USE OF MOLECULAR METHODS FOR IDENTIFYING CULTURE OF SOIL FUNGI FROM TROPICAL FORESTS OF VIETNAM

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1. INTRODUCTION

Soil microscopic fungi are always present in all biological communities and they are also one of the key elements in detrital food web, providing a circulation of nutrients. They grow on all possible substrates and play important role in the decay of organic matter. They are actively involved in the processes of soil formation and the formation of plant communities and also have an influence on the abundance and species composition of other groups of organisms in the soil [1]. The fungi provide rapid mineralization of organic residues, immobilization of macro- and micronutrients and plant nutrition, so it explains their great role in tropical forests [2].

Tropical regions are characterized by an exceptional diversity of biotic organisms, including fungi [3, 4]. Their species composition in the soil and on plant residues is a relatively little-studied component of ecosystems [5]. In this case, the main attention is usually focused on particular groups, which have practical importance. There are pathogens for plants or animals and humans [6] and producers of biologically active substances [7].

Estimates of species diversity of fungi vary widely, but experts are consentient that it has been insufficiently studied. There are described more than 1,200 species of fungi annually, a lot of them are associated with the soil [8]. Poorly studied areas of tropical forests have a huge potential of undescribed species inhabiting different substrates. There are a lot of features that make difficulties in studies of species diversity of microscopic fungi in the soil, like the impossibility of their observation directly in the nature conditions, complexity and difficulty of isolation of different groups of micromycetes on nutrient media, data comparability, obtained by different methods, and problems associated with species identification.

Identification is possible only by morphological characteristics associated with sporification, however, not all species of the microscopic fungi can form it in the culture and accordingly they can not be determined by the standard methods.

Also, there are many cryptic species or sibling species among microscopic fungi, which are morphologically similar, but have differences at the genetic level [8, 11].

Use of molecular genetic techniques helps in solving such problems. These techniques provide additional information in some cases and allow to identify or specify the species of fungi. However, this problem can not be solved completely by this method because of the incompleteness of the information in the database. Database of GenBank contains information of no more than about 20% of the presently described fungal species [8].

The objective of this research is a complete study of the species diversity of soil fungi of tropical forests in Vietnam with using morphological and molecular genetic methods for identification of the obtained cultures.

2. RESEARCH METHODS

Systematic research of the soil mycobiota of rainforest was organized in 2009 and based on the resources of Joint Russian-Vietnamese Tropical Research and Technology Center [10, 11, 12]. The research material (upper horizon soil samples and plant litter) is collected between 2009 and 2014 in the monsoon rainforests of the six especially protected areas in Vietnam: in national parks Cat Tien, Bi Doup - Nui Ba, Chu Yang Sin, Bu Gia Map, in national reserves Dong Nai and Loc Bac forestry. The samples of the upper soil horizon and leaf litter were taken by the standard method [13] in sterile packages and were quickly dried.

Laboratory work was carried out at the Department of mycology and algology of Biological faculty of Moscow State University. Isolation of micromycetes was performed by inoculation of serial dilutions of Z. Waxman on the solid nutrient media in the modification of D.G. Zvyagintsev [15]. There were two culture media for use in the research, that allow to reveal a wide range of micromycetes and provide easily differentiate the morphological types of colonies in samples: Czapek agar with 0.3% of sucrose and malt extract agar. Pure cultures of fungi were inoculated for identification on recommended for specific groups culture media [14].

Identification with using morphological features was performed according to generally accepted determinants and articles containing the research of individual genera and species description [17]. The names of species and systematic position were given in databases: The MycoBank Fungal databases (http://www.mycobank.org) and CABI Bioscience Databases (http://www.indexfungorum.org).

The molecular-genetic method was used to identify sterile cultures and cultures that can not form sporification and clarify the definition of species which morphological criteria required verification.

The sequences of the ribosomal gene cluster ITS1-5.8S-ITS2, including both variable regions of intergenic spacer sequences ITS 1 and ITS 2 and the conserved region of the ribosomal subunits 5.8S were determined by this method. For this sector in the GenBank were collected more information than for the other ones and it is offered to use it in the barcoding program unit [8, 9]. These sectors are recommended to a sequence for primary molecular identification, and only after that to continue work with sectors that give more precise results for specific groups of fungi [14].

Pure cultures of microscopic fungi were grown in Petri dishes on malt extract agar for 7÷10 days. Inoculating was produced by three injections into the culture medium. Colonies were aseptically removed from the surface of the medium and were placed into microtubes. DNA was isolated from the mycelium using Wizard® kit SV Genomic DNA Purification System A2361 (Promega, USA) according to the manufacturer's protocol.

ITS-nuclear rDNA portions (ITS1 and ITS2) were amplified by PCR, which divide the two macromolecular rRNA genes - 18S (SSU) and 25-28S (LSU) - and include low molecular weight rRNA sequence 5.8S, using an appropriate pair of primers ITS- 1F [16] and ITS-4 [17]. Amplification program was set through many experiments and carried out in specific mode: denaturation at 95°C for 5 minutes, annealing of primers 35 cycles at 95°C - 15 seconds, 55°C - 20 seconds, 72°C - 30 seconds, the final elongation step - 7 min. PCR was performed by using a kit for the amplification of DNA polymerase Colored Taq (art. K0132) of Sileks Company. The reaction products were analyzed by electrophoresis using a 2% agarose gel with ethidium bromide. After 30 minutes of the electrophoresis, the authors assessed the presence of DNA under UV light. The total yield of DNA was quite high. Sequencing was performed with the same primers in both directions on an automated sequencer ABI Prism 3100 Genetic Analyzer ("Applied Biosystems HITACHI", USA) using a kit of reagents BigDyev.1.1. The sequences were processed and analyzed by Codon Code Aligner programs (www.codoncode.com). Alignment is done manually at Codon Code Aligner program and the Clustal W 1.6 [20]. Comparison and identification of nucleotide sequences was carried out at Nucleotide Blast Search (www.ncbi.nlm.nih.gov/BLAST/) data. This results of the sequence have been deposited in the GenBank database (NCBI) at numbers KP074967 ÷ KR075007, KR747689 ÷ KR747712.

3. RESULTS AND DISCUSSION

The collection of pure cultures of micromycetes that isolated from samples of the soil and the leaf litter of tropical forests of specially protected areas of Vietnam was created as a result of this work. The collection includes 1149 isolates, and 252 of them are not formed sporulation in culture. There were 349 species identified as a result of morphological research.

ITS parts of rDNA were sequenced for 184 strains, only 65 strains (35%) were found in the GenBank Data Base as a closest match with existed strains, 42 were identified up to species level and 23 were identified up to genus level. As a result of this work, at a list of a collection of the soil micromycetes of Vietnam were added 30 species with a total amount of 379 species from 117 genera. The rest of the strains with no sufficiently close conformity could be the part of non-information species in the GenBank database or, as a supposition, they are new for the science undescribed species. The research on them will be continued.

Among the strains with match at the GenBank database, the vast majority was belonged to division Ascomycota (Table 1.) 31% of them owns to order Xylariales, to order Eurotiales - 15%, in order Botryosphaeriales and Pleosporales - 12%, Hypocreales - 11%, Diaporthales - 3%, and a group of uncertain taxonomic position (Incertae sedis) - 5%. Division Basidiomycota was presented by strains of orders Agaricales - 3%, Cantharellales - 3%, Hymenochaetales - 2% and Incertae sedis - 3%.

Table 1. Taxonomic position of strains of soil microscopic fungi isolated from the soil of tropical forests in Vietnam, for which a close match was found in the GenBank database

Division	Class	Order	Amount of strains	The proportion of the total number of strains obtained, %
Ascomycota	Dothideomycetes	Botryosphaeriales	8	12
		Pleosporales	8	12
	Eurotiomycetes	Eurotiales	10	15
	Sordariomycetes	Diaporthales	2	3
		Hypocreales	7	11
		Xylariales	20	31
		Incertae sedis	3	5
Basidiomycota	Agaricomycetes	Agaricales	2	3
		Cantharellales	2	3
		Hymenochaetales	1	2
		Incertae sedis	2	3
Total			65	100

Most of soil micromycetes cultures of tropical forests of Vietnam, that not forming sporulation in culture, belong to the order Xylariales (20 strains classified to 12 species): *Annulohypoxylon* sp. - in the collection of strains F138 number (number in GenBank KP747712); *Hypoxylon* sp. - № FV-13193 (KP747705); *Neopestalotiopsis* sp. - FV-13275 (KP747700); *Pestalotiopsis clavispora* (G.F. Atk.) Steyaert - № 362 (KR074999), 381 (KR075005); *P. mangiferae* (Henn.) Steyaert - № 297 (KR074988), 128 (KR074973), 139 (KR074975); *P. vismiae* (. Petr) J. Xiang Zhang & T. Xu - № FV-13 300 (KR747694), FV-13299 (KP747698), FV-13 47 (KP747709); *Pestalotiopsis* sp. - № FV-13213 (KP747695); *Pestalotiopsis* sp.1 - № 358 (KP074997); *Pestalotiopsis* sp.2 - № 359 (KP074998); *Pestalotiopsis* sp.3 - № 335 (KP074992), 376 (KP075003); *Pestalotiopsis* sp.4 - № 378 (KR075004); *Xylaria* sp. - № FV-13 120 (KP747692), FV-13 335 (KP747691), FV-13 89 (KP747707).

The genus *Pestalotiopsis* provides the largest abundance - 8 species, 5 of them possibly are newly identified. Species of this genus are widely distributed mainly in regions with tropical and subtropical climates. This genus in recent years attracts the close attention of mycologists. On the one side, these species are endophytes and phytopathogens which cause rot and leaf spot many grassy and woody plants [21]. On the other side, the species of this genus are very reactive - they produce a wide variety of metabolites that may be involved in the decomposition of complex organic substances [20].

Types of genera *Annulohypoxylon*, *Hypoxylon* and *Xylaria* are from the wood-destroying group of fungi, many of which form rather large fruiting bodies on wood, some of them can also be pathogenic for trees. On nutrient media they often produce stroma, but usually, do not form mature fruiting bodies required for identification.

The next in the richness of species among sterile cultures are orders Botryosphaeriales and Pleosporales, all of the identified species are saprotrophs on plant residues and potential phytopathogens:

Botryosphaeriales: *Endomelanconiopsis endophytica* E.I. Rojas & Samuels - № 127 (KP074972); *Lasiodiplodia theobromae* (Pat.) Griffon et Maubl. - 161 (KP074976), 276 (KP074984), 285 (KP074985); *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous - 124 (KP074970), № 125 (KP074971), № FV-13 69 (KP747702); *Microdiplodia* sp. - FV-13 341 (KP747699).

Pleosporales: *Leptosphaeria spegazzinii* Sacc. & P. Syd. - № 372 (KP075001); *Leptosphaeria* sp.1 - № FV-13 39 (KP747704); *Leptosphaeria* sp.2 - № FV-13 147 (KP747710); *Lewia infectoria* (Fuckel) M.E. Barr & E.G. Simmons - № 28 (KP074967); *Paradendryphiella salina* (G.K. Sutherl.) Woudenberg & Crous - №336 (KP074993); *Phoma tropica* R. Schneid. etBoerema - № 298 (KP074989); *Pseudocochliobolus eragrostidis* Tsuda et Ueyama - № 293 (KP074986).

Sterile cultures from order Hypocreales: *Emericellopsis* sp. - № 259 (KP074981); *Gibberella baccata* (Wallr.) Sacc - № 226 (KP074977); *Nectria mauriticola* (Henn.) Seifert et Samuels - № 233 (KP074978). The last two of them are phytopathogens.

Representatives of the order Diaporthales are also not form sporulation in culture: *Diaporthe eucalyptorum* Crous & R.G. - № FV-13340 (KP747690); *D. neotheicola* A.J.L. Phillips & J.M. Santos Shivas - № Ba8 (KP747693) are phytopathogens.

Sterile cultures related to the division Basidiomycota belong to two species of wood-agaricoid fungi: *Gymnopilus* sp.1 - № 121 (KP074969), *Gymnopilus* sp.2 - №374 (KP075002); two types of sponk wood-destroying fungi are: *Oxyporus corticola* (Fr.) Ryvarden - № FV-13279 (KP747703), *Phellinus noxius* (Corner) G. Cunn. - № FV-13219 (KP747708). № FV-13 30 (KP747689), FV-13243 (KP747696) - strains *Thanatephorus cucumeris* (A.B. Frank) Donk also identified. This is a very interesting fungus, better known as the asexual stage (Rhizoctonia solani Kühn), it can be phytopathogen for culture plants, causing root rot, in natural biocenoses it often acts as mycorrhiza forming species with a variety of plants, including orchids [21].

Among the strains that require clarification species identification by molecular methods were 13 strains of the genera Penicillium and Trichoderma.

Six species belong to genus *Penicillium*: *Penicillium citreonigrum* Dierckx - № 268 (KP074982), 269 (KP074983); *P. commune* Thom - № 344 (KP074995); *P.melinii* Thom - № 334 (KP074991); *P. sacculum* E.Dale - № 339 (KP074994); *Penicillium* sp. - № 252 (KP074980), 347 (KP074996), FV-13214 (KP747701); *Penicillium* sp.1 - № 364 (KP075000). At the present time the description of many new species of this genus is conducted exceptionally on the basis of molecular characteristics [22], so it is not possible to accurately identify *Penicilllium* species without using of a molecular method.

Genus *Trichoderma* is represented by 3 species: *Trichoderma gamsii* Samuels & Druzhin - № Psp6 (KP075006), *Trichoderma koningiopsis* Samuels, C. Suárez & HC Evans - № Psp4 (KP075007), *Trichoderma reesei* EG Simmons - № MDL1 (KP074990). Fungi of this genus are very common in nature, it could be found in the soil, on plant residues and in the wood. They are used as producers of cellulolytic enzymes and antibiotic substances, as an agent for the biological control of pathogenic fungi of plants [23].

4. CONCLUSION

As a result of this work, the authors could succeed in species identifying by the molecular-genetic method for only one-third of the cultures in the research. The rest of the strains with no sufficiently close conformity could be the non-information species in the GenBank database, or they may be the new for science and undescribed species.

In reality the study on identification of fungi culture allows to perform more focused search for producers of biologically active metabolites. The molecular genetic method is usefull to identify or clarify the species attachment for microscopic fungi when it is difficult or even not possible by morphological features. However, its use is restricted by the shotage and incompleteness of data of the fungi in the GenBank database.

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REFERENCES

1. Christensen M., *A view of fungal ecology*, Mycologia, 1989, **81**(1):1-19.
2. Lodge D. J., *Nutrient cycling by fungi in wet tropical forests*, in Aspects of tropical mycology (Isaac S., Frankland J. C., Walting R. and Whalley A. J. S., eds), Cambridge, British Mycological Society, 1993, p.37-57.
3. Hawksworth D. L., *Why Study Tropical Fungi*? in Tropical Mycology. Vol. 2. Micromycetes (Watling R., Frankland J. C., Ainsworth A. M., Isaac S. and Robinson C. H., eds), New York: CABI Publishing, 2002, p.1-11.
4. Mueller G. M., Schmit J. P., *Fungal biodiversity: what do we know? What can we predict,* Biodiversity and Consevation, 2007, **16**:1-5.
5. Hyde K. D., Bussaban B., Paulus B., Crous P. W., Lee S., McKenzie E. C., Photita W. and Lumyong S., *Diversity of saprobic microfungi*, Biodiversity and Conservation, 2007, **16**:7-35.
6. Evans E. G. V. and Ashbee H. R., *Tropical mycoses: hazards to travellers* in Tropical Mycology. Vol. 2. Micromycetes (Watling R., Frankland J. C., Ainsworth A. M., Isaac S. and Robinson C. H., eds), New York: CABI Publishing, 2002, p.145-163.
7. Bills G. F., Dombrowski A., Peláez F., Polishook J. D. and Zhiqiang An., *Recent and Future Discoveries of Pharmacologically Active Metabolites from Tropical Fungi in Tropical Mycology. Vol. 2. Micromycetes* (Watling R., Frankland J. C., Ainsworth A. M., Isaac S. and Robinson C. H., eds), New York: CABI Publishing, 2002, p.165-194.
8. Hibbett D. S., Ohman A., Glotzer D., Nuhn M., Kirk P. and Nilsson R. H., *Progress in molecular and morphological taxon discovery in fungi and options for formal classification of environmental sequences*, Fungal Biology Reviews, 2011, **25**:38-47.
9. Seifert K. A. *Progress towards DNA barcoding of fungi*, Molecular Ecology Resources, 2009, **9**(1):83-89, http://dx.doi.org/10.1111/j.1755-0998.2009.02635.x.
10. Alexandrova A. V., Sidorova I. I., Tiunov A. V., *Microfungi of soil and litter of the National Park Cat Tien (South Vietnam)*, Mikologiya i fitopatologiya, 2011, **45**:12-25 (in Russian).
11. Kalashnikova K. A., Alexandrova A. V., *Soil inhabited microscopic fungi of the national park Bi Dup - Nui Ba (South Vietnam)*, Mikologiya i fitopatologiya, 2014, **48**:355-364 (in Russian).
12. Kalashnikova K. A., Alexandrova A. V., *Soil inhabiting microscopic fungi of the piedmont tropical forest (Loc Bac Forest Enterprise, South Vietnam)*, Mikologiya i fitopatologiya, 2015, **49**:91-101 (in Russian).

13. *Methods of soil microbiology and biochemistry* (Ed. Zvyagintsev D. G.), Moskva: Izdatelstvo MGU, 1991, 304 p. (in Russian).

14. *Fungal Biodiversity*, CBS Laboratory Manual Series 1 (Crous P.W. et al., eds), Utrecht, Netherlands: CBS, 2009, 270 p.

15. Domsch K. H., Gams W. and Anderson T. H., *Compendium of soil fungi*, Second edition, IHW Verlag Ehing, 2007, 672 p.

16. Gardes M. and Bruns T. D., *ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts*, Molecular Ecology, 1993, **2**(2):113-118.

17. White T. J., Bruns T., Lee S. and Taylor J., *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics,* in PCR Protocols: a guide to methods and applications (Innis M. A, Gelfand D. H, Sninsky J. J, White T. J, eds.), New York, USA: Academic Press, 1990, p.315-322.

18. Thompson J. D., Higgins D. G. and Gibson T. J., *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice*, Nucleic Acids Res, 1994, **22**:4673-4680.

19. Maharachchikumbura S. N., Guo L. D., Cai L., Chukeatirote E., Wu W., Sun X., Crous P., Bhat D. J., McKenzie E. C., Bahkali A. and Hyde K., *A multi-locus backbone tree for Pestalotiopsis, with a polyphasic characterization of 14 new species*, Fungal Diversity, 2012, **56**(1):95-129.

20. Xu J., Ebada S. S. and Proksch P., *Pestalotiopsis a highly creative genus: chemistry and bioactivity of secondary metabolites*, Fungal Divers, 2010, **44**(1)**:**15-31.

21. Srinivasachary, Willocquet L. and Savary S., *Resistance to rice sheath blight (Rhizoctonia solani Kühn) [(teleomorph: Thanatephorus cucumeris (A.B. Frank) Donk.] disease: current status and perspectives*, Euphytica, 2011, **178**(1):1-22.

22. Visagie C. M., Houbraken J., Frisvad J. C., Hong S. B., Klaassen C. H. W., Perrone G., Seifert K. A., Varga J., Yaguchi T. and Samson R. A., *Identification and nomenclature of the genus Penicillium*, Studies in Mycology, 2014, **78**:343-371.

23. Druzhinina I. S., Seidl-Seiboth V., Herrera-Estrella A., Horwitz B. A., Kenerley C. M., Monte E. and Kubicek C. P., *Trichoderma: the genomics of opportunistic success*, Nature Reviews Microbiology, 2011, **9**(10):749-759.

TÓM TẮT

SỬ DỤNG PHƯƠNG PHÁP SINH HỌC PHÂN TỬ TRONG ĐỊNH LOẠI CÁC CHỦNG NẤM ĐẤT CỦA RỪNG NHIỆT ĐỚI VIỆT NAM

Vi nấm đất luôn hiện diện trong tất cả các quần xã sinh vật và là một trong những thành phần cơ bản của lưới thức ăn mùn bã, góp phần vào chu trình dinh dưỡng. Chúng đóng vai trò quan trọng trong việc phân hủy các chất hữu cơ trong các quá trình hình thành đất và các quần xã thực vật. Hiện chưa có thông tin về thành phần loài vi nấm đất trong nhiều vùng khác nhau do có sự khó khăn trong nghiên cứu đa dạng loài của của chúng - không thể quan sát chúng trong tự nhiên, mà chỉ có thể phân lập bằng môi trường nhân tạo. Việc định loại vi nấm được tiến hành dựa trên các đặc điểm hình thái của chúng kết hợp với sự hình thành bào tử. Tuy nhiên, không phải tất cả các loài có thể hình thành bào tử trong môi trường nuôi cấy, vì thế không thể định loại hết được bằng các phương pháp truyền thống. Việc phát triển các kỹ thuật sinh học phân tử trong ngành nấm học có thể giúp giải quyết vấn đề này. Bài báo đề cập đến việc nghiên cứu nấm đất trong rừng nhiệt đới Việt Nam. Từ kết quả nghiên cứu, đã ghi nhận được 1.149 chủng, trong đó có 252 chủng không có khả năng hình thành bào tử (chủng vô sinh). Các trình tự của gen rDNA ITS 1 - ITS 2 đã được xác định bằng phương pháp phân tử đối với 184 chủng trong số 1.149 chủng thu thập được và chỉ có 65 chủng từ 184 chủng nêu trên được tìm thấy trong cơ sở dữ liệu của GenBank, trong đó 42 chủng được định loại tới loài, 23 chủng được định loại tới chi. Đã bổ sung 30 loài vi nấm cho khu hệ vi nấm đất Việt Nam, đưa tổng số loài hiện tại của khu hệ vi nấm đất Việt Nam lên 379 loài thuộc 117 chi.

Từ khóa: Vi nấm đất, rừng nhiệt đới, vùng đệm được sao mã, ITS, microscopic soil fungi, tropical forests, internal transcribed spacer.

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