of 85 haplotypes representing 151 ITS sequences from *Pleurotus* taxa indicated 12 groups, as shown in Fig. 2. The determination of *Pleurotus* species for these groups and the application of species names were based on results of molecular phylogeny and mating compatibility analyses (13, 43, 50), although various names have been used for the sequences submitted to GenBank. In particular, there was considerable sequence variation in groups 5, 6, and 10 (Fig. 2; see also the supplemental material). Although the degree of molecular variation among the group 5 sequences was higher than that among sequences of other groups, *P. tuber-regium* is morphologically distinctive owing to its tuberous sclerotium, from which the basidiomata are pro-

TABLE 2. Representative sequences of ITS haplotypes used for the phylogenetic analyses and the autoscreening of restriction endonucleases for PCR-RFLP

Species	GenBank voucher	Origin	GenBank accession no.	Taxon name used in GenBank
P. abalonus Han, Chen, et Cheng	CBS 61580	India	AY315792	*P. cystidiosus* subsp. *abalonus*
	ASIK 3	Taiwan	AY315794	*P. cystidiosus* subsp. *abalonus*
	FCUP 661	Philippines	AY315801	*P. cystidiosus* subsp. *abalonus*
	VT 2476	Hawaii	AY315802	*P. cystidiosus* subsp. *abalonus*
	IFO 31074	Japan	AY315804	*P. cystidiosus* subsp. *abalonus*
	CBS 80391	China	AY315806	*P. cystidiosus* subsp. *abalonus*
	DSM 5340	Thailand	AY315808	*P. cystidiosus* subsp. *abalonus*
	ZA 472	Thailand	AY315809	*P. cystidiosus* subsp. *abalonus*
	CBS 61580	India	AY315810	*P. cystidiosus* subsp. *abalonus*
	Blao	Vietnam	DQ882571	*P. cystidiosus* subsp. *abalonus*
	S 396	Japan	DQ882573	*P. cystidiosus* subsp. *abalonus*
P. calyptratus (Lindblad) Saccardo	HMAS 63355	China	AY562495	*P. djamor* f. *calyptratus*
	CBS 325.85	Unknown	AY265814	*P. calyptratus*
	TENN 57451	Austria	AY450338	*P. calyptratus*
P. cornucopiae (Paulet) Rolland	ATCC 42045	Unknown	AB115037	*P. cornucopiae*
	wc 608	Unknown	AF079582	*P. cornucopiae*
	IFO 30528	Unknown	AY265816	*P. cornucopiae*
	CBS 383.80	Unknown	AY265817	*P. cornucopiae*
	ASI 2011	Unknown	AY265852	*P. citrinopileatus*
	S 033	China	AY540318	*P. citrinopileatus*
	HMAS 63344	China	AY696301	*P. citrinopileatus*
	PHZAU 1	Unknown	DQ077889	*P. citrinopileatus*
P. cystidiosus Mill	ATCC 28598	Unknown	AY265818	*P. cystidiosus*
	D 420	United States	AY315767	*P. cystidiosus*
	VT 1780	United States	AY315769	*P. cystidiosus*
	D 412	United States	AY315770	*P. cystidiosus*
	D 417	United States	AY315773	*P. cystidiosus*
	CBS 297.35	United States	AY315776	*P. cystidiosus*
	ATCC 28598	South Africa	AY315777	*P. cystidiosus*
P. djamor (Rumphius ex Fries) Boedijn	IFO 9573	Unknown	AB115053	*P. djamor*
	IFO 31859	Unknown	AY265843	*P. salmoneostramineus*
	ASI 2104	Unknown	AY265844	*P. salmoneostramineus*
	ASI 2172	Unknown	AY265845	*P. salmoneostramineus*
P. dryinus (Persoon) Kummer	7947	Denmark	AY450343	*P. dryinus*
P. eryngii (Lanzi) Saccardo	TFM-M-E 856	Unknown	AB115042	*P. eryngii*
	IFO 32798	Unknown	AY265825	*P. eryngii*
	WU 13414	Austria	AY450347	*P. eryngii*
	S 607	China	AY540333	*P. eryngii*
	J 2	Unknown	AY589046	*P. eryngii*
	tw 1	Unknown	AY589047	*P. eryngii*
P. nebrodensis (Inzenga) Quélet	S 498	China	AY540331	*P. nebrodensis*
	W	Unknown	AY581426	*P. nebrodensis*
	W 1	Unknown	AY581427	*P. nebrodensis*
	W 3	Unknown	AY581429	*P. nebrodensis*
	W 4	Unknown	AY581430	*P. nebrodensis*
	No. 4 Bailing	Unknown	AY720935	*P. nebrodensis*

Continued on following page

TABLE 2—*Continued*

Species	GenBank voucher	Origin	GenBank accession no.	Taxon name used in GenBank
P. ostreatus (Jacquin: Fries) Kummer	wc 534	Unknown	AF079583	*P. ostreatus*
	ATCC 38538	Unknown	AY265828	*P. ostreatus* f. *florida*
	ASI 2029	Unknown	AY368665	*P. ostreatus*
	TENN 53662	Austria	AY450345	*P. ostreatus*
	S 039	China	AY540322	*P. floridanus*
	S 474	China	AY540332	*P. ostreatus*
	OE-43	Unknown	AY636055	*P. ostreatus*
	TENN 53662	Unknown	AY854077	*P. ostreatus*
P. pulmonarius (Fries) Quélet	TFM-M-C960	Unknown	AB115046	*P. pulmonarius*
	IFO 31345	Unknown	AB115052	*P. pulmonarius*
	HMAS 76474	China	AY696298	*P. pulmonarius*
	HMAS 76672	China	AY696299	*P. pulmonarius*
	HMAS 86396	China	AY696300	*P. pulmonarius*
	NZFRI 3528	New Zealand	U60648	*P. pulmonarius*
P. smithii Guzmán	CBS 689.82	Unknown	AY265851	*P. smithii*
	IE 74	Mexico	AY315779	*P. smithii*
	ATCC 46391	Mexico	AY315781	*P. smithii*
	ATCC 46391	Mexico	AY315782	*P. smithii*
	ATCC 46391	Mexico	AY315783	*P. smithii*
	ATCC 46391	Mexico	AY315784	*P. smithii*
	ATCC 46391	Mexico	AY315786	*P. smithii*
P. tuber-regium (Rumphius ex Fries) Singer	TFM-M-D 779	New Caledonia	AB115045	*P. tuber-regium*
	RV 95/174.15	Australia	AF109964	*P. tuber-regium*
	RV 95/175.1	Australia	AF109965	*P. tuber-regium*
	RV 95/947.1	Papua New Guinea	AF109966	*P. tuber-regium*
	RV 95/949.2	Papua New Guinea	AF109970	*P. tuber-regium*
	RV 95/950.2	Papua New Guinea	AF109971	*P. tuber-regium*
	Pt 5	New Caledonia	AF109972	*P. tuber-regium*
	Pt 5.1	New Caledonia	AF109973	*P. tuber-regium*
	NedaS 467	Indonesia	AF109975	*P. tuber-regium*
	PTR 5	Ghana	AF109976	*P. tuber-regium*
	PTV 2	Ghana	AF109978	*P. tuber-regium*
	Pt 3	Nigeria	AF109982	*P. tuber-regium*
	Pt 1	Nigeria	AF109983	*P. tuber-regium*
	Pt 8	Nigeria	AF109986	*P. tuber-regium*
	Pt 9	Nigeria	AF109987	*P. tuber-regium*
	PtWat	Gameroon	AF109988	*P. tuber-regium*
	PTR 1	Ghana	AF109989	*P. tuber-regium*
	DSH-92-155	Papua New Guinea	AY450344	*P. tuber-regium*

duced. It is treated here as one species according to Isikhuemhen et al. (13), who were able to demonstrate that the molecular variations are associated with geographic isolation. A similar situation was applicable to group 10, which is treated here as *P. cornucopiae* (see also references 43 and 49). The paraphyletic status of group 6, *P. ostreatus*, in the phylogenetic analysis was also found by Vilgalys and Sun (43) with the support of mating test results. *P. ostreatus* was considered as a species for the selection of restriction endonucleases to test the tolerability and sensitivity of the PRE Auto program. In addition, *P. floridanus* in group 6 was considered to be synonymy for *P. ostreatus* (18), *P. salmoneostramineus* was treated as synonymous with *P. djamor* in group 12 (18), and *P. citrinopileatus* was considered to be a morphological variant of *P. cornucopiae* in group 10 (28). More data on the taxonomic and nomenclatural treatment of these species will be reported in a separate paper on the molecular phylogeny of *Pleurotus*.

Autoscreening and selection of restriction endonucleases for PCR-RFLP by the PRE Auto program. The ITS sequence haplotypes were input into the PRE Auto program and divided into 12 groups based on the phylogenetic analysis. The restriction endonucleases were autoscreened in the PRE Auto program. The ranges of coefficients and degrees of cleavage site coverage for all 2,992 endonucleases were 0.08 to 0.24 and 1.2 to 100%, respectively, in the autoscreening of single enzymes. The predicted band patterns of 569 restriction enzymes with a coefficient above 0.1 and a degree of cleavage site coverage up to 100%, sorted into 23 different groups based on recognition sites, were examined by eye. The enzyme group of HaeIII, with 179 enzymes in total, was found to have the highest resolution among all the 23 groups, distinguishing 6 of the 12 *Pleurotus* species and dividing the remaining 6 species into two groups. As a single enzyme could not discriminate among all the *Pleurotus* species, further screening of combinations of two enzymes was performed. There were 221 enzyme combinations with coefficients above 0.1 and a degree of cleavage site coverage of 50%. The combination of AluI and HpyCH4IV was found to

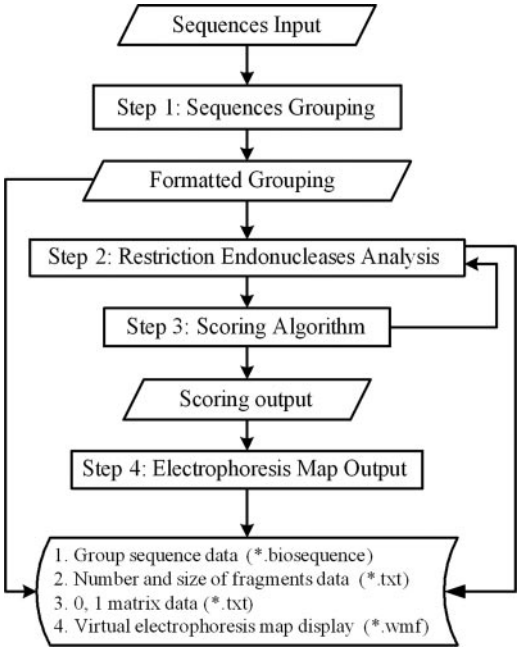

FIG. 1. The PRE Auto work flow includes steps 1 through 4, as shown in the figure. Rectangles show the tasks that the program performs. Parallelograms show the data that the user supplies or that every step produces. The program may produce four files, as shown: *.biosequence in step 1, *.txt in steps 2 and 3, and *.wmf in step 4, where * represents an optional string for writing the file name.

have the highest score, 0.33, distinguishing the remaining six species, which could not be identified by digestion with the single enzyme HaeIII. With the criteria for the selection of restriction endonucleases described above, a two-stage digestion using the single enzyme HaeIII followed by the combination of AluI and HpyCH4IV was finally designed to obtain the best species-specific band patterns with minimal testing.

The expected enzyme digestion band patterns for HaeIII are shown in Fig. 3, with representative sequences from the 12 species. Among the patterns, six were species-specific profiles, representing *P. calyptratus* (lanes 1 to 3), *P. smithii* (lanes 4 to 6), *P. cystidiosus* (lanes 7 to 9), *P. abalonus* (lanes 10 to 12), *P. dryinus* (lane 13), and *P. cornucopiae* (lanes 14 to 16). There were some differences in band patterns of *P. pulmonarius* (lanes 17 to 19), *P. tuber-regium* (lanes 20 to 22), and *P. djamor* (lanes 23 to 25), but the sizes of the fragments were very similar. The band patterns of the remaining three species, *P. eryngii* (lanes 26 to 28), *P. nebrodensis* (lanes 29 to 1), and *P. ostreatus* (lanes 32 to 34), were identical.

The further digestion with the combination of two endonucleases, AluI and HpyCH4IV, differentiated the three species with the similar HaeIII bands, i.e., *P. pulmonarius* (Fig. 4, lanes 1 to 3), *P. tuber-regium* (lanes 4 to 6), and *P. djamor* (lanes 7 to 9), and the other three species with the identical HaeIII band profiles, i.e., *P. eryngii* (lanes 10 to 12), *P. nebrodensis* (lanes 13 to 15), and *P. ostreatus* (lanes 16 to 18). Through the virtual sequence analysis generated by the PRE Auto program, the 12 *Pleurotus* species could be distinguished by the two-stage digestion of ITS fragments. The procedures for the detection and identification of *Pleurotus* species are summarized in Fig. 5.

PCR-RFLP identification of *Pleurotus* species and validation by ITS sequencing. PCR amplification using the primers ITS4 and ITS5 resulted in products of approximately 720 bp (Fig. 6) from 28 tested *Pleurotus* samples. According to the flow chart for the identification of *Pleurotus* species (Fig. 5), PCR products of the tested samples were first digested with HaeIII. The RFLP band patterns on an agarose gel are shown in Fig. 7. The observed band patterns of 24 samples were in accordance with those predicted from ITS sequences by the PRE Auto program (Fig. 3.). These samples included *P. calyptratus* (Fig. 7, lanes 1 and 2), *P. smithii* (lane 3), *P. cystidiosus* (lane 4), *P. abalonus* (lanes 7 and 8), *P. dryinus* (lanes 9 and 10), *P. cornucopiae* (lanes 12 and 13), *P. pulmonarius* (lanes 15 to 17), *P. tuber-regium* (lanes 18 and 19), *P. djamor* (lanes 20 and 21), *P. eryngii* (Fig. 7, lanes 22 and 23), *P. nebrodensis* (lanes 24 and 25), and *P. ostreatus* (lanes 26 and 28). However, two of the three *P. cystidiosus* samples (lanes 5 and 6), used as negative controls, produced the species-specific band pattern of *P. abalonus* (lanes 7 and 8), in agreement with the pattern predicted for the sequences (AY540320 and AY540321) by the PRE Auto program. The species in these two samples were renamed, as noted in Table 1. Additionally, one of the three samples of *P. dryinus* (lane 11) produced a band pattern similar to that of *P. pulmonarius* (lanes 15 to 17), and one of the three *P. cornucopiae* samples (lane 15) produced the same pattern as *P. eryngii*, *P. nebrodensis*, and *P. ostreatus* (lanes 22 to 28). Using HaeIII, six species, *P. calyptratus*, *P. smithi*, *P. cystidiosus*, *P. abalonus*, *P. dryinus*, and *P. cornucopiae*, were successfully identified based on the species-specific PCR-RFLP band profiles predicted by the PRE Auto program (Fig. 3), and four samples misidentified as *P. cystidiosus*, *P. dryinus*, and *P. cornucopiae* were also detected (Fig. 7, lanes 5 and 6, 11, and 14, respectively).

Because samples in lanes 15 to 21 of Fig. 7 (*P. pulmonarius*, *P. tuber-regium*, and *P. djamor*) produced similar patterns, as predicted by the PRE Auto program (Fig. 3), these samples were further treated with the combination of AluI and HpyCH4IV, as were the samples in lanes 22 to 28 of Fig. 7 (*P. eryngii*, *P. nebrodensis*, and *P. ostreatus*), which displayed the same band profiles, also as predicted in Fig. 3, to obtain better resolution for species identification. In addition, two of the four misidentified samples (Fig. 7, lanes 11 and 14), which could not be identified at the species level after the digestion by HaeIII, were also further digested with the two enzymes. Figure 8 shows the resulting band patterns after digestion by the combination of the two selected enzymes. Samples with similar HaeIII band patterns in lanes 11 and 15 to 21 of Fig. 7 were clearly distinguished from one another in three groups in Fig. 8, representing *P. pulmonarius* (lanes 1 to 4), *P. tuber-regium* (lanes 5 and 6), and *P. djamor* (lanes 7 and 8). Samples with the same HaeIII band patterns in lanes 14 and 22 to 28 of Fig. 7 were also separated without any ambiguity into *P. eryngii* (Fig. 8, lanes 9 and 10), *P. nebrodensis* (Fig. 8, lanes 11 and 12), and *P. ostreatus* (Fig. 8, lanes 13 to 16), as predicted from the sequence information (Fig. 4).

To confirm PCR-RFLP species identification, the PCR products of ITS regions from nine samples, including two misidentified samples of *P. pulmonarius* (previously misidentified as

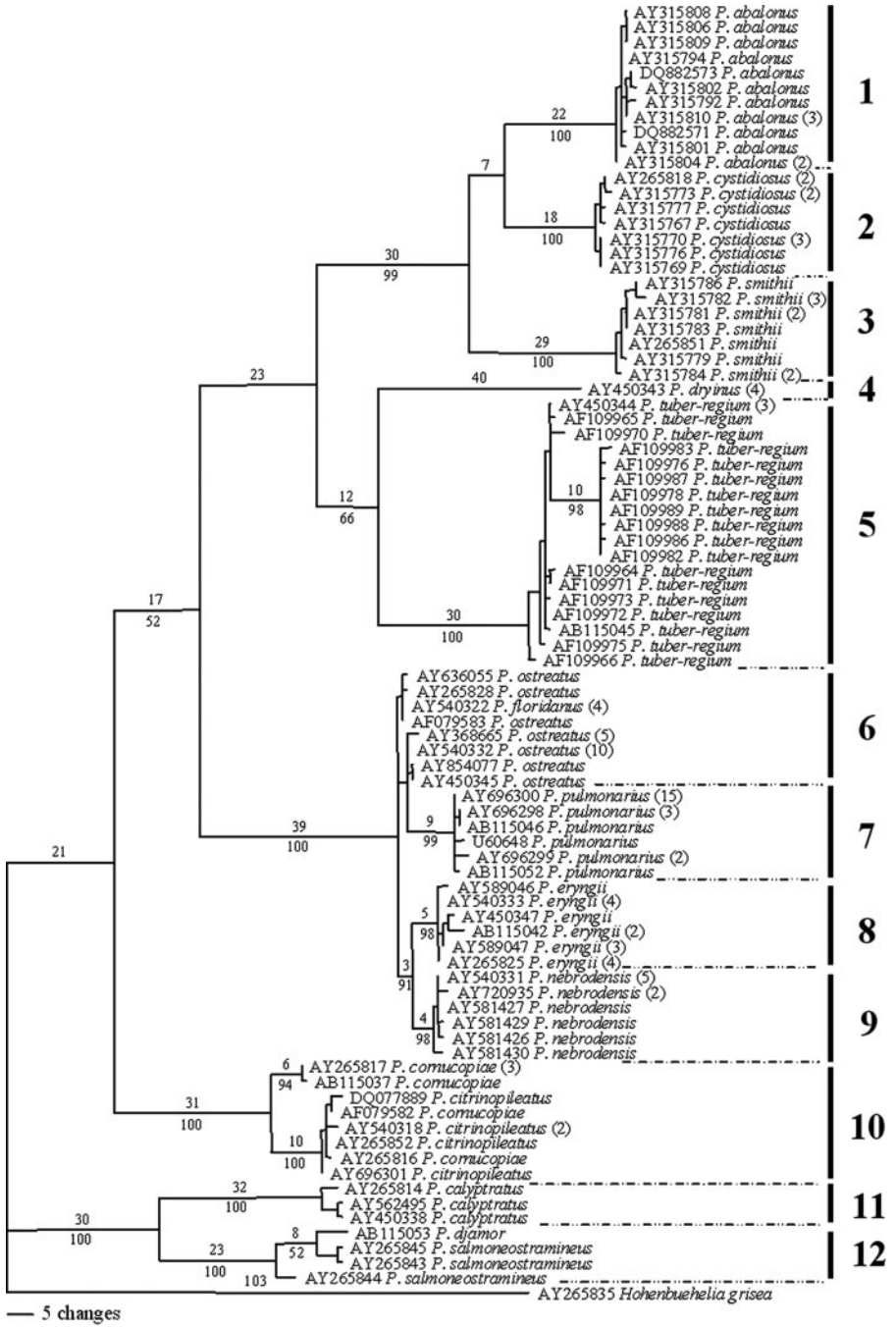

FIG. 2. One of the 1,288 most parsimonious trees obtained from the analysis of nucleotide sequences of ITS regions (nuclear rDNA). The upper and lower numbers on each branch denote the number of estimated substitutions and the percentage of bootstrap replicates, respectively. Only bootstrap values higher than 50% are shown. Numbers in the brackets after species names are the numbers of sequences that the haplotypes represented. The length of the tree is 701 steps, with a consistency index of 0.6904 and a retention index of 0.9469. Grouping is as follows: group 1, *P. abalonus*; group 2, *P. cystidiosus*; group 3, *P. smithii*; group 4, *P. dryinus*; group 5, *P. tuber-regium*; group 6, *P. ostreatus*; group 7, *P. pulmonarius*; group 8, *P. eryngii*; group 9, *P. nebrodensis*; group 10, *P. cornucopiae*; group 11, *P. calyptratus*; and group 12, *P. djamor*.

P. dryinus, in lane 11 of Fig. 7 and lane 1 of Fig. 8) and *P. ostreatus* (previously misidentified as *P. cornucopiae*, in lane 14 of Fig. 7 and lane 16 of Fig. 8), were subjected to DNA sequencing. The GenBank accession numbers for ITS regions from the sequenced samples are listed in Table 1. The results of sequencing showed the same conclusion obtained by the PCR-RFLP analysis described here. The redetermination of fungal names for the four previously misidentified samples, based on the results of both PCR-RFLP analysis and ITS sequencing, is indicated in Table 1.

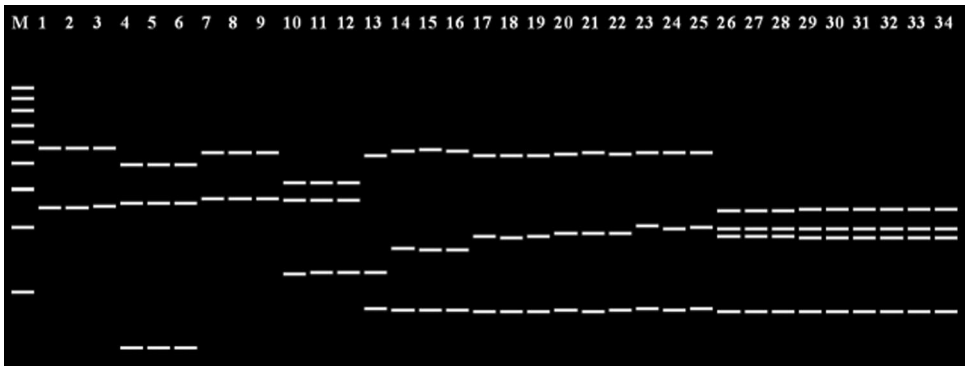

FIG. 3. Predicted band patterns produced by the PRE Auto program after digestion with HaeIII. Lane 1, *P. calyptratus* AY562495; lane 2, *P. calyptratus* AY265814; lane 3, *P. calyptratus* AY450338; lane 4, *P. smithii* AY315779; lane 5, *P. smithii* AY315781; lane 6, *P. smithii* AY315786; lane 7, *P. cystidiosus* AY315767; lane 8, *P. cystidiosus* AY315770; lane 9, *P. cystidiosus* AY315773; lane 10, *P. abalonus* AY315794; lane 11, *P. abalonus* AY315806; lane 12, *P. abalonus* AY315808; lane 13, *P. dryinus* AY450343 (one haplotype with four sequences); lane 14, *P. cornucopiae* AB115037; lane 15, *P. cornucopiae* AY265817; lane 16, *P. citrinopileatus* DQ077889; lane 17, *P. pulmonarius* AB115046; lane 18, *P. pulmonarius* AY696300; lane 19, *P. pulmonarius* U60648; lane 20, *P. tuber-regium* AB115045; lane 21, *P. tuber-regium* AF109983; lane 22, *P. tuber-regium* AY450344; lane 23, *P. djamor* AB115053; lane 24, *P. salmoneostramineus* AY265843; lane 25, *P. salmoneostramineus* AY265845; lane 26, *P. eryngii* AB115042; lane 27, *P. eryngii* AY450347; lane 28, *P. eryngii* AY540333; lane 29, *P. nebrodensis* AY540331; lane 30, *P. nebrodensis* AY581427; lane 31, *P. nebrodensis* AY720935; lane 32, *P. ostreatus* AY368665; lane 33, *P. ostreatus* AY540332; and lane 34, *P. ostreatus* AY636055. Lane M, 100 bp DNA ladder.

DISCUSSION

The method developed in this study for the autoscreening of restriction endonucleases for the PCR-RFLP technique using sequence analyses and especially the PRE Auto software is considerably efficient and accurate within a short period of time. It took less than 1 min for the PRE Auto program to perform the calculations for and select candidate endonucleases from a total of 2,992 endonucleases for 85 haplotype sequences. The display of the predicted band patterns from digestion with an enzyme made the selection of a particular enzyme and the estimate of results more intuitive. In this study, every band of DNA fragments predicted by the PRE Auto program after digestion by the selected enzymes was confirmed by actual sample experiments (compare Fig. 3 with Fig. 7 and Fig. 4 with Fig. 8). The unequal densities of DNA in the bands in Fig. 7 and 8 reflect the different sizes of the fragments after digestion. The smaller the fragment, the fainter the band appears under UV illumination after ethidium bromide staining.

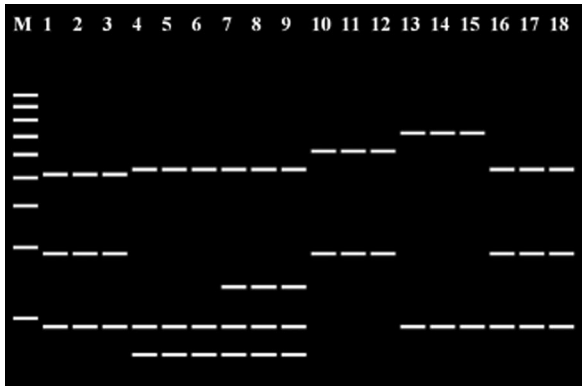

FIG. 4. Predicted band pattern after digestion with AluI and HpyCH4IV produced by the PRE Auto program. Lane 1, *P. pulmonarius* AB115046; lane 2, *P. pulmonarius* AY696300; lane 3, *P. pulmonarius* U60648; lane 4, *P. tuber-regium* AB115045; lane 5, *P. tuber-regium* AF109983; lane 6, *P. tuber-regium* AY450344; lane 7, *P. djamor* AB115053; lane 8, *P. salmoneostramineus* AY265843; lane 9, *P. salmoneostramineus* AY265845; lane 10, *P. eryngii* AB115042; lane 11, *P. eryngii* AY450347; lane 12, *P. eryngii* AY540333; lane 13, *P. nebrodensis* AY540331; lane 14, *P. nebrodensis* AY581427; lane 15, *P. nebrodensis* AY720935; lane 16, *P. ostreatus* AY368665; lane 17, *P. ostreatus* AY540332; and lane 18, *P. ostreatus* AY636055. Lane M, 100-bp DNA ladder.

Although the existing design tools for the PCR-RFLP assay, e.g., DNA CLUB (47), NEBcutter version 2.0 (44), Genetyx version 6.1 (26), and SNP Cutter (51), have been used to search for cleavage sites and to calculate the sizes of restriction fragments, the selection of restriction endonucleases by manual comparison of restriction fragment profiles of different sequences with various restriction enzymes is still very time-consuming. For example, using the DNA CLUB and Origin 5.0 computer packages, Wright and Pimm (47) had to manually compare the restriction fragment patterns of 29 sequences with 55 different restriction enzymes and finally selected 10 endonucleases to digest the 16S gene for the molecular identification of methanogens. The PRE Auto software reported here is a much more efficient and informative program than the other computer packages for PCR-RFLP experiment design, especially for the functions of multiple-sequence input, autoscreening of endonucleases, and production of an electrophoresis gel map. After the autocomparison and scoring of restriction fragment patterns of each restriction enzyme for all the input ITS sequences, the selection of restriction endonucleases was very efficient with the guidance of the electrophoresis map. Further, the treatment of grouped sequences is a novel function that makes it possible to consider the sequence variation within a species for the autoscreening of restriction enzymes. Although the haplotype sequences from the *Pleurotus* species used were considerably variable, e.g., groups 5 and 10 (Fig. 2), or very conservative, e.g., groups 8 and 9 (Fig. 2), within a group, the PRE Auto program could still find the restriction endonucleases to differentiate the groups, which represent different species. This result will avoid the possible restriction sites within

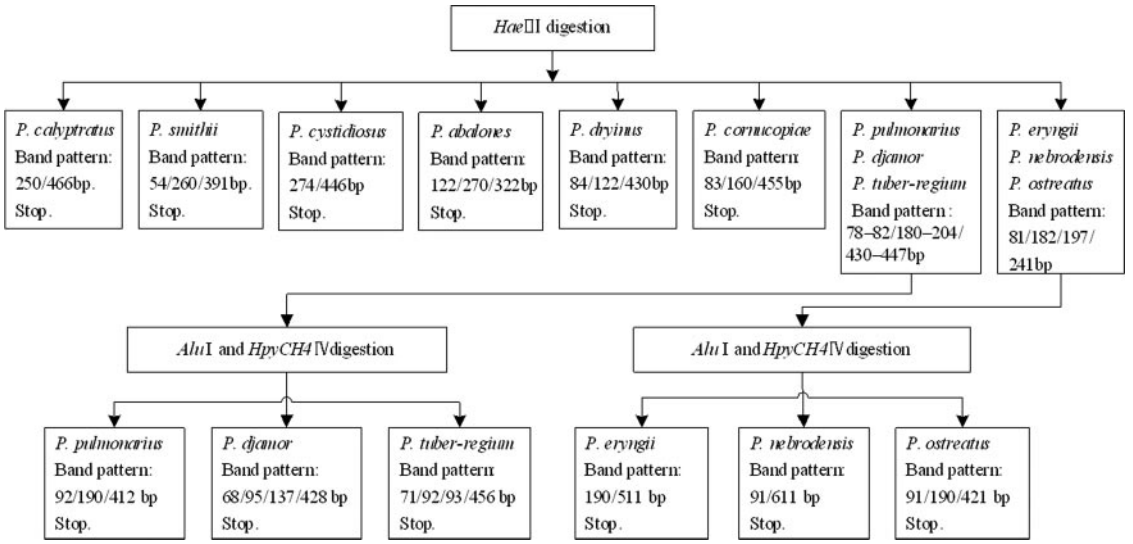

FIG. 5. Optimized flow chart for identification of *Pleurotus* species using three restriction endonucleases.

an individual sequence but without the capability to separate species. The new method developed in this study for the rapid selection of restriction enzymes can be applied widely in the molecular identification of living organisms using the PCR-RFLP technique when a large number of target sequences are considered. However, it is worth notice that group 6, representing *P. ostreatus*, is a paraphyletic group in the ITS sequence analysis (Fig. 2), closely related to group 7, *P. pulmonarius*. Because groups 6 and 7 were input into PRE Auto as separate groups, they were successfully distinguished from each other by the restriction digestion by HaeIII. As a computer program, the PRE Auto program is able to search for the most effective enzymes to separate the sequence groups input by the researcher, but it cannot make the taxonomic decision on the grouping of the sequences.

In addition, all other available computer packages, except the SNP Cutter, take only an individual sequence representative of a species to select restriction endonucleases. It is an advantage of both the PRE Auto program and the SNP Cutter to be able to analyze multiple sequences from a species for searching the cleavage sites of restriction enzymes. However, SNP Cutter is unable to do the autoscreening of restriction endonucleases and to output the predicted electrophoresis gel map after digestion.

The newly developed technique for the rapid selection of restriction enzymes has been validated by experiments using 17 living strains and 11 dried specimens, representing 12 *Pleurotus* species. This method could unequivocally identify all the 28 *Pleurotus* samples, including 24 in conformity with the previous species determination based on the morphological method and four in discordance with the original identification. Among the 28 tested samples, the five samples used as controls (three as positive and two as negative controls) based on ITS sequences previously submitted to GenBank were verified by the PCR-RFLP method. ITS sequences from two misidentified samples detected here (HMAS 76520 and HKAS 17450) (Table 1) and from seven correctly named samples (ATCC 28597, CBS 44977, HMAS 25978, HMAS 86357, HMAS 66080, CGMCC 5.37, and HMAS 84647) also confirmed the determination of

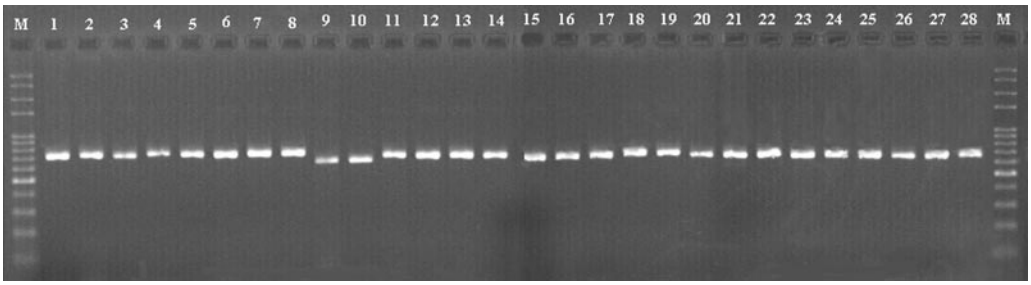

FIG. 6. PCR amplification products from *Pleurotus* species. Lane 1, *Pleurotus calyptratus* HMAS 63355; lane 2, *P. calyptratus* HMAS 77117; lane 3, *P. smithii* IE 74; lane 4, *P. cystidiosus* ATCC 28597; lane 5, *P. cystidiosus* CGMCC 5.467; lane 6, *P. cystidiosus* CBS 80391; lane 7, *P. abalonus* MG 005; lane 8, *P. abalonus* CGMCC 5.409; lane 9, *P. dryinus* F 14011; lane 10, *P. dryinus* CBS 44977; lane 11, *P. dryinus* HKAS 17450; lane 12, *P. cornucopiae* ATCC 38547; lane 13, *P. cornucopiae* MG 504; lane 14, *P. cornucopiae* HMAS 76520; lane 15, *P. pulmonarius* MG 502; lane 16, *P. pulmonarius* HMAS 76672; lane 17, *P. pulmonarius* HMAS 72869; lane 18, *P. tuber-regium* MG 506; lane 19, *P. tuber-regium* HMAS 84647; lane 20, *P. djamor* CGMCC 5.600; lane 21, *P. djamor* CGMCC 5.407; lane 22, *P. eryngii* MG 497; lane 23, *P. eryngii* HMAS 25978; lane 24, *P. nebrodensis* MG 500; lane 25, *P. nebrodensis* HMAS 86357; lane 26, *P. ostreatus* CGMCC 5.344; lane 27, *P. ostreatus* CGMCC 5.37; and lane 28, *P. ostreatus* HMAS 66080. Lane M, 100-bp DNA ladder.

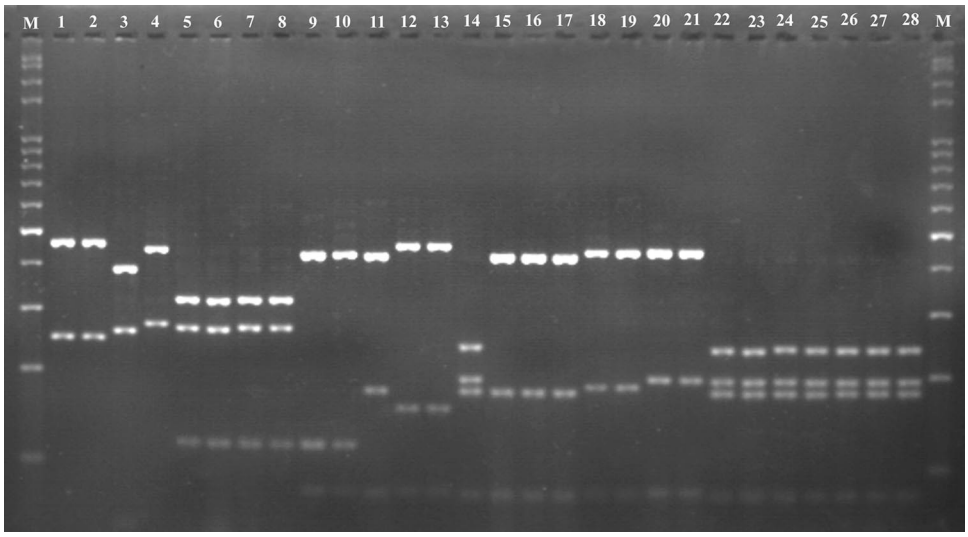

FIG. 7. PCR-RFLP band patterns observed with HaeIII. Lane 1, *Pleurotus calyptratus* HMAS 63355; lane 2, *P. calyptratus* HMAS 77117; lane 3, *P. smithii* IE 74; lane 4, *P. cystidiosus* ATCC 28597; lane 5, *P. cystidiosus* CGMCC 5.467; lane 6, *P. cystidiosus* CBS 80391; lane 7, *P. abalonus* MG 005; lane 8, *P. abalonus* CGMCC 5.409; lane 9, *P. dryinus* F 14011; lane 10, *P. dryinus* CBS 44977; lane 11, *P. dryinus* HKAS 17450; lane 12, *P. cornucopiae* ATCC 38547; lane 13, *P. cornucopiae* MG 504; lane 14, *P. cornucopiae* HMAS 76520; lane 15, *P. pulmonarius* MG 502; lane 16, *P. pulmonarius* HMAS 76672; lane 17, *P. pulmonarius* HMAS 72869; lane 18, *P. tuber-regium* MG 506; lane 19, *P. tuber-regium* HMAS 84647; lane 20, *P. djamor* CGMCC 5.600; lane 21, *P. djamor* CGMCC 5.407; lane 22, *P. eryngii* MG 497; lane 23, *P. eryngii* HMAS 25978; lane 24, *P. nebrodensis* MG 500; lane 25, *P. nebrodensis* HMAS 86357; lane 26, *P. ostreatus* CGMCC 5.344; lane 27, *P. ostreatus* CGMCC 5.37; and lane 28, *P. ostreatus* HMAS 66080. Lane M, 100-bp DNA ladder.

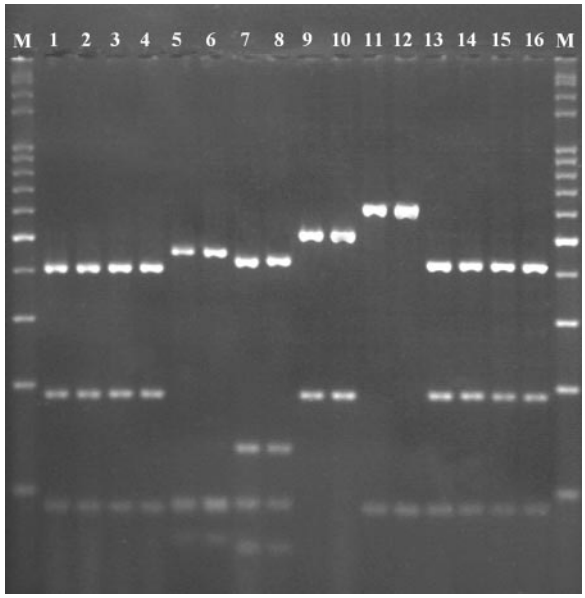

FIG. 8. PCR-RFLP band patterns observed after digestion with the combination of AluI and HpyCH4IV. Lane 1, *P. dryinus* HKAS 17450; lane 2, *P. pulmonarius* MG 502; lane 3, *P. pulmonarius* HMAS 76672; lane 4, *P. pulmonarius* HMAS 72869; lane 5, *P. tuber-regium* MG 506; lane 6, *P. tuber-regium* HMAS 84647; lane 7, *P. djamor* CGMCC 5.600; lane 8, *P. djamor* CGMCC 5.407; lane 9, *P. eryngii* MG 497; lane 10, *P. eryngii* HMAS 25978; lane 11, *P. nebrodensis* MG 500; lane 12, *P. nebrodensis* HMAS 86357; lane 13, *P. ostreatus* CGMCC 5.344; lane 14, *P. ostreatus* CGMCC 5.37; lane 15, *P. ostreatus* HMAS 66080; lane 16, *P. cornucopiae* HMAS 76520; and lane M, 100-bp DNA ladder.

species using the PCR-RFLP method. The results indicate that the method developed in this study is accurate and reliable.

Many efforts have been devoted to the identification of *Pleurotus* species, including the application of molecular methods. In the work on RFLP and PCR-RFLP by Bao et al. (2) and Iracabal et al. (12), extensive experiments were conducted to screen the restriction enzymes from a very limited set of enzyme candidates based on a few DNA sequences. The autoscreening method reported here, supported by the PRE Auto program and based on the analysis of a large number of sequences, can select restriction enzymes from thousands of candidate endonucleases, and the 12 *Pleurotus* species were unambiguously identified with only two steps and three restriction endonucleases. In comparison with previous studies identifying *Pleurotus* species by the PCR-RFLP technique, this study used a minimal number of restriction endonucleases and required minor costs and a minor amount of time for the selection of enzymes. Further, there is much scope for increase in the number of species for identification and the number of endonucleases for screening in the PRE Auto program.

In conclusion, a new method for the rapid selection of restriction endonucleases with the aid of the PRE Auto computer program was established for the molecular identification of living organisms using the PCR-RFLP technique. The method is based on the sequence analysis of target DNA fragments of species for screening of restriction enzymes from a large number of candidate endonucleases, and the autoscreening is performed by the PRE Auto program. This is a genotypic identification approach that can be applied for the discrimination of a large number of species, confirmed with at least 12 *Pleurotus* species in this study, and is flexible to take more species and more endonucleases into its database. The computer autoscreening of restriction enzymes for PCR-RFLP

identification of species is a very efficient and time- and cost-saving method for the characterization of living organisms at the species level.

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