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Speciation of cultivated temperate and tropical *Pleurotus* species – An *in silico* prediction using conserved sequences

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ABSTRACT

Conserved sequences are molecular fossil record of any organism and their evolution can be traced using these sequences. Genus *Pleurotus* is one of the most cultivated mushroom species for food purposes. To trace the evolution in *Pleurotus* species, two conserved sequences (ITS and LSU) of 23 species were analysed and phylogenetic relationship was studied to see evolution pattern linked with thier domestication. The sequencse were analysed using the Molecular Evolutionary Genetics Analysis (MEGA) and Bayesian phylogenetic analysis (Mr. Bayes). Analysis of LSU and ITS categorized the *Pleurotus* species into two groups. The differences in evolutionary rate among various species were ascertained. Grouping of sequences confirmed that cultivation requirements of *Pleurotus* species might be due to allopatric variation occurred during the evolution. Our results suggested that summer and winter species of *Pleurotus* might be evolved at different evolutionary rate.

Keywords: Bioinformatics, DNA sequence, domestication, evolution

Evolutionary biology is important to understand the origin, divergence and development of any organism in course of time. Evolutionary studies can answer classical questions regarding the development of characters, speciation and the genomic diversity present in the organism. The evolutionary history and distribution helps to reveal the adaptability of the organism to favourable and adverse climate. Fungi in general and mushrooms in particular are potential organisms that harbour enormous diversity with regard to metabolism, development, fruiting and life forms. Evolution of gilled mushroom was around lower cretaceous period approximately 130 million year ago (Chang, 1993). Globally, mushroom production has increased by more than 200 times in last five decades from 0.17 million ton in 1961 to 34.8 MT in 2013 (Royse *et al*. 2017). Genus *Pleurotus* {(Jacq. Fr.) Kumm. (Pleurotaceae)} is the 2nd most widely cultivated mushroom in India (Sharma *et al.,* 2017) and its cultivation is emerging as an important agribusiness activity (Upadhyay, 2011; Shirur *et al.,* 2017). The genus is one of the most diverse groups of cultivated mushrooms distributed through temperate, subtropical and tropical regions (Cohen *et al*. 2002). Growth and fructification of this mushroom across a wide variety of agro-industrial lignocellulosic wastes is due to their diverse lignolytic and hydrolytic enzymes system (Mikiashvili *et al*., 2006). *Pleurotus* mushroom also plays important role in carbon sequestration in environment by its lignin metabolism properties (Elhuyar, 2006). There are approximately 50 species recognized in genus *Pleurotus* (Guzman, 2000) while Kirk *et al*. (2008) reported only 20 species for this genus (Menolli *et al.,* 2010). Low level of species delineation in genus *Pleurotus* is because of large variation in morphology and inappropriate characteristics to infer the relationships of taxa within the genus (Gonzalez and Labare 're, 2000). The phenotypic plasticity and huge genetic variation leads to the confusing taxonomy of

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Pleurotus. Thus, change in morphology due to an allopatric mode of speciation is main cause of development of new species in this genera (Petersen and Hughes, 1999)..

In recent times, molecular phylogeny has emerged as powerful tool in complementing the identification of the species. In kingdom fungi, six universal gene regions are currently used for phylogenetic studies. ITS in eukaryotic rRNA coding gene consists of two transcribed spacer fragment between LSU and SSU including 5.8S. These ribosomal RNA genes (rDNA) of the fungus are mainly located on a single chromosome and are present as repeated subunits of a tandem array of transcribed and non-transcribed stretches of DNA, which appeared highly conserved. ITS are most commonly used in phylogenetic analysis of fungi for interspecific and intraspecific comparisons (Shnyreva and Shnyreva, 2015). LSU is another conserved sequence of 28S large subunit of rDNA. Although the discriminating power of LSU is comparatively lesser than the ITS but LSU in combination with ITS is used for high precision phylogenetic studies. The differentially evolved *Pleurotus* species are grown in the different parts of world. The primitive distribution and speciation might be linked with domesticated conditions. The conserved sequence analysis was utilized to understand the linkages of evolutionary pattern with occurrence of temperate and subtropical cultivated species in *Pleurotus*. Moreover, the study also aimed to seek the answer of classical questions of speciation and its causes in the different *Pleurotus* spp.

MATERIAL AND METHODS

Sequence retrieval from databases

ITS and LSU Sequences of different 23 *Pleurotus* spp. were accessed from the NCBI and BOLD databases (BOLD 2016). Accession numbers of ITS sequence and of respective *Pleurotus* spp. are listed in the Table 1.

Table 1. ITS and LSU sequences retrieved from NCBI and BOLD database

S. No	GenBank Accession for ITS	GenBank Accession For LSU	Species
1	JN671965.1	MG282532.1	*Pleurotus abalonus*
2	AF345656	KP867909.1	*Pleurotus abieticola*
3	AY450342	KY963066.1	*Pleurotus australis*
4	JQ837486.1	KY963071.1	*Pleurotus calyptratus*
5	AY540318	KY963081.1	*Pleurotus citrinopileatus*
6	AF079582.1	KY963088.1	*Pleurotus cornucopiae*
7	EF514244	KY963086.1	*Pleurotus cystidiosus*
8	GU722273	KY963091.1	*Pleurotus djamor*
9	JF908617.1	MG282498.1	*Pleurotus dryinus*
10	EU233979.1	KY963084.1	*Pleurotus eryngii*
11	JN234837	MG282551.1	*Pleurotus floridanus*
12	FJ545251	MG282545.1	*Pleurotus fossulatus*
13	GU722279	MG282535.1	*Pleurotus levis*
14	FJ904765	MG282550.1	*Pleurotus nebrodensis*
15	AY450340	MG282523.1	*Pleurotus opuntiae*
16	FN391585	KY963093.1	*Pleurotus ostreatus*
17	AY450346	KY963083.1	*Pleurotus populinus*
18	MG819743.1	KY963039.1	*Pleurotus pulmonarius*
19	MG282442.1	MG282501.1	*Pleurotus purpureo-olivaceus*
20	AY728273	KY963015.1	*Pleurotus salmoneostramineus*
21	JN234847	KY963080.1	*Pleurotus sapidus*
22	GU722288	MG282504.1	*Pleurotus smithii*
23	EU908191	EU908175.1	*Pleurotus tuber-regium*

Phylogenetic analysis of ITS DNA sequence

Sequences of different accessions were analyzed by MEGA7 and Mr Bayes to establish a molecular phylogenetic relationship. All the twenty three ITS sequences *Pleurotus* species were aligned using MUSCLE. The Phylogenetic analysis was done using the MEGA7 and phylogram was generated. The Neighbor-Join and BioNJ algorithms were used to obtain the initial tree(s) for the heuristic search. The Maximum Composite Likelihood (MCL) approach was used to obtain the matrix of pairwise distances. All positions with less than 95% site coverage were eliminated. The time tree of evolution was analysed using Reltime method. Nucleotide substitution model was taken as Kimura 2-parameter model. The phylogenetic tree was prepared using the Bio Neighbor joining, (BioNJ) analysis. To reconfirm the results obtained in the MEGA analysis, Mr. Bayes phylogenetic analysis was performed using the maximum likelihood analysis, while the test of phylogeny was done by the bootstrap method with 100 replication of the analysis. A total of 100000 generation was used to calculate posterior probability using Markov Chain Monte Carlo (MCMC) method for Bayesian inference of phylogeny(Huelsenbeck and Ronquist 2001).

RESULTS AND DISCUSSION

Phylogenetic analysis using ITS sequences

The Kimura 2-parameter model was used to ascertain the evolutionary history and to get the molecular clock of *Pleurotus* spp through maximum likelihood approach using ITS sequences. The obtained phylogenetic tree showed the highest log likelihood (-2914.68) (Fig 1a). In Maximum Composite Likelihood (MCL) approach, a total of 463 positions were considered in final data set. The results suggested the significant differential evolutionary rates for selected species of *Pleurotus*. The phylogenetic tree obtained based on ITS sequence of divided different species of *Pleurotus* into two main groups, i.e. Group A and Group B (Fig 1a). The Group A contained 15 and B contained 8 species. The branch length of the phylogenetic tree was found to be 0.02, which indicated amount of genetic change in terms of nucleotide substitutions per site (that is the number of changes or 'substitutions' divided by the length of the sequence). *Pleurotus levis* was taken as out group in the analysis.

The Bayesian phylogeny was studied to examine the results of ITS using posterior probability. Average standard deviation was found to be 0.0044. The total of 699 trees was sampled and out of which 299 was found credible up to 99%. The phylogenetic tree obtained based on ITS sequence divided different species of *Pleurotus* into two main groups, i.e. Group A and Group B. The Group A contain 17 and B contains 6 species (Fig 1b).

Phylogenetic analysis using LSU sequences

The phylogenetic tree obtained using LSU sequences through maximum likelihood approach showed the highest log likelihood (-1270.40) (Fig 2a). A total of 512 positions were considered for Maximum Composite Likelihood (MCL) analysis to obtain the matrix of pair wise distances in the final data set. The phylogenetic tree obtained based on LSU sequence divided different species of *Pleurotus* into two main groups, i.e. Group A and Group B. The Group A contain 15 and B contains 8 species. The genetic change in terms of nucleotide substitutions per site was 0.02. The *Pleurotus purpureo-olivaceus* was taken as out group.

The Bayesian inference of phylogeny was also studied to examine the results of LSU using posterior probability. Average standard deviation was found to be 0.0030. A total of 26170 trees were sampled and out of which 24220 was found credible up to 99%. The phylogenetic tree obtained based on LSU sequence formed two major groups, i.e. Group A and Group B. The Group A contain 13 and B contains 10 species. The genetic change in terms of nucleotide substitutions per site was 0.02 (Fig 2b).

Understanding the phylogenetic relationship using both ITS and LSU

Pleurotus species taken in the analysis were grouped into two clades in both the analysis. Classification showed divergence of species according to habitat, domestication and distinctness. MEGA analysis of ITS sequences showed two group A and

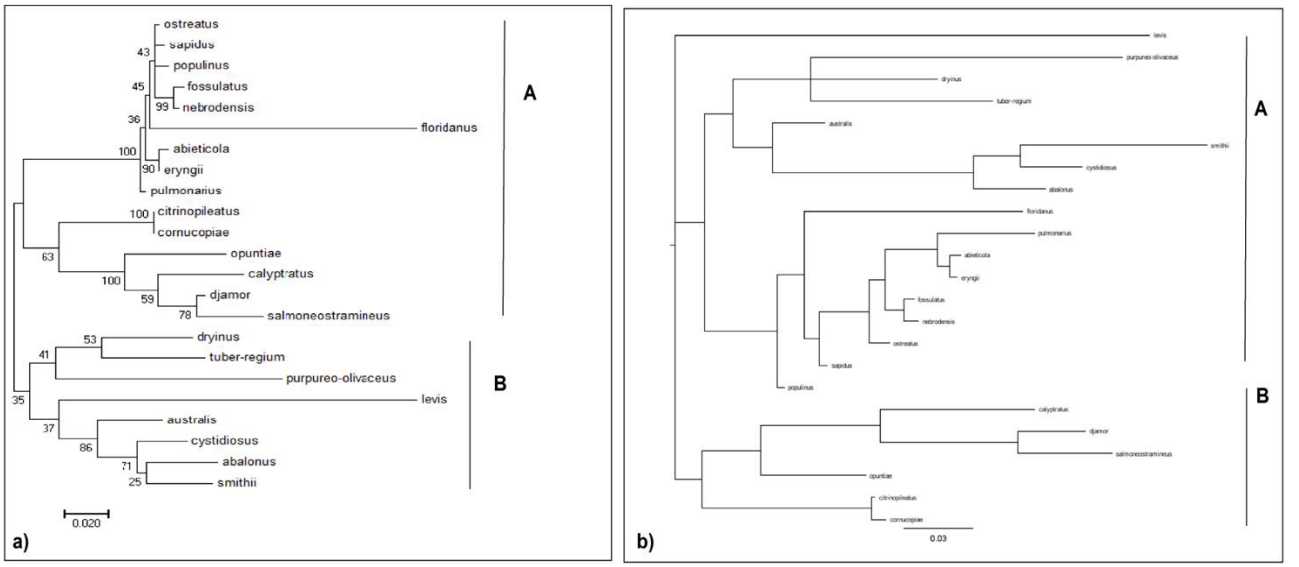

Fig. 1. ITS sequences of 23 Pleurotus spp. analysed a) MEGA7 and b) Mr.Bayes

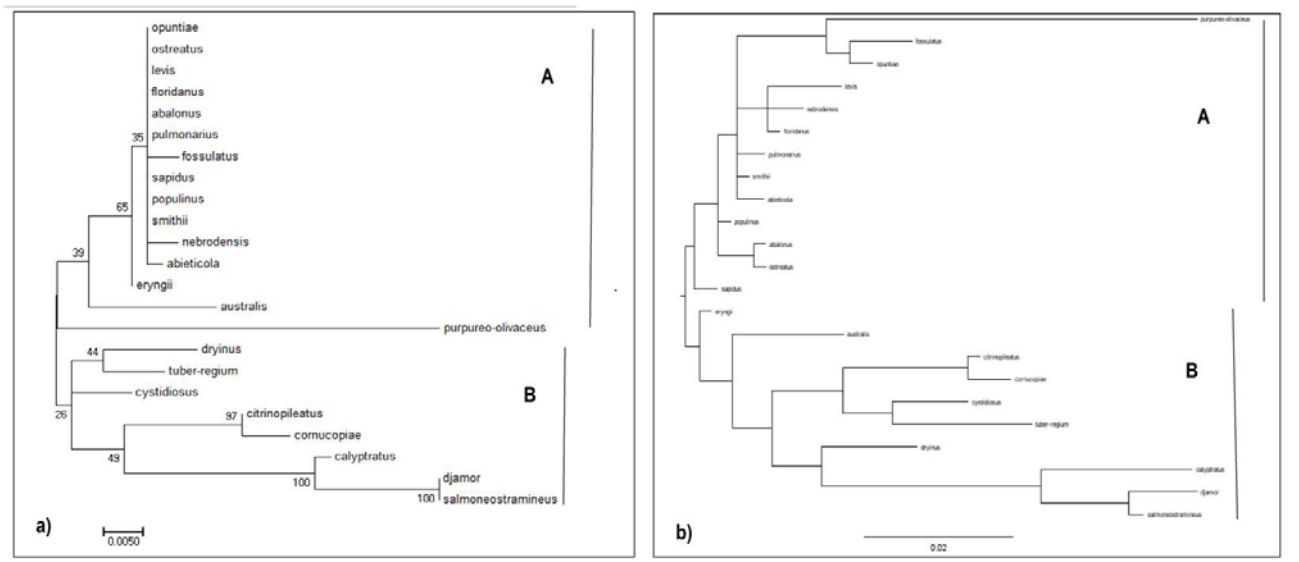

Fig. 2. LSU sequences of 23 Pleurotus spp. analysed a) MEGA7 and b) Mr.Bayes

B. Group A is further divided into A1 and A2 (Fig 1).

Group A consisted mainly the cultivated species. The sub-group A1 contains species (*P. oestratus, P. sapidus, P. populinus, P. fossulatus, P. nebrodensis, P. floridnas, P. abeticola, P. eryngii* and *P. Pulmonarius*) with average growing temperature of 17.6°C. The analysis showed that most of the species in sub group A1 are winter cultivated or temperate species. The sub group A2 contained 6 species (*P. citrinopileatus, P. cornucopiae, P. opuntiae, P. calyptratus, P. djamor* and *P. salmoneostramineus*) with average growing temperate of 26.5°C.

Group B comprised of least domesticated species of *Pleurotus* (*P. drynius, P. tuber-regium*, *P. purpureo-olivaceus, Pleurotus levis, P. australis, P. cystidiosus, P. abalonus, P. Smithi*). Similar results were also obtained from analysis done by Bayesian method.

LSU sequences analysis also showed two groups A and B (Fig 2). The groups classified by LSU sequences do not clearly delineated the domesticated and lesser domesticated species but it differentiated temperate and tropical *Pleurotus* species. The group A consisted of 15 species (*P. oestratus, P. purpureo-olivaceus, Pleurotus levis, P. australis, P. cystidiosus, P. abalonus, P. smithi P. sapidus, P. populinus, P. fossulatus, P. nebrodensis, P. floridnas, P .abeticola, P. eryngii* and *P. Pulmonarius*) with average growing temperature of 21.9°C, which is greater than ITS sequence result. This shows that the most species in group A may be winter cultivated or temperate species but the results are not very clear as in ITS sequences analysis.

Group B consisted 8 species (*P. citrinopileatus, P. cornucopiae, P. drynius P. tuber-regium, P. opuntiae, P. calyptratus, P. djamor* and *P. salmoneostramineus*) with average growing temperate of 23.1°C. The results suggested that the delineation of summer and winter species might have evolved at different evolutionary rate. The differences in the analysis using two different conserved sequences suggested the lower efficiency of LSU sequences compared to ITS in resolving the species metabolic and physiological requirements.

Bayesian analysis of LSU sequences also showed formation of two groups. The group A consisted of 13 species (*P. purpureo-olivaceus, P. fossulatus, P. opuntiae, Pleurotus levis, P. nebrodensis, P. floridnas, P. pulmonarius, P. smithi, P .abeticola, P. populinus, P.abalonus, P. ostreatus* and *P. sapidus*) with average growing temperature of 22.3 °C. The group B contained 10 species (*P. eryngii, P. australis P. citrinopileatus, P. cornucopiae, P.cystidiosus, P. tuber-regium, P. drynius, P. calyptratus, P. djamor* and *P. salmoneostramineus*) with average growing temperature of 24.8 °C. It is quite evident that species with high cultivation temperature and species with low cultivation are segregating in different clades of the tree. These results suggested the possibility of evolution for acclimatization of species in different climate.

Speciation is development or evolution of new species from existing one by evolutionary forces like selection, isolation, etc. The biological concept of species depends on mating compatibility. The compatibility tests are done to categorize the species on the basis of inter-compatibility or inter-sterility. Mating behavior studies in *Pleurotus* species contribute significantly to phylogenetic studies as the compatibility status between two known taxa represents the degree of gene flow between them. Various workers identified inter-sterility and mating groups in the *Pleurotus* population (Bao *et al.,* 2004). *Pleurotus* mating system is bi-factorial tetrapolar genetic system (Larraya *et al.,* 2001), in which a haploid dikaryotic mycelium occurs only when two compatible monokaryotic hyphae fuse together (Alam *et al.,* 2010). The genetic control of mating depends on two loci A and B. The compatibility between species occurs only when the alleles at both loci differ. If we compare the formation of inter-sterility group (IG) as reviewed by Bao *et.al*, the compatibility might be the criteria for the demarcation of species only when both pre-zygotic and post-zygotic isolation mechanisms are studied together. It means that after mating two diverse strains the formation of dikaryons is successful only when normal fruit body is and viable spores are formed. However, most of the workers have taken the formation of clamp connections as a criterion for compatibility as conjugate nuclear division needs a delicate balance of the two nuclei from two parents which is possible only if clear relationship exists.

The relative evolution of the species in *Pleurotus* (Fig 3) presents two distinct groups with marked difference with respect to their period of evolution. The grouping of *Pleurotus* species into two distinct groups indicates that summer and least domesticated species evolved earlier than winter cultivating species. The hypotheses behind this distribution may be continental drift theory, which ultimately caused the allopatric speciation and development of two groups. The theory believed that the ancient species were prevalent before the Pangean continent break up while some recent species are result of Laurasia breakup occurring in the Northern Hemisphere (Maftoun *et al*., 2015). The study also suggests the possibility of newly evolved group being restricted in its distribution mostly to the northern hemisphere. This can answer many questions as why some of the species of this

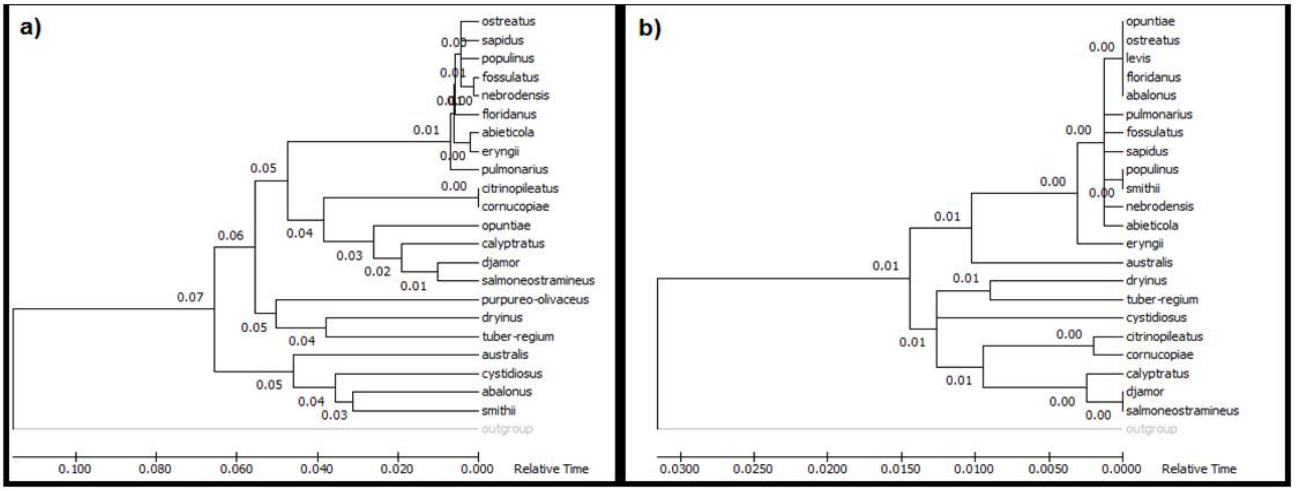

Fig. 3. Time tree generated by MEGA 7 a) ITS sequence time tree b) LSU sequence time tree

group like *P. eryngii* grows well when subjected to low temperature. On the contrary, the species from the earlier evolved group are widely distributed across both the hemispheres and therefore some species of this group such as *P. djamor* are well suited for warmer climate. The reports of geographic distribution of the oyster mushroom for example, *P. cystidiosus* are known to be distributed in the tropical and subtropical region, while *P. eryngii* are found in southern Europe, North Africa and central Asia which is similar in our findings. Similarly, *P. ostreatus* is widespread in the temperate zones such as Korea and Japan because it forms fruit-bodies at relatively low temperature compared to other species(Singer 1986). The study also suggests that the allopatric speciation caused the development of rare alleles and creates the bottlenecks for the population ultimately leading to speciation.

CONCLUSION

Species concept is defined on basis of classes, order and genera. If we consider the concept of gene exchange and reproduction barriers, it is the mating experiments that designate groups as species. Huge diversity of *Pleurotus* species prevails in nature therefore, additional and supporting data is required to study the evolution pattern of the species. A lot of taxonomic confusion prevails in the literature with regard to speciation in *Pleurotus* species, molecular phylogeny have helped to resolve such confusion to some extent but linking of molecular sequences with the taxonomic descriptors need to be ascertained. It is needed to integrate morphological traits data, taxonomic microscopic studies, conserved molecular sequences and inter-breedability studies to resolve the taxonomic chaos existing in genus *Pleurotus* effectively. As if now until such studies are not conducted, the conserved regions sequences can be the best strategy to trace the evolutionary history of *Pleurotus* species. In our study, it seems that the origin and distribution of *Pleurotus* is somehow linked with evolutionary pattern. Inter-compatability or inter-sterility seems to be related to the evolutionary pattern to some extent but still more study with different conserved regions are required to establish such relations. The application of such studies are useful in determining evolution of the different enzymatic genes such as laccase and their alleles which might had developed for utilization of different substrate in *Pleurotus*. Moreover, these studies lead us to ascertain the genetic relatedness of the species and help breeders and biotechnologists to augment the work on breeding and genetic improvement of *Pleurotus* spp.

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