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Strain Improvement and Molecular Characterization of Mushrooms

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ABSTRACT

Mushrooms are valued as highly tasty and nutritional food and considered to be a rich source of proteins, amino acids, carbohydrates, vitamins and minerals. Therefore, mushrooms are called as functional food. The breeding of new strains has significantly improved, allowing the use of strains with high yield, quality, texture, colour and taste, increasing productivity and diminishing the use of chemicals for pest control. Strain improvement of mushroom species thus far has been based on conventional breeding methods. However, breeding of improved strains by conventional methods is a labour intensive and time-consuming procedure. Hence, the protoplast fusion has been used as a method to create mushroom hybrids, especially when using conventional methods cannot achieve this result. This technique opened up an new area of study, which has both fundamental and applied importance. Protoplast fusion is an appropriate method in creating hybrids of interspecific hybridization in mushrooms Strain improvement is one of the important methods in mushroom technology to improve its potential towards industrial and pharmaceutical point of view. The protoplast fusion is potentially important technique for genetic manipulation of industrially important fungi, obtaining valuable hybrids with disease resistance and for production of quality products in cultivated plants in agriculture. Combinations of desirable traits are possible in recent days through protoplast fusion.

Key Words: Mushrooms, Strain improvement, Conventional techniques, Protoplast fusion, Isolation of viable fungal protoplast, Fusion methods of protoplast, Regeneration and reversion of protoplast, Molecular characterization

INTRODUCTION

Strain improvement is one of the important methods in mushroom technology to improve its potential towards industrial and pharmaceutical point of view (Selvakumar *et al.*, 2015). The protoplast fusion is potentially important technique for genetic manipulation of industrially important fungi, obtaining valuable hybrids with disease resistance and for production of quality products in cultivated plants in agriculture. Combinations of desirable traits are possible in recent days through protoplast fusion. Transformation of protoplast opens up genetic revolution of transgenic plants and genetically improved transformants in microorganisms. In addition, it has been widely used in genetic transformation, karyotype, genetic and physiological analysis, and selection of asexual clones with desired properties (Homolka *et al.,* 1995; Woestemeyer and Woestemeyer 1998; Eichlerova *et al*., 2000). This technique also makes it possible to study somatic genetics. To date, most of the successful genetic transformation of mushrooms has also adopted with protoplast-mediated techniques (Noel and Labarere, 1994; Jia *et al.,* 1997; Yan *et al.,* 2002). When conventional methods cannot achieve the required results, protoplast fusion has been used as a method to create mushroom hybrids. In *Lentinula edodes*, a recombination of mitochondrial genomes only occurred after protoplast fusion and was not found in conventional method or sexual crosses (Fukuda *et al.,* 1995). Mitochondria had a pronounced influence on the growth characteristics of these fungi, industrial strains with vigorous growth might be produced after recombination of mitochondrial genomes through protoplast fusion (Woestemeyer and Woestemeyer, 1998). Therefore, protoplast fusion can be performed intraspecifically (Kiguchi and Yanagi, 1985; Tokimoto *et al*., 1998; Fukuda *et al*., 2007a; Maeta, *et al*., 2008), interspecifically (Takehara *et al*., 1993; Matsumoto *et al*., 1997; Dhitaphichit and Pornsuriya, 2005; Fukuda *et al*., 2007b; Singh, *et al*., 2007; Selvakumar *et al*., 2015), intergenerically (Liang and Chang, 1989; Eguchi *et al.*, 1993; Zhao and Chang, 1996; Chakraborty and Sikdar, 2008) and even interheterogenerically (Toyomatsu and Mori, 1987; Eguchi and Higaki, 1995). In the 1950s, protoplast fusion technology originated from cell-cell fusion technology and

was originally applied to animal cells (Yue and Alessandra, 2024). Nevertheless, protoplast fusion opened up an important area of study, which has both fundamental and applied research in mushrooms (Peberdy, 1987; Woestemeyer and Woestemeyer, 1998).

Fukuda *et al*. (2007b) have reported the successful mtDNA transmission in interspecific fusion protoplasts of edible mushrooms *Pleurotus ostreatus* (a methionine auxotrophic and chloramphenicol- resistant strain) and *P. pulmonarius* (wild-type strain), thus increasing the genetic variability of economically important mushrooms. This possibility is useful in mushroom breeding, because the mitochondrial genome may affect phenotypical properties of edible mushrooms. Interspecific hybridization from different species may dramatically influence the course of biological processes compared to their occurrence in the parental species. It has been established as a new genetic manipulation tool in fungi. Protoplast preparation, fusion and regeneration are essential steps in protoplast technology.

Molecular systematics has been shown to be a valuable tool in modern fungal taxonomy (Bruns *et al.,* 1991; Mitchell *et al*., 1995). Various molecular methods, including DNA-DNA hybridization, RFLP and sequence analyses, phylogenetic analyses of amino acid or DNA sequences are known to have the maximum resolving powers (Bruns *et al*., 1991). Due to the ubiquitous occurrence and essential functions, DNA sequence data of 18S, 26S, Internal Transcribed Spacer (ITS) and mitochondrial rDNAs are most frequently used in recent phylogenetic studies of eukaryotic cells. The 18S rDNAs sequences are conserved and they have been used in phylogenetic analyses of higher taxonomic ranks of fungi such as orders or classes (Swann and Taylor, 1993, Wilmotte *et al*., 1993). However, ITS rDNAs are so variable that they often cannot be aligned accurately between genera and are now commonly used in the systematics of species within a genus (Moncalvo *et al*., 1995a; 1995b; Yan *et al*., 1995). However, mitochondrial small subunit (SSU) rDNAs have been reported to evolve 16 times faster than 18S rDNAs (Bruns and Szaro, 1992) but are less variable than ITS rDNA. Thus, they are believed to have a potential to fill the phylogenetic gaps at a family level between those available from ITS rDNAs. Moreover, the identification and comparison of hybrids and parental strains were resolved using molecular techniques.

STRAIN IMPROVEMENT

Strain improvement method lays primary emphasis on identifying or incorporating genetic potentials for higher yield, since the production performance of any crop variety is the outcome of its genotype and the growing environment-generally manipulated by way of crop management practices. Attempts to identify/ incorporate the genotypes with high yielding potentials to life cycles/sexuality of the edible mushrooms were worked out in the seventies (Elliott, 1972; Raper *et al.,* 1972). However, once these mysteries were unfolded, significant breakthrough were made on this front e.g. release of two high yielding hybrids U1 and U3 of *Agaricus bisporus* by Fritsche in the year 1991. Besides, there is a definite spurt in activities related to strain improvement of various cultivated mushroom, and much more is expected in the years to come with more and more application of molecular techniques for breaking the yield barriers. Nevertheless, the following breeding approaches are considered useful for improvement the genetic yield potential of edible mushrooms.

Conventional techniques

There has been a continuing attempt to identify and use improved strains of cultivated mushrooms particularly in the button mushroom right from the day when its indoor cultivation became a commercial enterprise. Due to limited knowledge of reproduction of these fungi as well as want to improved techniques, the earlier workers (Eger *et al*., 1976) relied mostly on traditional breeding such as raising tissue culture, multispore culture, strain mixing and single spore isolation (Verma *et al.,* 2000).

Tissue culture

Raising of tissue culture from the sporophores of wild collection of edible mushrooms and their use for germplasm conservation and spawn production have been in vogue for many years. However, their uses to obtain improved strains have not been found very rewarding. Although tissues from

different parts of the sporophore have been found to differ in their growth rates and even yield performance, yet these have not been considered very dependable and are also known to carry some undesirable parental traits (Verma *et al.,* 2000). The performance of isolated strains taken from stipe and the gills were still inferior. Obviously, tissue isolates can hardly be useful for getting improved strains but its usefulness for raising and conservation of cultures of wild germplasm cannot be overemphasized, since this technique ensures their conservation in an unchanged form.

Multispore culture

Multispore isolates may yield some variability due to anastomosis of the hyphae originating from different spores, which may prove useful for further breeding purposes, but this technique is more in use for rejuvenating the old and degenerated cultures due to repeated subculturing, particularly by spawn makers (Verma *et al.,* 2000). As regards the yield potentials of multispore cultures, there are already reports that they may perform better, worse or at par with the parent strain. In addition, Metha (1991) reported that multispore isolates exceeded the yield of the parent strain, but the former degenerated faster than the later. It could thus be concluded that instead of using this technique directly for breeding purposes, it could be more useful for rejuvenation of old cultures and isolation and conservation of wild germplasm.

Strain mixing

Interstrainal fusion of hyphae have been tried by many researchers by allowing the mycelia of different strains to intermingle and anastomose (Verma *et al.,* 2000). Even exchange and fusion of nuclei of different strains have been claimed due to intermixing and the isolations made from the meeting zone have been supposed to perform superiorly due to complementary/ supplementary action of the nuclei derived from the two parental strains. However, there are either contradictory reports of such possibility or examples of such an improvement are too few to be of any practical consequence (Verma *et al.,* 2000).

Single spore isolation

Strain improvements directly achieved by isolating single spore isolates (SSIs) are possible only in homothallic mushroom species, such as *Agaricus bisporus* and *Volvariella volvacea* and are not in heterothallic species. In both of these mushrooms, SSIs are self-fertile, although basidiospore of *A. bisporus* derived from 2 spored basidia contain two nuclei of different parental types and those of *V. volvacea* are uninucleate receiving only one of the four meiotic nuclei in each basidiospore. Monosporous cultures of both these mushrooms exhibit variability in terms of growth rate, morphology and yield potential and provide ample opportunity to select improved strains of both these homothallic mushrooms through this technique (Verma *et al.,* 2000).

Mutation

Spontaneous and induced mutation have been reported in *A. bisporus* but it has not yet found any major application in the genetic improvement of cultivated mushrooms, except the chance of spotting the natural pure white and smooth capped individuals in a bed of cream strain of *A. bisporus*. Since then, the pure white strain has attained the status of the most popular commercial culture of button mushroom. Both physical and chemical mutagens such as X-rays, UV-rays and nitrosomethyl urea, methyl uracil, thio-uracil, chloroethyl phosphoric acid and uranium nitrate have been used for obtaining induced mutants of mushroom (Verma *et al.,* 2000).

PROTOPLAST FUSION

Several techniques are available for strain improvement such as conventional breeding (sexual hybridization) (Ball and Azevedo, 1976), mutagenesis (Elander *et al.*, 1973), recombinant DNA technology, transformation (Merrick, 1976) and protoplast fusion (Peberdy, 1976, 1983). Transfer of nuclei by protoplast fusion has been demonstrated as an efficient method to improve the industrially important microorganism (Peberdy, 1980; Kabuki *et al*., 1984.). Protoplast fusion has potential value in strain in which sexual (or) parasexual mechanisms are lacking or not observed and is the only means by which hybridization can be achieved for strain improvement procedure (Fournier *et al.,* 1977). The induced fusion of protoplast is an artificial system by with novel strains with new characteristics can be obtained. These new characteristics cannot be achieved by the

conventional methods. A number of reports have suggested the possibility of breeding of microorganisms such as *Aspergillus* sp. (Kirimura *et al.*, 1988) and *Trichoderma* sp. (Anne and Peberdy, 1976) through induced protoplast fusion. Gold *et al.* (1983) first reported protoplast fusants in higher Basdiomycetes between different auxotrophic strains of *Phanerochaete chrysosporium*. In the majority of the reports available, fusion experiments have utilized auxotrophic mutant strains which after treatment with polyethylene glycol are plated on minimal medium and the fused strains are selected by nutritional complementation (Peberdy, 1980). In particular, interspecific protoplast fusion has made possible the development of new strains carrying novel properties that could not have been created by traditional breeding techniques such as artificial mating within intraspecific compatible combinations (Fukuda *et al.,* 2007b). Interspecific hybrids constructed by protoplast fusions in several species of edible mushrooms have been reported; the control of their vegetative and generative phenotypes by nuclear genes has been investigated by culturing tests, isozyme characterization and DNA fingerprinting analysis (Toyomatsu *et al*., 1986; Sunagawa and Miura, 1992; Peberdy and Fox, 1993; Yoo and Cha, 1993; Eguchi and Higaki, 1995; Sunagawa *et al.,* 1995).

Among the biotechnological techniques, cell fusion is becoming barrier existing between strains/ species/ genera to mate and exchange the genes in each other. To achieve such cell fusion, however, protoplast are required to be isolated, tested for reversion and then made to fuse with another type (both monokaryotic types) to produce a somatic hybrid protoplast. The parental types to be fused are carefully selected and are fully characterized by creating a selection marker, which helps to identify the fusants. The fusants are later put to identical tests to compare them among themselves and with the parent strains which together help to identify and evaluate the fusants. Further, the fusants are cultured in pairs with the parent monokaryons to test for antagonistic reaction a barrage like phenomenon, which suggest that the fusants are different from the original test to identify those having hybrid vigour and high yielding capacity (Verma *et al.,* 2000).

The protoplast isolation and reversion have been attempted in a number of Basidiomycetes, including *Agaricus bisporus, Armillaria mellea, Coprinus* spp., *Ganoderma* spp., *Hericium coralloides, Lentinula edodes, Morchella* sp., *Pholiota* spp., *Pleurotus* spp., *Schizophyllum commune, Volvariella* spp. and *Auricularia* spp. (Sunagawa *et al*., 1989; Garcia 1992; Zhang *et al*., 1994; Selvakumar *et al.*, 2015; Raman *et al*., 2021). Also protoplast fusion, intra and interspecific heterokaryons have been obtained in several edible mushrooms such as *Auricularia auricular*, *A. polytricha* (Sunagawa 1992; Sunagawa and Mura 1992; Sunagawa *et al*., 1992), *Pleurotus* spp. (Toyomatsu *et al.,* 1987) and intergeneric protoplast fusion between *Lentinula edodes* and *Pleurotus* spp. (Zang *et al*., 1992). Protoplast fusion can be performed intraspecifically, intergenerically and introheterogenerically (Kiguchi and Yanagi, 1985; Toyomatsu and Mori, 1987; Takehara *et al*., 1993; Eguchi and Higaki, 1995; Zhao and Chang, 1997; Tokimoto *et al*., 1998; Matsumoto *et al*., 1997; Singh, *et al*., 2007; Fukuda *et al*., 2007a; Fukuda *et al*., 2007b; Chakraborty and Sikdar, 2008; Maeta, *et al*., 2008).

A. Intraspecies protoplast fusion

Conventional parasexual techniques, attempted so far to construct the strains producing higher titers of industrially important metabolites, are not always successful (Peberdy, 1980). Intraspecies protoplast fusion, an alternative method to induce the parasexual cycle for strain improvement program is found to be successful (Fukuda *et al*., 2007a; Maeta *et al*., 2008). The first successful experiment covering protoplast fusion was carried out with auxotrophic mutants of *Geotrichium candidum* (Ferency *et al*., 1974). Protoplast fusion between *Aspergillus niger* strains resulted in heterodiploid and production of citric acid was found to be intermediate between those of parent strain (Kirimura *et al*., 1988). Hyphal anastomosis formation is very difficult in *Penicillium chrysogenum* by conventional method (Anne *et al.*, 1976) but protoplast fusion has been applied with great success. The use of colour mutants of *Trichoderma reesei* monitor the fusion of protoplasts instead of auxotrophs or labeled strains which produced heterokaryons and the enzyme activities of these heterokaryons are found to be as

in the parents (Ogawa *et al*., 1988; 1989). Intraspecies fusions of *T. reesei* non-parental protrophic hybrids have shown enhanced cellulolytic activity (Ogawa *et al*., 1988). In *Cephalosporium* sp., recombinants procedure significantly increase the yield of cephalosporin than the parental strains (Peberdy, 1980).

B. Interspecies protoplast fusion

In nature, different species do not often exchange genetic material. However, in the recent days, new genetic techniques including transformation and protoplast fusion have become available making genetic transfer between species more feasible. Interspecies fusion has been extensively investigated in *Agaricus* (Sonnenberg *et al.,* 1988). *Pleurotus* spp. (Fukuda *et al*., 2007b), *Volvariella* spp. (Zhao and Chang, 1997), *Penicillium* spp. (Anne, 1977), *Aspergillus* spp. (Das *et al*., 1989), *Mucor* spp. (Lasker and Borgia, 1980) and *Trichoderma* sp. (Manczinger and Ferenczy 1985; Stasz and Harman, 1990). Heterokaryon formation is the first step in determining the stability of the heterokaryon state. The heterokaryon so formed might be heterodiploid or partially heterodiploid (Dales and Croft, 1977). Viability of heterokaryon depends on species relatedness and somatic (or) nuclear compatibility. Heterokaryons derived from the crosses between various species have shown retarded growth, irregular colony morphology and unusual segregating pattern. The hybrids derived from *Aspergillus nidulans* and *A. rugulosus* gave segregants both spontaneously and following induction by growth on a haploidizing agent (Ferenczy *et al*., 1975) but induction was necessary to produce segregants of *Penicillium* hybrids (Dales and Croft, 1977). Interspecific hybridization from different species may dramatically influence the course of biological processes compared to their occurrence in the parental species. Positive or negative deregulation of gene control may occur or metabolic pathways may be mixed, resulting in the production of novel metabolites and it has been established as a new genetic manipulation tool in fungi (Perberdy, 1991; Yoo and Cha, 1993). Aswini et al (2014) studied the protoplast fusion between wild and UV mutant of *Pleurotus* species and they found the fusants were proved to be hybrids of wild and UV mutant strain.

Through protoplasmic fusion between *Pleurotus ostreatus* var. *florida* and *Pleurotus djamor* var. *roseus*, Selvakumar *et al*. (2015) obtained 2 hybrid strains, and they showed that the hybrid strains had intermediate morphology to the parents. The hybrid *Pleurotus* sp. HS showed improved biological efficiency. According to Porselvi and Ramasamy (2020), the fusant strain between *Pleurotus eous* and *P. florida* showed maximum biological efficiency than the native strains. According to Keqing *et al.* (2023) the fusants of *Auricularia cornea* cv. Yu Muer and *Auricularia heimuer* cv. Bai Muer contained polymorphic bands, which indicated the rearrangement and deletion of DNA in the fusants. Higher yield of fruit body of the fusants between *Ganoderma lingzhi* and *G. lingzhi* was observed (Jintao *et al.* 2023).

C. Intergeneric fusion

A successful intergeneric protoplast fusion has been carried out between the two edible mushroom strains *Volvariella volvacea* and *Pleurotus florida* (Chakraborty and Sikdar, 2008). Protoplast fusion between *Aspergillus niger* and *Trichoderma viride* has been successfully fused and the strains were formed colonies showing mixed morphologies and have grown more slowly than the prototrophic parent strains (Kirimura *et al*., 1990). The fusants were proved to be heterokaryons based on conidial analysis. This showed that viable fusions can be obtained between *niger* and *T. viride* suggesting some compatibility is likely to exist between the two species (Kirimura *et al*., 1989). The fusants obtained from the intergeneric fusion between *Trichoderma viride* and *Neurospora crassa* exhibited cellulobiose activities and are able to synthesize carotinoid pigment in amounts analogous to those produced by *Neurospora crassa*.

Intergeneric hybridization between *Monascus anka* and *Aspergillus oryzae* by protoplast fusion produced heterokaryon fusions (Kiyohara *et al*., 1990). The fusion resulted in suitable heterozygous diploid that grow more rapidly and produce higher amount of ethanol than the parents. The enzyme activities of protease, amylase and kojic acid by the fusant was higher than the two parents (Iizuka and Mineki, 1977). Yamada *et al.* (1983) studied the regeneration of mycelial protoplasts on *Collybia velutipes* and *Pleurotus*

ostreatus. Protoplast fusion between *Calocybe indica* var. APK2 and *Pleurotus florida* resulted in γ-linoleic acid content in hybrid lines (Chakraborty and Sikdar, 2009). The protoplasmic fusion of white oyster mushroom (*Pleurotus floridae*) and brown oyster mushroom (*Pleurotus cystidiosus*) resulted a strain with higher productivity (Djajanegara and Masduki, 2010). Kang *et al.* (2019) studied the protoplast fusion of *Flammulina velutipes* and *Pleurotus ostreatus*.

ISOLATION OF VIABLE FUNGAL PROTOPLAST

The fungal protoplast limited by the cell wall can be isolated by mechanical non-enzymatic method or enzymatic method. The cell wall has to be removed without damaging the cell membrane to obtain intact protoplast. Removal of cell wall is possible through digestion using different lytic enzymes or through mechanical rupture.

A. Non-enzymatic method

This procedure is economical compared to the cost of commercially available enzymes. The mechanical rupture of the cell wall is carried out by using a French pressure cell. The pressure (lb/ inch^2) extracted and the exposures rate varies with different fungi (Vijayapalani and Lalithakumari, 1999).

B. Enzymatic method

Microbial enzymes have been reported to liberate protoplasts from bacteria, yeast, filamentous fungi and higher plant cells (Peberdy, 1987). Gascon *et al.* (1965) reported that *Micromonospora chalcea* grown in a variety of complex media synthesized a complex lytic enzyme system, able to release protoplasts from a number of fungi. These enzymes are released into the growth medium during exponential growth. Numerous factors concerned with optimal growth conditions have been studied with a view to obtain consistent and maximal yields of the lytic material. The techniques for isolation of protoplasts from fungi and higher plants using lytic enzymes have been well established. The first enzyme described to dissolve fungal cell walls was the digestive juice of the snail (*Helix pomatia*) in 1919 by Giaja, though it was not until 1957 that its lytic properties were used to induce protoplast formation in yeast (Eddy and Williamson, 1957). The snail digestive juice preparation was reported to be effective against *Neurospora crassa* (Backmann and Bonner, 1959), *Cephalosporium acremonium* (Fawcett *et al.,* 1973), *Aspergillus nidulans* (Frenczy *et al.,* 1974) and *Geotrichum candidum* (Ruiz- Herreva and Bartnicki- Garcia, 1976). An enzyme derived from *Cytophaga* is also effective against *C. acremonium* (Fawcett *et al.,* 1973; Hamlyn and Ball, 1979). For protoplast formation and improvement of protoplast regeneration from various fungi, an enzyme from *Trichoderma harzianum* was used (Kitamoto *et al*., 1988).

FUSION METHODS OF PROTOPLAST

Virus induced cell fusion

Okada *et al.* (1957) first discovered that the animal cells in suspension can be rapidly fused under the influence of hem agglutinating virus of Japan (HVJ) or sendai virus. Harris and Watkins (1965) using UV inactivated sendai virus detected the practical application of virus induced cell fusion to produce heterokaryons between different animal cells. A number of enveloped DNA and RNA viruses have been reported to produce syncytia (Hoekstra and kok, 1989) but certain RNA viruses such as those that belonging to the paramyxovirus groups which induce mumps and new cattle disease and parainfluenze viruses are better suited as fusion reagents.

Chemically induced cell fusion

A. Sodium nitrate with Ca^{2+} ions

Power *et al.* (1970) devised first controlled conditions using high concentrations of $NaNO_3$ (0.25M) to fuse plant protoplasts from different taxa in order to produce hybrid somatic cells. Later, Lazar (1983) reported that it yield only very low fusion frequency. Anne (1977) reported that it was not useful for microbial protoplast fusion. Improved results were observed when the cells were treated with Ca^{2+} ions under alkaline conditions. Calcium under alkaline condition led to the fusion of all cell types, including plant, liverwort, bacterial and fungal protoplasts.

B. Polyethylene Glycol (PEG)

Improved results have been obtained with a new class of fusogen, independently detected by Wallin *et al*. (1974) and Kao and Michayluk (1974) who observed that the non-ionic water soluble surfactant PEG, could efficiently agglutinate plant

protoplasts and that these protoplasts subsequently were fused at high frequency. PEG is an efficient fusogen for all kind of cells (Ferenczy, 1981). Since the discovery of fusogenic properties of PEG/ Ca^{2+} by Kao and Michayluk (1974), it has been used to induce the fusion of many types of cells and organelles and continues to be most widely used method of fusion. PEG has also been used to fuse recombinant *Agrobacterium tumefaciens* protoplast with plant protoplasts (Cocking, 1972), in addition, the artificially constructed liposomes containing DNA with bacterial, plant and fungal protoplasts (Radford *et al.,* 1981; Makins and Holt, 1981; Nagata, 1984). Ferenczy *et al*. (1975) found that PEG solution is a good osmotic stabilizer if the concentration is at least 25% (w/v) and that the effect of Ca^{2+} on fusion is significant. Despite the good aggregation of protoplast at high PEG concentrations, the fusion frequency was negligible without the addition of Ca^{2+}. The addition of 1 mM $CaCl_2$ was effective to stimulating the fusion process. Prior to the discovery of the fusogenic properties of PEG/ Ca^{2+}, high Ca^{2+} concentration under alkaline pH condition caused fusion (Keller and Melchers, 1973; Binding and Weber, 1974; Anne and Peberdy, 1975; 1976).

PEG may act as a polycation, inducing the formation of small aggregation of protoplasts (Stewart, 1981). Constabel and Kao (1974), working with plant protoplasts, assumed that PEG acted as a molecular bridge between adjacent membranes, either directly by hydrogen bonds or indirectly via Ca^{2+}. Anne and Peberdy (1975) found that lower pH promotes formation of linkage via hydrogen bonds if Ca^{2+} is not present. Presence of other cations, namely K^+, Na^+ and Mg^{2+}, greately diminishes the stimulatory effect of Ca^{2+} on fusion and attributed this effect to decrease in the amount of Ca^{2+} bound to the membranes (Anne and Peberdy, 1975). They postulated that high pH might promote Ca^{2+} links with the (-O-CH_2-) ends of the PEG molecules. The molecular weight of PEG used is critical for the fusion frequency and in most protocols the molecular weight for PEG is 4000 or 6000 (Ferenczy *et al*., 1975). Ferenczy (1976) studied PEG solutions with molecular weight of 400, 1540, 4000, 6000 and 20,000 for their ability to promote fusion. The best fusion was obtained with 25% (w/v) PEG 4000 or 6000 in 10 - 100 ml $CaCl_2$. Besides for somatic cell fusion, PEG treatment could be used as an efficient means to bring about the uptake of macromolecules such as DNA (Hopwood, 1981) and organelles such as mitochondria or nuclei (Ferenczy, 1984; Sivan *et al*., 1990) or even whole cells into larges cell types such as bacteria into fungal protoplast (Guerra *et al*., 1991) or into plant protoplasts (Hasegawa *et al*., 1983) or bacteria into mammalian cells. This method is applied for direct gene transfer bacteria to mammalian cells (Sandri *et al*., 1983). Krishnapriya and Geetha (2020) have reported PEG mediated protoplast fusion between *Pleurotus opuntiae* and *P. cystidiosus*.

C. Polyvinyl alcohol (PVA)

Polyols have been used successfully to induce protoplast fusion. Nagata (1978) used a solution containing 15% PVA (MW 500), 50 mM $CaCl_2$ and 0.3 M mannitol to induce fusion between plant protoplasts and found that PVA/ Ca^{2+} induce fusion at least comparable to that of the PEG/ Ca^{2+} system, and has also been used to successfully fuse artificial liposomes containing tobacco mosaic virus (TMV) RNA with protoplast of *Micotinana tabacum* and *Vinca rosea* (Nagata, 1984).

D. Lipids in cell fusion

Several lipids and phospholipids were shown to induce cell fusion and have at least a positive effect on the fusion process (Lucy *et al*., 1971). Lysolecithin and glycerol mono oleate (Croce *et al*., 1971; Cramp and Lucy, 1974) have been used in mammalian cell fusion. For plant protoplasts, positively charged synthetic phospholipids has been used for fusion (Nagata *et al*., 1979).

E. Liposomes

Liposomes have been also used in combination with PEG forming lipid crystal structures. Liposomes are also synergistic fusogen in combination with PEG. In the presence of liposomes, the requirement for high concentration of the polymer is lowered for efficient protoplast fusion (Makins and Holt, 1981). Liposomes are highly used in transformation and transfection experiments with mammalian cells (Felgner *et al*., 1987), bacterial protoplasts (Makins and Holt, 1981; Boizet *et al*., 1988) and plant protoplasts (Lurquin, 1979).

Electrical Field mediated protoplast fusion

The electrofusion technique has been successfully applied to animal cells, plant, yeast and fungal protoplasts (Delorme, 1989; Chakraborty and Kapoor, 1990). In this method, protoplasts of parental strains are mixed and subjected to high frequency alternating electric field which causes the formation of pearl chains of protoplasts and this process called is dielectrophoresis. The fusion is induced by the application of one to several short direct current pulses, which causes reversible membrane breakdown at sites of membrane contacts (Gokhale, 1992).

REGENERATION AND REVERSION OF PROTOPLAST

Protoplasts are suspended in osmotically stabilized nutrient medium, part of the population starts to synthesize new cell wall and eventually return to normal hyphal form, termed as regeneration. The process of germination or regeneration of protoplast is an important factor for protoplast fusion. The fungal protoplast more rapidly regenerates a wall (regeneration) and reverts to the original mode of growth (reversion). The protoplasts are round and spherical in shape when they are subjected to lytic enzymes in the presence of osmotic stabilizer. The first and foremost important process in protoplast regeneration is the synthesis of rigid cell wall. Most studies on the regeneration of protoplasts concerned simple microscopical observations (Peberdy and Gibson, 1971; Anne *et al.,* 1974; Toyoda *et al*., 1984; Revathi and Lalithakumari, 1992; Mrinalini, 1993; Reyes *et al.,* 1998; Sun *et al.,* 2001; Selvakumar *et a*l., 2015; Chandrappa, and Basavarajappa, 2017).

Factors affecting regeneration of protoplasts: Important characters in the regeneration of protoplast is viability, the capacity to synthesize the cell wall and the retention of properties of the parent cell. Factors influencing regeneration of protoplast are the nature of osmotic stabilizers, pH, temperature and the physical state of regeneration medium. Studies with *Fusarium culmorum* (Lopez *et al.,* 1966) showed that reversion frequency was influenced by the carbon sources in regeneration medium. In *A. nidulans,* Issac and Gokule (1982) reported that reversion frequencies were in the range of 10 – 30% and no difference was found with defined and complex media. Optimum conditions for protoplasts regeneration of *T. longibrachiatum* were glucose mineral regeneration medium with 0.5 M KCl and pH 6.0 at 30°C (Kumari and Panda, 1993). Osmotic stabilizers such as glucose, NaCl and KCl in regeneration medium were found to induce high regeneration percentage of protoplasts 65%, 65 - 75% and 70 - 75% respectively in *Talaromyces flavus* (Santos and Demelo, 1991). Large amounts of regeneration of protoplasts were recorded by using 0.7 M mannitol as stabilizers in *T. harzianum* (Tashpultov *et al.,* 1991). Revathi and Lalithakumari (1992) reported that PDYEA amended with 0.6 M sorbitol – sucrose was effective for regeneration of protoplasts of *Verticellium inaequalis*. Yan *et al.* (2004) reported protoplast regeneration faster and higher when sugars (mannitol and sucrose) served as osmotic stabilizer than when inorganic salt ($MgSO_4$) served as osmotic stabilizer. The regeneration of protoplasts of *Trametes hirsuta* 072 was 2 times higher in the presence of β-carotene and quercetin (Mosunova *et al*. 2016).

MOLECULAR CHARACTERIZATION

Molecular techniques are sensitive to detecting the soil-borne fungi. The internal transcribed spacers (ITS) of nuclear rDNA (rRNA genes) are widely used to analyze the molecular differences between fungi; because inter- and intraspecific variations have been observed in the ITS of related fungal species. The ITS of rDNA is nested between conserved sequences of the 18S, 5.8S and 28S subunits, moreover, it is present in multiple, tandemly arranged copies in fungal genomes (Takamatsu, 1999). In eukaryotes, each cluster contains three genes that coded rDNA small subunit (SSU, 18S), the 5.8S subunit, the large subunit (LSU, 25–28S) and two ITS (Takamatsu, 1999). The intergenic spacer (IGS) contains the 5S rRNA gene, found between the gene clusters (Takamatsu, 1999). The different regions of rDNA evolve at variable rates, making them useful for phylogenetic studies among closely or distantly related organisms. Molecular phylogenetic studies in mushrooms are mainly based on RFLP and sequence data from LSU rDNA, especially the 5′ portion of LSU rDNA (Takamatsu, 1999; Hibbett, 1992). Dahlman *et al.,* (2000) performed phylogenetic analysis of 5′ end LSU rDNA sequence data to distinguish the identity of species between the genera *Cantharellus* and

Craterellus. A similar region of rDNA was also used to study the phylogenetic relationships in *Agaricus* (Mitchell and Bresinsky, 1999), *Amanita* (Drehmel *et al.,* 1999), *Coprinus* (Hopple and Vilgalys, 1994), *Ganoderma* (Moncalvo *et al.,* 1995a) and *Pleurotus* (Bunyard *et al.,* 1996).

MOLECULAR SYSTEMATICS

Ribosomal DNA genes (rDNA) are the primary focus on investigation of new taxonomic approaches in fungal molecular systematics. These genes are arranged in tandemly repeated units (**Figure 1**) with each unit containing the genes for the small subunit 18S, 5.8S and large subunit (25-28S). Each unit is separated by one or more IGS regions and these regions may contain a separate transcribed coding region for 5S RNA.

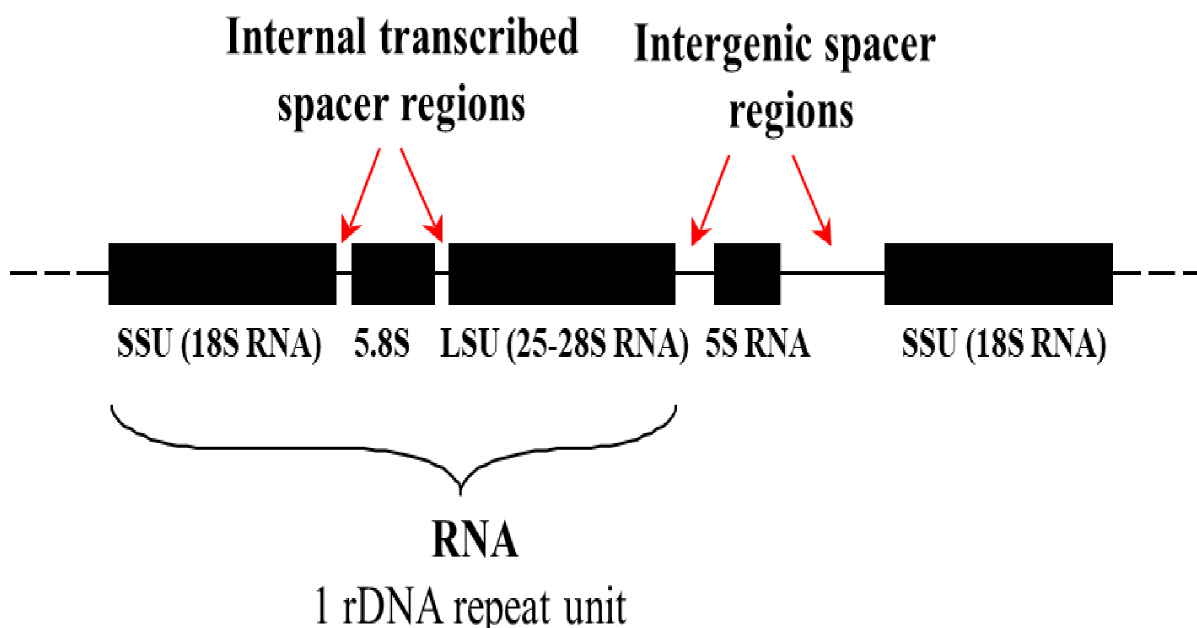

Figure 1: A ribosomal DNA repeat unit showing the ITS and IGS regions. The diagram shows the location of the ribosomal subunits within the repeat unit. The small subunit (18S RNA) to the large subunit (25-28S RNA) consists of one major rRNA transcript (Bruns *et al.*, 1991).

The coding regions of the 18S, 5.8S and 28S nuclear rDNA genes are highly conserved among fungi and they show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Berbee and Taylor, 1992; Swann and Taylor, 1993, 1995; Binder and Hibbett, 2002). Within each repeat unit, the conserved regions are separated by two internal transcribed spacers, ITS I and ITS II, which show higher rates of divergence (Moncalvo *et al.*, 1995b; Perlin and Park, 2001). These ITS regions are now widely sequenced DNA regions in fungi. The variable sequence regions in large (25S) and small (18S) subunits of rDNA genes have also resulted in many molecular approaches which made possible of quick identification of fungal species (Perlin and Park, 2001). Molecular taxonomy of the Ganodermataceae was first performed by Moncalvo *et al.* (1995a, 1995c). They believed that most of the controversy which has been associated with *Ganoderma* systematics in the past might be resolved with the use of molecular techniques to generate novel taxonomic characters and with the use of phylogenetically based classification methods.

Internal transcribed spacer (ITS) region

For environmental sampling and phylogenetic analyses of fungi, plants and animals, both the ITS1 and ITS2 regions of ribosomal DNA have been widely used (Campbell *et al.,* 1993; Downie *et al.,* 2000; Madani *et al.,* 2005; O'Brien *et al.,* 2005). For environmental sampling, ITS is a convenient marker because several taxonomic group-specific primer sets exist for this gene region (Gardes and Bruns 1993; Martin *et al.,* 2004) and it is easily amplified by polymerase

chain reaction (PCR) (Bruns and Shefferson, 2004). For fungi and other microorganisms, DNA-based detection techniques are particularly well suited because of these techniques can identify microbes that exhibit few morphological characters or grow embedded within opaque substrates (Bruns and Shefferson, 2004; O'Brien *et al.*, 2005). Furthermore, DNA-based techniques have the potential to avoid some of the biases associated with community sampling based on reproductive structures or culture-based assays (Bidartondo and Gardes, 2005). The ITS region has been choice for molecular analysis of fungal communities since reported by Gardes and Bruns (1996) who used restriction digests (RFLP) of this region to differentiate species of ectomycorrhizal fungi colonizing individual roots. The derivative fingerprinting techniques, in combination with DNA sequencing of the ITS have been employed to examine the diverse fungal communities such as from roots, soil, air, wood and animal tissues (Jasalavich *et al.*, 2000; Buchan *et al.*, 2002; Dickie *et al.*, 2002; Redecker, 2002; Allmer *et al.*, 2006; Scupham *et al.*, 2006). For molecular diagnosis of diseases caused by fungi and other eukaryotes, the ITS region has become a popular target region (Reiss *et al.,* 1998; Bensoussan *et al.,* 2006; Petti, 2007).

Even though multicopy genes, such as the ITS, are used largely as approximately species-specific markers, yet little is known about the natural variation of these genes within individuals or populations (Avis *et al.,* 2006). Theoretically, concerted evolution homogenizes multicopy genes such as ITS, over time, resulting in low or non-existent variation within individuals and species (Elder and Turner, 1995; Liao, 1999). However, the paradigm of concerted evolution does not always apply (Rooney and Ward, 2005) and ITS variation has been noted within species and individuals from a wide variety of organisms including crustaceans (Harris and Crandall, 2000; Gandolfi *et al.*, 2001), angiosperms (Buckler *et al.*, 1997; Feliner *et al.*, 2004), insects (Fairley *et al.,* 2005), nematodes (Hugall *et al.,* 1999), marine sponges (Worheide *et al.,* 2004) and fungi (Karen *et al.,* 1997; O'Donnell *et al.,* 1998; Aanen *et al.,* 2001; Horton, 2002).

The ITS regions are most important region in fungi for molecular systematics within a genus. The highly conserved ribosomal genes, which flank the ITS regions, are ideal for universal primer targeting and as a result the ITS regions can be amplified by PCR, the sequences can be analysed and compared and evolutionary trees can be produced. The ITS regions in fungi are highly variable and for this reason are useful in differentiating between *Ganoderma* species (Moncalvo *et al.*, 1995a; 1995b). In contrast, the coding regions from nucleotide sequence data from nuclear and mitochondrial rDNA, do not offer enough variation to infer phylogenetic relationships between *Ganoderma* species (Moncalvo, 2000) and therefore only useful at the genus level. There has been many reports available on the analysis of the ITS regions to establish taxonomic relationships within the *Ganoderma* species (Gottlieb *et al.*, 2000; Moncalvo *et al.*, 1995a; Smith and Sivasithamparam, 2000a).

The specific segments of the ITS regions have greater variability than other segments. Moncalvo *et al.* (1995a) have observed that the frequency of nucleotide substitutions was similar in both ITS regions but found that variations were mostly located in the central region of ITS I and close to the terminal in ITS II. They also reported that nucleotide divergence between recently diverged taxa was usually in the ITS II region. This was also observed by Gottlieb *et al.* (2000), who reported that a lower level of resolution of internal phylogenetic branches was obtained from the ITS I region.

Primers for ITS region

Because of its higher degree of variation than other genetic regions of rDNA (SSU and LSU), ITS region is most useful for molecular systematics at the species level and even within species. Within both the ITS and IGS regions, variation among individual rDNA repeats can sometimes be observed. In addition to the standard ITS1+ITS4 primers used by most laboratories, several taxon-specific primers have been described that allow selective amplification of fungal sequences (**Figure 2, Table 1**). Amplification of basidiomycete ITS sequences from mycorrhiza samples was described by Gardes and Bruns (1993).

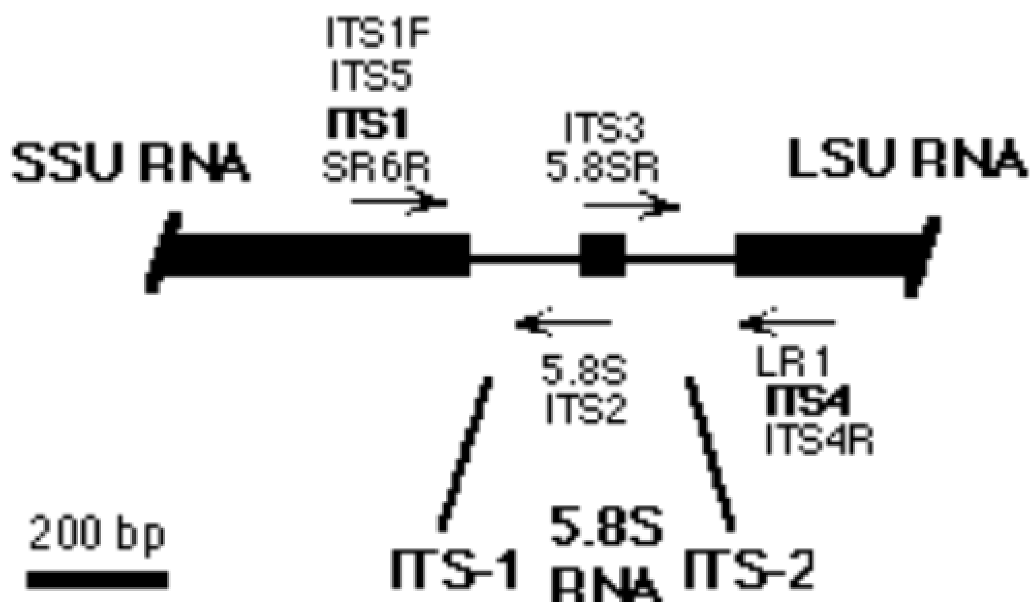

Figure 2: Primers for routine sequencing were showed in bold

Table 1: Different primers for fungal ITS region amplification.

Primer	Sequence (5'-3')	Comments	Reference
ITS1	TCCGTAGGTGAACCTGCGG		White *et al.,* 1990
ITS2	GCTGCGTTCTTCATCGATGC	(similar to 5.8S below)	White *et al.,* 1990
ITS3	GCATCGATGAAGAACGCAGC	(is similar to 5.8SR below)	White *et al.,* 1990
ITS4	TCCTCCGCTTATTGATATGC		White *et al.,* 1990
ITS5	GGAAGTAAAAGTCGTAACAAGG	(is similar to SR6R)	White *et al.,* 1990
ITS1-F	CTTGGTCATTTAGAGGAAGTAA		Gardes and Bruns, 1993
ITS4-B	CAGGAGACTTGTACACGGTCCAG		Gardes and Bruns, 1993
5.8S	CGCTGCGTTCTTCATCG		Vilgalys lab
5.8SR	TCGATGAAGAACGCAGCG		Vilgalys lab
SR6R	AAGWAAAAGTCGTAACAAGG		Vilgalys lab

Endonuclease restriction digestions: Two important techniques that involve digestion of DNA with restriction endonucleases are restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). While restriction enzyme treatment of the PCR amplified ITS regions can discriminate between most *Ganoderma* species (Park *et al.*, 1996), RAPD can be used to differentiate between isolates having identical ITS sequences (Hseu *et*

al., 1996). PCR coupled with RFLP is a useful tool in phylogenetic studies and is now widely used for fungal phylogeny and taxonomy (Hughes *et al.*, 1998; Magee *et al.*, 1987; Miller *et al.*, 1999). Moreover, PCR-RFLP was one of the first molecular approaches to approximate the differences in species. This technique, which cleaves chromosomal DNA using restriction endonucleases followed by separation of the fragments by gel electrophoresis, provides a measurement of the genetic difference and relatedness between the organisms (Perlin and Park, 2001). RAPD is another powerful tool in molecular biology especially in fungal biotechnology. Park *et al*. (1996) analysed twenty eight isolates of *Ganoderma* by PCR-RFLP and RAPD. They found that resulting phenograms of genetic relatedness showed similar patterns by the two different methods, although slightly different bands were observed within the *G. lucidum* by RAPD. Further, Hseu *et al*. (1996) attempted to different isolates of the *G. lucidum* complex using RAPD analysis and found that groupings based on this technique did not distinguish the same clades as ITS data and they suggested that RAPD might be helpful for systematics at the lower taxonomic level that are unresolved by ITS sequence data. Single stranded conformational polymorphism (SSCP) is another technique that also used for taxonomic analysis of *Ganoderma* species. It is advantageous for over restriction analysis because it is less time consuming and more economical (Gottlieb *et al.*, 2000). In spite of being useful in determining variability, taxonomic names determined using SSCP does not corresponding to the current taxonomic status, particularly within the subgenus *Elfvingia* and these groupings were more or less similar with isoenzyme data (Gottlieb *et al.*, 1998).

Isoenzymes

Isoenzymes have been investigated as a means of taxonomic identification of *Ganoderma* species, although this method is not as popular as DNA based techniques because of the need to use comparatively large amounts of fresh material (Smith and Sivasithamparam, 2000b). Gottlieb and Wright (1999a, 1999b) used isoenzymic data to distinguish between groups of *Ganoderma* from the subgenus *Ganoderma* and *Elfvingia* from South America. They observed that many isolates belonging to the same taxa failed to cluster together in the gene trees and in general, correlation between morphological features and isoenzymic patterns could not be established. Therefore the groupings determined by both sets were different (Gottlieb and Wright, 1999a). They concluded that some *Ganoderma* species were misidentified (Gottlieb *et al.*, 1998). However, groupings obtained by phylogenetic analyses of the ITS regions by Gottlieb *et al.* (2000) were almost the same as those determined using isoenzyme data (Gottlieb *et al.*, 1998). Smith and Sivasithamparam (2000b) used phenetic analysis of isoenzymes to examine population and species relationships of *Ganoderma* species in Australia. Their results confirmed the same taxonomic conclusion derived upon analysis of the ITS region (Smith and Sivasithamparam, 2000a), although, two species could not be distinguished from each other highlighting the limitations of using limited numbers of enzyme systems to differentiating the taxa (Smith and Sivasithamparam, 2000b).

Phylogenetic analysis

Taxonomy aims to reflect a natural classification of taxa and molecular data offer a set of objective characters on which to base taxonomic decisions (Buchanan, 2001). The use of phylogenetic programs to analyse such molecular data has rapidly become popular and it is the resulting of phylograms (genetic evolutionary trees) that display monophyletic groups, the members of which share a common ancestor (Buchanan, 2001). The practice of phylogenetic analysis should be conceived as a search for a correct model and as much as a search for the correct tree (Brinkman and Leipe, 2001). The method of phylogenetic inference can be classified into three major groups such as distance methods, maximum likelihood methods and parsimony methods (Nei, 1996). In distance methods, an evolutionary distance is computed from all pairs of sequences and a phylogenetic tree (phylogram) is constructed from the pairwise distances (Nei, 1996). In the case of maximum likelihood methods, maximization of the likelihood is performed for each topology separately and the topology with the maximum likelihood is chosen as an estimate of the true tree topology (Nei, 1996). In maximum parsimony methods, in a

given set of nucleotide sequences the mutational changes occur in all directions among the 4 different nucleotides. The smallest numbers of nucleotide substitutions that explain the entire evolutionary process for the given topology are computed. The topology that requires the smallest number of substitutions is chosen to be the best tree (Nei, 1996). Das *et al.* (2021) carried out the protoplasmic fusion between *Calocybe indica* and *Pleurotus sajor-caju* and molecular characterization of hybrid mushrooms. They found that some of the hybrids contain some non-parental bands confirming the hybridity of newly developed strains. The genome shuffling technique results a specific phenotype improvement by inducing mutation and recursive protoplast fusion (Hospet et al., 2023).

There are reviews available on the statistical methods developed for different models and how these models may affect data sets (Brinkman and Leipe, 2001; Nei, 1996). The reliability and practicality of phylogenetic algorithms, procedures and computational programs are dependent on the structure and size of the data (Brinkman and Leipe, 2001). Some of the debates have been summarised in reviews (Saitou, 1996; Swafford *et al.*, 1996). The particular importance is the issue of phylogenetic analysis of large molecular data sets (Hillis, 1996; Graybeal, 1998; Poe, 1998) and the suggestions that statistical reliability is sensitive to sample size (Felsenstein, 1985; Sanderson and Donoghue, 1989; Bremer, 1994; Farris *et al.*, 1996). Evidence from various studies suggest that increasing sample size generally increases the phylogenetic accuracy (Hillis, 1996; Moncalvo *et al.*, 2000).

CONCLUSION

Protoplast fusion offers a great potential for the improvement of industrially important microorganisms. Protoplast is a naked cell whose cell wall has been usually digested by enzymes; thereby it can be applied to the cell fusion or transformation of nucleus. It is one of the genetic tools to produce hybrids especially when conventional method cannot be achieved. Protoplast fusion can be interspecifically, intraspecifically, intergenerically and even inter-hetero-generically. The stable interspecific protoplasts can successfully developed between Mushroom species. Interestingly, the hybrids are remarkably fast growing and produced high mycelial biomass in culture medium compared to parental strains. The molecular techniques are exact choice to easily identify the genetic relationship and systematic position of the hybrids and parents of mushroom species. In phylogenetic analysis, ITS sequence data of hybrid strains can closely clad with variety of mushroom species. The outcome of an evolutionary study can vary depending on the input data. The consensus phylogenic tree produced by both complete deletion and pairwise deletion models had the same topology and only differed in the statistical support of internal branches.

REFERENCES

Aanen, D.K., Kuyper, T.W., Hoekstra, R.F. 2001. A widely distributed ITS polymorphism within a biological species of the ectomycorrhizal fungus *Hebeloma velutipes*. *Mycological Research,* **105:**284-290.

Allmer, J., Vasiliauskas, R., Ihrmark, K., *et al.*, 2006. Wood-inhabiting fungal communities in woody debris of Norway spruce (*Picea abies* (L.) Karst.), as reflected by sporocarps, mycelial isolations and T-RFLP identification. *FEMS Microbiology Ecology,* **43:**329-340.

Anne, J. 1977. Somatic hybridization between *Penicillium* species after induced fusion of their protoplasts. *Agricultura,* **25:**1-117.

Anne, J. and Peberdy, J.F. 1975. Conditions for induced fusion of fungal protoplasts in polyethylene glycol solutions. *Archives of Microbiology,* **105:**201-205.

Anne, J. and Peberdy, J.F. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. *The Journal of General Microbiology,* **92:**413-417.

Anne, J., Eyssen, H., Desomer, P. 1974. Formation and regeneration of *Penicillium chrysogenum* protoplasts. *Archives of Microbiology,* **98:**159-166.

Anne, J., Eyssen, H., Somer, P. 1976. Somatic hybridisation of *Penicillium roquefortii* with *P. chrysogenum*. *Nature,* **262:**719-721.

Aswini, L., Arunagirinathan, N., Kavitha, M. 2014. Strain improvement of *Pleurotus* species by protoplast fusion. *International Journal of Advancements in Research and Technology,* **3**:32-38.

Avis, P.G., Dickie, I.A., Mueller, G.M. 2006. A 'dirty' business: testing the limitations of terminal restriction length polymorphism (TRFLP) analysis of soil fungi. *Molecular Ecology,* **15:**873-882.

Backmann, B.J. and Bonner, D.M. 1959. Protoplast of *Neurospora crassa*. *Journal of Bacteriology,* **78:**550-556.

Ball, C. and Azevedo, J.L. 1976. Genetic instability in parasexual fungi. In: *Second international symposium on the genetics of industrial microorganisms* (Ed.: Macdonald, K.D.). Academic press, London, pp.243-251.

Berbee, M.L. and Taylor, J.W. 1992. Detecting morphological convergence in true fungi, using 18S rRNA gene sequence data. *Biosystems,* **28:**117-125.

Bensoussan, E., Nasereddin, A., Jonas, F., *et al*., 2006. Comparison of PCR assays for diagnosis of cutaneous Leishmaniasis. *Journal of Clinical Microbiology,* **44:**1435-1439.

Bidartondo, M.I. and Gardes, M. 2005. Fungal diversity in molecular terms: profiling, identification, and quantification in the environment. In: *The fungal community-its organization and role in the ecosystem*. Third edition. (Eds.: Dighton. J., White, J.F., Oudemans, P.). Taylor and Francis, New York. pp.215-239.

Binder, M. and Hibbett, D.S. 2002. Higher-level phylogenetic relationships of homobasidiomycetes (mushroom-forming fungi) inferred from four rDNA regions. *Molecular Phylogenetics and Evolution,* **22:**76-90.

Binding, H. and Weber, H.J. 1974. The isolation, regeneration and fusion of *Phycomyces* protoplasts. *Molecular Genetics and Genomics,* **135:**273-276.

Boizet, B., Flickinger, J.L., Chassy, B.M. 1988. Transformation of *Lactobacillus bulgaricus* protoplasts by bacteriophge DNA. *Applied and Environmental Microbiology,* **54:**3014-3018.

Bremer, K. 1994. Branch support and tree stability. *Cladistics,* **10:**295-304.

Brinkman, F.S.L. and Leipe, D.D. 2001. Phylogenetic Analysis. In: *Bioinformatics: A pratical guide to the analysis of genes and proteins*, Volume 2. (Eds.: Baxevanis, A.D. and Ouellette, B.F.F.). John Wiley & Sons Ltd., Vancouver, pp.323-358; doi: 10.1002/0471223921.ch14.

Bruns, T.B. and Shefferson, R.P. 2004. Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Canadian Journal of Botany,* **82:**1122-1132.

Bruns, T.D. and Szaro, T.M. 1992. Rate and mode differences between nuclear and mitochondrial small subunit rRNA genes in mushrooms. *Molecular Biology and Evolution,* **9:**836-855.

Bruns, T.D., White, T.J., Taylor, J.W. 1991. Fungal Molecular Systematics. *Annual Review Ecology, Evolution and Systematics*, **22:**525-564; doi: 10.1146/annurev.es.22.110191.002521.

Buchan, A., Newell, S.Y., Moreta, J.I.L., *et al.,* 2002. Analysis of internal transcribed spacer (ITS) regions of rRNA genes in fungal communities in a southeastern US salt marsh *Microbial Ecology,* **43:**329-340.

Buchanan, P.K. 2001. A taxonomic overview of the genus *Ganoderma* with special reference to species of medicinal and neutriceutical importance. In: Proceedings of the International Symposium on Ganoderma Science, pp.27-29.

Buckler, E.S., Ippolito, A., Holtsford, T.P. 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics,* **145:**821-832.

Bunyard, B.A., Chaichuchote, S., Nicholson, M.S., *et al*., 1996. Ribosomal DNA analysis for resolution of genotypic classes

of *Pleurotus. Mycological Research*, **100(2)**:143-150.

Campbell, B.C., Steffen-Campbell, J.D., Werren, J.H. 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Molecular Biology,* **2:**225-237.

Chakraborty, B.N. and Kapoor, K. 1990. Transformation of filamentous fungi by electroporation. *Nucleic Acids Research,* **18:**6737.

Chakraborty, U. and Sikdar, S.R. 2008. Production and characterization of somatic hybrids raised through protoplast fusion between edible mushroom strains *Volvariella volvacea* and *Pleurotus florida*. *World Journal of Microbiology and Biotechnology,* **24:**1481-1492.

Chakraborty, U. and Sikdar, S.R. 2009. Intergeneric protoplast fusion between *Calocybe indica* (milky mushroom) and *Pleurotus florida* aids in the qualitative and quantitative improvement of sporophore of the milky mushroom. *World Journal of Microbiology and Biotechnology*, **26**:213-225.

Chandrappa, B.P. and Basavarajappa, M.P. 2017. Enhancement of antagonism through protoplast fusion of *Trichoderma* spp. *Journal of Pure and Applied Microbiology,* **11(1)**:277-284; doi: 10.22207/JPAM.11.1.35.

Cocking, E.C. 1972. Plant cell protoplasts - isolation and development. *Annual Review of Plant Physiology,* **23:**19-50.

Constabel, F. and Kao, K.N. 1974. Agglutination and fusion of protoplasts by polyethylene glycol. *Canadian Journal of* Botany, **52:**1603-1606.

Cramp, F.C. and Lucy, J.A. 1974. Glycerol monooleate as a fusogen for the formation of heterokaryons and interspecific hybrids. *Experimental Cell Research,* **87:**107-110.

Croce, C.M., Sawichi, W., Kritchevsky, D., *et al.*, 1971. Induction of heterokaryocyte, heterokaryocyte and hybrid formation by lysolecithin. *Experimental Cell Research,* **67:**427-435.

Dales, R.B.G. and Croft, J.H. 1977. Protoplast fusion and the isolation of heterokaryons and diploids from vegetatively incompatible strains of *Aspergillus nidulans. FEMS Microbiology Letters,* **1:**201-204.

Das, A., Gokhale, D.V., Peberdy, J.F. 1989. Protoplast fusion and genetic recombination in intra strain and inter-strain crossing in *Aspergillus niger. Enzyme and Microbial Technology,* **11:**2-5.

Das, P., Sikdar, S.R., Samanta, A. 2021. Nutritional analysis and molecular characterization of hybrid mushrooms developed through intergeneric protoplast fusion between *Pleurotus sajor-caju* and *Calocybe indica* with the purpose to achieve improved strains. *World Journal of Microbiology and Biotechnology,* **37**:1-12; doi: 10.1007/s11 274-021-03032-3.

Delorme, D. 1989. Transformation of *Saccharomyces cerevisiae* by electroporation. *Applied and Environmental Microbiology,* **55:**2242-2246.

Dhitaphichit, P. and Pornsuriya, C. 2005. Protoplast fusion between *Pleurotus ostreatus* and *P. djamor*. *Songklanakarin Journal of Science and Technology,* **27:**975-982.

Dickie, I.A., Xu, B., Koide, R.T. 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. *New Phytologist,* **156:**527-535.

Djajanegara, I. and Masduki, A. 2010. Protoplast fusion between white And Brown Oyster Mushrooms. April 2010; doi: 10.21082/ijas. v11n1.2010.p16-23.

Downie, S.R., Katz-Downie, D.S., Spalik, K. 2000. A phylogeny of Apiaceae tribe Scandiceae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany,* **87:**76-95.

Drehmel, D. Moncalvo, J.M., Vilgalys, R.1999. Molecular Phylogeny of Amanita Based on Large-Subunit Ribosomal DNA Sequences: Implications for Taxonomy and Character Evolution. *Mycologia*, **91(4)**:610-618.

Eddy, A.A. and Williamson, D.H. 1957. A method for isolating protoplasts from yeast. *Nature,* **183:**1101-1104.

Eger, G., Eden, G., Wissig, E. 1976. *Pleurotus ostreatus* breeding potential of a new cultivated mushroom. *Theoretical and Applied Genetics,* **47:**155-163.

Eguchi, F. and Higaki, M. 1995. Production of New Species of Edible Mushrooms by Protoplast Fusion Method III. Protoplast Fusion and Analysis of the Fusant between *Pleurotus sajor-caju* and *Mycoleptodonoides aitchisonii*. *Mokuzai Gakkaishi,* **41:**342-348.

Eguchi, F., Fukuzumi, T., Higaki, M. 1993. Production of new species of edible mushrooms by protoplast fusion method. II. Analysis of mycelia and basidiospores of a fusant between *Pleurotus ostreatus* and *Agrocybe cylindracea* (in Japanese). *Mokuzai Gakkaishi,* **39:**825-830.

Eichlerova, I. K., Ruel, L., Homlka, J.P., *et al*., 2000. Ligninolytic characteristics of *Pleurotus ostreatus* strain F6 and its monokaryotic protoplast derivative P19. *Canadian Journal of Microbiology*, **46:**1153-1158.

Elander, R.P., Espenshade, M.A., Pathak, G.G., *et al*., 1973. The use of parasexual genetics in an industrial strain improvement programme with Penicillium chrysogenum. In: *Genetics of industrial microorganisms* (Eds.: Vanek, Z., Hostalex, Z., Cudlin, J.). Elsevier publishing Co., Amsterdam, pp.239-253.

Elder, J.F. and Turner, B.J. 1995. Concerted evolution of repetitive DNA sequences in eukaryotes. *The Quarterly Review of Biology,* **70:**297-320.

Elliott, T.J. *1972*. Sex and the single spore. *Mushroom Science,* **8:**11-18.

Fairley, T.L., Kilpatrick, C.W., Conn, J.E. 2005. Intragenomic hetereogeneity of internal transcribed spacer rDNA in neotropical malaria vector *Anopheles aquasalis* (Diptera: Culicidae). *Journal of Medical Entomology,* **42:**795-800.

Farris, J.S., Albert, V.A., Kallersjo M., *et al*., 1996. Parsimony jackknifing outperforms neighbor-joining. *Cladistics,* **12:**99-124.

Fawcett, P.A., Loder, P.B., Duncan, M.J., *et al*., 1973. Formation and properties of protoplasts from antibiotic producing strains of *Penicllium chrysogenum* and *Cephalosporium acremonium. Journal of General Microbiology,* **79:**293-309.

Felgner, P.L., Gadek, T.R., Holm, M., *et al*., 1987. Lipofection: a highly efficient, lipid mediated DNA transfection procedure. *Proceedings of National Academy of Science* USA, **84:**7413-7417.

Feliner, G., Larena, B.G., Aguilar, J.F. 2004. Fine-scale geographical structure, intra-individual polymorphism and recombination in nuclear ribosomal internal transcribed spacers in *Armeria* (Plumbaginaceae). *Annals of Botany,* **93:**189-200.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* **39:**783-791.

Ferenczy, L. 1976. Some characteristics of intra- and interspecific protoplast fusion products of *Aspergillus nidulans* and *Aspergillus fumigatus*. In: Cell genetics in higher plants (Eds.: Dudits, D., Farkas, G.L., Maliga, P.). Akademiai kiado, Budapest, pp.171-182.

Ferenczy, L. 1981. Microbial protoplasts fusion. In: Genetics as a tool in Microbiology. (Eds.: Glover, S.W. and Hopwood, D.A.). Cambridge University Press, Cambridge, pp.1-134.

Ferenczy, L. 1984. Fungal Protoplast fusion: basic and applied aspects in cell fusion: In: Gene Transfer and Transformation (Eds.: Beer Jr., R.F. and Bassett, E.G.). Raven Press, New York, pp.145-169.

Ferenczy, L., Kavei, F., Szegedi, M. 1975. High frequency fusion of fungal protoplasts. *Experientia,* **31:**1028-1029.

Ferenczy, L., Kavei, F., Zsolt, J. 1974. Fusion of fungal protoplasts. *Nature,* **248:**793-794.

Fournier, P., Provost, A., Bourguignon, C., *et al*., 1977. Recombination after protoplast fusion in the yeast *Candida tropicalis*. *Archives of Microbiology,* **115:**143-149.

Fritsche, G. 1991. A personal view on mushroom breeding from 1957–1991. Genetics and breeding of Agaricus. In: Genetics and breeding of Agaricus, Proceedings of the 1st International Seminar on Mushroom Science (Ed. Van Griensven, L.). Wageningen, pp.3-20.

Fukuda, M., Harada, Y., Imahori, S., *et al*., 1995. Inheritance of mitochondrial DNA in sexual crosses and protoplast cell fusions in *Lentinula edodes*. *Current Genetics,* **27:**550-554.

Fukuda, M., Sendo, I., Imahori, S., *et al*., 2007a. Transmission of mitochondrial plasmids in protoplast cell fusion between compatible monokaryons of *Lentinula edodes*. *Journal of Wood Science,* **53:**264-267.

Fukuda, M., Wakayama, M., Uchida, M., *et al*., 2007b. Introduction of mitochondrial DNA from *Pleurotus ostreatus* into *Pleurotus pulmonarius* by interspecific protoplast fusion. *Journal of Wood Science,* **53:**339-343.

Gandolfi, A., Bonilauri, P., Rossi, V., *et al*., 2001. Intraindividual and intraspecies variability of ITS1 sequences in the ancient asexual *Darwinula stevensoni* (Crustaceae: Ostracoda). *Heredity*, **87:**449-455.

Gardes, M. and Bruns, T.D. 1993. ITS primers for enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rust. *Molecular Ecology,* **2:**113-118.

Gardes, M. and Bruns, T.D. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. *Canadian Journal of Botany,* **74:**1572-1583.

Garcia, M.C. 1992. Cell wall structure and in basidiomycetes protoplast reversion. *World Journal of Microbiology and Biotechnology,* **8:**36-38.

Gascon, S., Ochoa, A.G., Villaneuva, J.R. 1965. Production of yeast and mould protoplasts by treatment with the strepzyme of *Micronospora. Canadian Journal of Microbiology,* **11:**573-580.

Giaja, J. 1919. Emploi des ferments dans les etudes de physiologie cellulaire: Le globule de levure depouille de sa membrane. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales,* **82:**719-720.

Gokhale, D.V. 1992. Fungal protoplast fusion - A tool for breeding industrial strains. *Journal of Scientific and Industrial Research,* **51:**497-506.

Gold, M.H., Cheng, T.M., Alic, M. 1983. Formation, fusion, and regeneration of protoplasts from wild-type and auxotrophic strains of the white rot basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology,* **46:**260-263.

Gottlieb, A.M., Ferrer, E., Wright, J.E. 2000. rDNA analyses as an aid to the taxonomy of species of *Ganoderma*. *Mycological Research,* **104:**1033-1045.

Gottlieb, A.M., Saidman, B.O., Wright, J.E. 1998. Isoenzymes of *Ganoderma* species from southern South America. *Mycological Research,* **102:**415-426.

Gottlieb, A.M. and Wright, J.E. 1999a. Taxonomy of *Ganoderma* from southern South America: subgenus *Elfvingia*. *Mycologica Research,* **103:**1289-1298.

Gottlieb, A.M. and Wright, J.E. 1999b. Taxonomy of *Ganoderma* from southern South America: subgenus *Ganoderma*. *Mycological Research,* **103:**661-673.

Graybeal, A. 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? *Systematic Biology,* **47:**9-17.

Guerra T.I., Martin, I., Gonzales, M.T. 1991. Polyethylene glycol induced

internationalization of bacteria into fungal protoplasts: Electron microscopic study and optimization of experimental conditions. *Applied and Environmental Microbiology,* **57:**1516-1522.

Hamlyn, P.F. and Ball, C. 1979. Recombination studies with *Cephalosporium acremonium*. In: Genetics of industrial microorganisms (Eds.: Sebek, O.K. and Laskin, A.I.). American Society for Microbiology, Washington, pp.185-191.

Harris, D.J. and Crandall, K.A. 2000. Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae); implications for phylogenetic and microsatelite studies. *Molecular Biology and Evolution,* **17:**284-291.

Harris, H. and Watkins, J.F. 1965. Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. *Nature,* **205:**640-646.

Hasegawa, S., Matsui, C., Nagata, T., *et al*., 1983. Cytological study of the introduction of *Agrobacterium tumefaciens* spheroplasts into *Vinca rosea* protoplasts. *Canadian Journal of Botany,* **61:**1052-1057.

Hillis, D.M. 1996. Inferring complex phylogenies. *Nature,* **383:**130-131.

Hoekstra, D. and Kok, J.W. 1989. Entry mechanisms of enveloped viruses. Implications for fusion intracellular membranes. *Bioscience Reports,* **9:**273-305.

Homolka, L., Volakova, I., Nerud, F. 1995. Variability of enzymatic activities in lignolytic fungi *Pleurotus ostreatus* and *Letinus tigrinus* after protoplasting and UV-mutagenization. *Biotechnology Techniques,* **9:**157-162.

Hopple, J.S. and Vilgalys, R. 1994, Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. *Mycologia*, **86:**96-107; doi: 10.1080/00275514.1994.12026378.

Hopwood, D. 1981. Genetic studies with bacterial protoplasts. *Annual Reviews of Microbiology,* **35:**237-272.

Horton, T.R. 2002. Molecular approaches to ectomycorrhizal diversity studies: variation in ITS at a local scale. *Plant Soil,* **244:**29-39.

Hospet, R., Thangadurai, D., Cruz-Martins, N., *et al*., 2023. Genome shuffling for phenotypic improvement of industrial strains through recursive protoplast fusion technology. *Critical reviews in food science and nutrition* **63(17)**:2960-2969; doi: 10.1080/10408398.2 021.1983763.

Hseu, R.S., Wang, H.H., Wang, H.F., *et al.*, 1996. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Applied and Environmental Microbiology,* **62:**1354-1363.

Hugall, A., Stanton, J., Moritz, C. 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution,* **16:**157-164.

Hughes, K.W., Toyohara, T.L., Petersen, R.H. 1998. DNA sequence and RFLP analysis of *Pleurotopsis longinqua* from three disjunct populations. *Mycologia,* **90:**595-600.

Iizuka, H. and Mienki, S. 1977. Studies on the genus *Monascus*. Purification and properties of two forms of glucoamylase from *Monascus kaoliang* Nov. SP. F-l. *Journal of General and Applied Microbiology,* **23:**217-230.

Issac, S. and Gokule, A.V. 1982. Autolysis: A tool for protoplast production from *Aspergillus nidulans*. *Transactions of the British Mycological Society*, **3**:389-394; doi: 10.1016/s0007-1536(82) 80147-2.

Jasalavich, C.A., Ostrofsky, A., Jellison, J. 2000. Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. *Applied and Environmental Microbiology,* **66:**4725-4734.

Jia, J.H., Buswell, J.A., Peberdy, J.F. 1997. Transformation of the edible fungi, *Pleurotus ostreatus* and *Volvariella volvacea. Mycological Research,* **7:**876-880.

Jintao, L., Linling, L., Lin, X., *et al.,* 2023. Interspecific Hybridization between *Ganoderma lingzhi* and *G. resinaceum* by PEG-Induced Double-Inactivated Protoplast Fusion. *Horticulturae 2023*, **9(10)**:1129; doi: 10.3390/horticulturae9101129.

Kabuki, M., Kasai, Y., Ando, A., *et al.,* 1984. Rapid method for converting fungal cells into protoplasts with high regeneration frequency. *Experimental Mycology,* **8:**386-390.

Kang, L., Xiong, R., Jin, L., *et al*., 2019. The research of protoplast fusion of *Flammulina velutipes* and *Pleurotus ostreatus*. *Food Research and Development*, **40(19)**:210-214; doi: 10.12161/j .issn.1005-6521.2019.19.038.

Kao, K.N. and Michayluk, M.R. 1974. A method for high frequency intergeneric fusion of plant protoplasts. *Planta* (Berl.), **115:**355-367.

Karen, O., Hogberg, N., Dahlberg, A., *et al.,* 1997. Inter and intra-specific variation in the ITS region of ectomyocrrhizal fungi in Fennoscandia as detected by endonuclease analysis. *New Phytologist,* **136:**313-325.

Keller, W.A. and Melchers, G. 1973. The effect of high pH and calcium on Tobacco leaf protoplast fusion. *Zeitschrift für Naturforschung,* **28:**737-741.

Keqing, Q., Zhengxiang, Q., Anran X., *et al*., 2023. Interspecies hybridization between *Auricularia cornea* cv. Yu Muer and *Auricularia heimuer* cv. Bai Muer through protoplast fusion. *Frontiers in Microbiology*, **14**; doi: 10.3389/fmicb.2023.128 0420.

Kiguchi, T. and Yanagi, S.O. 1985. Intraspecific Heterokaryon and Fruit Body Formation in *Coprinus macrorhizus* by Protoplast fusion of Auxotrophic Mutants. *Applied Microbiology and Biotecnology,* **22:**121-127.

Kirimura, K., Imura, M., Lee, S.P., *et al*., 1989. Intergeneric hybridization between *Aspergillus niger* and *Trichoderma viride* by protoplast fusion. *Agricultural and Biological Chemistry,* **53:**1589-1596.

Kirimura, K., Itohiya, Y., Matsno, Y., *et al*., 1990. Production of cellulase and citric acid by the intergeneric fusants obtained via protoplast fusion between *Aspergillus niger* and *Trichoderma viride. Agricultural and Biological Chemistry,* **54:**1281-1283.

Kirimura, K., Nakajima, J., Lee, S., *et al*., 1988. Citric acid production by the diploid strains of *Apergillus niger* obtained by protoplast fusion. *Applied Microbiology and Biotechnology,* **27:**504-506.

Kitamoto, Y., Mori, N., Yamamoto, M., *et al*., 1988. A simple method for protoplast formation and improvement of protoplast regeneration from various fungi using an enzyme from *Trichoderma harzianum*. *Applied Microbiology and Biotechnology,* **28:**445-450.

Kiyohara, H., Watanabe, T., Imai, J., *et al*., 1990. Intergeneric hybridization between *Monascus anka* and. *Aspergillus oryzae* by protoplast fusion. *Applied Microbiology and Biotechnology,* **33:**671-676.

Krishnapriya, P. J. and Geetha, D. 2020. Protoplast fusion between *Pleurotus opuntiae* and *Pleurotus cystidiosus*. *Mushroom Research*, **29(2)**:181-194; doi: 10.36036/MR.29.2.2020. 116258.

Kumari, J. and Panda, T. 1993. Improved technique on reversion of mycelial protoplast of *Trichoderma reesei* into mycelia. *Bioprocess Engineering,* **8:**287-294.

Liao, D. 1999. Concerted evolution: molecular mechanism and biological implications. *The American Journal of Human Genetics*, **64:**24-30.

Lasker, B.A. and Borgia, P.T. 1980. High frequency heterokaryon formation by

Mucor racemosus. Journal of Bacteriology, **141:**565-569.

Lazar, G.B. 1983. Recent developments in plant protoplast fusion and selection technology. *Experientia Supplementum*, **46:**61-68.

Liang, Z.R. and Chang, S.T. 1989. A study on intergeneric hybridization between *PIeurotus sajor-caju* and *Schizophyllum commune* by protoplast fusion. *Mushroom Science*, **12:**125-137.

Lopez B.F., Garcia, A.I., Villaneuva, J.R. 1966. Observation on the protoplasts of *Fusarium culmorum* and on their fusion. *Journal of General Microbiology,* **45:**127-134.

Lucy, J.A., Ahkong, Q.F., Cramp, F.C., *et al.,* 1971. Cell fusion without viruses. *Biochemical Journal,* **124:**469-478.

Lurquin, P.F. 1979. Entrapment of plasmid DNA by liposomes and their interaction with plant protoplasts. *Nucleic acids Research,* **6:**3773-3784.

Madani, M., Subbotin, S.A., Moen, M. 2005. Quantitative detection of the potato cyst nematode, *Globodera pallida*, and the beet cyst nematode, *Heterodera schachtii*, using real-time PCR with SYBR green I dye. *Molecular Cell Probes,* **19:**81-86.

Maeta, K., Koike, A., Mukaiyama, H., *et al.,* 2008. Preparation and crossing of mating-capable monokaryons via protoplasting of the dikaryotic mycelia of a mycorrhizal mushroom, *Lyophyllum shimeji*. *Journal of Wood Science,* **54:**337-340.

Magee, B.B., D'Souza, T.M., Magee, P.T. 1987. Strain and species identification by restriction fragment length polymorphisms in the ribosomal DNA repeat of *Candida* species. *Journal of Bacteriology,* **169:**1639-1643.

Makins, J.F. and Holt, G. 1981. Liposome mediated transformation of *Streptomycetes* by chromosomal DNA. *Nature,* **293:**671-673.

Manczinger, L. and Ferenczy, L. 1985. Somatic cell fusion of *Trichoderma reesei r*esulting in new genetic combinations. *Applied Microbiology and Biotechnology,* **22:**72-76.

Martin, F.N., Tooley, P.W., Blomquist, C. 2004. Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. *Phytopathology,* **94:**621-631.

Matsumoto, M., Eguchi, F., Higaki, M. 1997. Polyethylene Glycol Centrifugal Fusion between *Pleurotus ostreatus* and *Pleurotus sajor-caju*. *Mokuzai Gakkaishi,* **43:**215-219.

Merrick, M.J. 1976. Hybridization and selection for penicillin production in *Aspergillus nidulans* - a biometrical approach to strain improvement. In: Second international symposium on the genetics of industrial micro- organisms (Ed.: Macdonald, K.D.). Academic press, London. pp.229-242.

Miller, R.N.G., Holderness, M., Bridge, P.D, *et al*., 1999. Genetic diversity of *Ganoderma* in oil palm plantings. *Plant Pathology,* **48:**595-603.

Mitchell, A.D. and Bresinsky, A. 1999. Phylogenetic relationships of *Agaricus* species based on ITS-2 and 28S ribosomal DNA sequences. *Molecular Mycologia*, **91**:811-819; doi: 10.1080/002755 14.1999.12061086.

Mitchell, J.I., Robertsm P.J., Moss, S.T. 1995. Sequence or structure?: A short review on the application of nucleic acid sequence information to fungal taxonomy. *Mycologist,* **9:**67-75.

Moncalvo, J.M., Lutzoni, F.M., Rehner, S.A., *et al.,* 2000. Phylogenetic relationships of agaric fungi based on nuclear large subunit ribosomal DNA sequences. *System Biology,* **49:**278-305.

Moncalvo, J.M., Wang, H.F., Hseu, R.S. 1995a. Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences. Comparison with traditional taxonomic characters. *Mycological Research*, **99**:1489-1499.

Moncalvo, J.M., Wang, H.F., Hseu, R.S. 1995b. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia,* **87:**223-238.

Mosunova, O.V., Vasina, D.V., Tyazhelova, T.V., *et al*., 2016. Preparation of protoplasts of the fungus *Trametes hirsuta* 072 and study of the effect of antioxidants on their formation and regeneration. *Applied Biochemistry and Biochemistry*, **52(3)**:311-315.

Nagata, T. 1984. Interaction of liposomes and protoplasts as a model system of protoplast fusion. In: Cell fusion: Gene transfer and transformation (Eds.: Beers, R.F. and E.G. Bassett). Raven Press, New York, pp.217-226.

Nagata, T., Eibl, H., Melchers, G. 1979. Fusion of plant protoplasts induced by positively charged synthetic phospholipids. *Zeitschrift für Naturforschung*, **34:**460-462.

Nagata, T. 1978. A novel cell-fusion method of protoplast by polyvinyl alcohol. *Naturwissenschafte,* **65:**263.

Nei, M. 1996. Phylogenetic analysis in molecular evolutionary genetics. *Annual Review of Genetics,* **30:**371-403.

Noel, T. and Labarere, J. 1994. Homologous transformation of the edible basidiomycete *Agrocybe aegerita* with the URA1 gene: characterization of integrative events and of rearranged free plasmids in transformants. *Current Genetics,* **25:**432-437.

O'Brien, H.E., Parrent, J.L., Jackson, J.A. *et al*., 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied and Environmental Microbiology,* **71:**5544-5550.

O'Donnell, K., Cigelnik, E., Nirenberg, H.I. 1998. Molecular systematic and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia,* **90:**465-493.

Ogawa, K., Ohara, H., Toyama, N. 1988. Interspecific hybridization of *Aspergillus awamori* Var. *kawachi* and *Aspergillus oryzae* by protoplast fusion. *Agricultural and Biological Chemistry,* **52:**1985-1991.

Ogawa, K., Ohara, H., Koide, T. *1989*. Intraspecific hybridization of *Trichoderma reesei* by protoplast fusion. *Journal of Fermentation and Bioengineering,* **67**:207-209.

Okada, Y., Suzuki, T., Hosaka, Y. 1957. Interaction between influenza virus and Ehrilch's tumor cells. III. Fusion phenomenon of Ehrlich's tumor cells by the action of HVJ strain. *Medical Journal of Osaka University,* **7:**709-717.

Park, D.S., Ryu, Y.J., Seok, S.J., *et al*., 1996. Rapid PCR-RFLP. The genetic relationship analysis of *Ganoderma* spp. using the PCR-RFLP and RAPD. *Journal of Agricultural Science,* **38:**251-260.

Peberdy, J.F. 1976. Isolation and properties of protoplast from filamentous fungi. In: Microbial and Plant protoplast. (Eds.: Peberdy, J.F., Rose, A.H., Rogers H.J., *et al*.,). Academic press, London, pp.39-50.

Peberdy, J.F. 1980. Protoplast fusion: A new approach to interspecies genetic manipulation and breeding in fungi. In: Advances in protoplast research (Eds.: Ferenczy I. and Farkas, G.L.). Proceedings of 5th International Protoplast Symposium, Pergamon, Oxford, pp.63-72.

Peberdy, J.F. 1983. Genetic recombination in fungi following protoplast fusion and transformation. In: Fungal differentiation (Ed.: Smith, J.E.). Marcel Dekker, New York, pp.559-581.

Peberdy, J.F. 1987. Development in protoplast fusion in fungi. *Microbiological Sciences,* **4:**108-114.

Peberdy, J.F. 1991. Fungal protoplasts. In: More Gene Manipulations in Fungi (Eds.: Bennett, J.W. and Lasure, L.L.). Academic Press, Inc. California, pp.307-318.

Peberdy, J.F. and Fox, H.M. 1993. Protoplast technology and edible mushrooms. In: Genetics and breeding of edible mushrooms (Eds.: Chang, S.T., Buswell J.A., Miles, P.G.). Gordon and Breach, USA, pp.125-155.

Peberdy, J.F. and Gibson, R.K. 1971. Regeneration of *Aspergillus nidulans* protoplast. *Journal of General Microbiology,* **69:**325-330.

Perlin, D.S. and Park, S. 2001. Rapid identification of fungal pathogens: molecular approaches for the new millennium. *Reviews in Medical Microbiology,* **12:**S13-S20.

Petti, C.A. 2007. Detection and identification of microorganisms by gene amplification and sequencing. *Clinical Infectious Disease,* **44:**1108-1114.

Poe, S. 1998. The effect of taxonomic sampling on accuracy of phylogeny estimation: test case of a known phylogeny. *Molecular Biology and Evolution,* **15:**1086-1090.

Porselvi, A. and Ramasamy, V. 2020. Strain improvement of *Pleurotus eous* and *Pleurotus florida* by protoplast fusion. *Journal of Information and Computational Science,* **10(3)**:1537-1547.

Power, J.B., Cummins S.E., Cocking, E.C. 1970. Fusion of isolated plant protoplasts. *Nature,* **225:**1016-1018.

Radford, A., Pope, S., Sazci, A., *et al.,* 1981. Liposome mediated genetic transformation of *Neurospora crassa*. *Molecular and General Genetics,* **184:**567-569.

Raman, J., Jang, K.Y., Oh, Y.L., *et al.,* 2021. Interspecific hybridization between *Ganoderma lingzhi* and *G. applanatum* through protoplast fusion. *World Journal of Microbiology and Biotechnology,* **37**:1-17; doi: 10.1007/s11274-021- 03084-5.

Raper, C.A., Raper, J.R., Miller, R.E. 1972. Genetic analysis of the lifecycle of *Agaricus bisporus*. *Mycologia,* **64:**1088-1117.

Redecker, D. 2002. Molecular identification and phylogeny of arbuscular mycorrhizal fungi. *Plant Soil,* **244:**67-73.

Reiss, E., Tanaka, K., Bruker, G., *et al.,* 1998. Molecular diagnosis and epidemiology of fungal infections. *Medical Mycology,* **36:**249-257.

Revathi, R. and Lalithakumari, D. 1992. *Venturia inaequalis*: A novel method for protoplast isolation and regeneration. *Journal of Plant Diseases and Protection,* **100:**211-219.

Reyes, R.G., Eguchi, F., Higaki, T.I.M. 1998. Regeneration of protoplasts from hyphal strands of *Volvariella volvacea. Journal of Wood Science,* **44:**401-407.

Rooney, A.P. and Ward, T.J. 2005. Evolution of a large ribosomal RNA multigene family in filamentous fungi: birth and death of a concerted evolution paradigm. *Proceedings of National Academy of Science,* **102:**5084-5089.

Ruiz-Herrara, J. and Bartnicki-Garcia, S. 1976. Proteolytic activation and inactivation of chitin synthetase from *Mucor rouxii*. *Journal of General Microbiology,* **97:**241-249.

Saitou, N. 1996. Reconstruction of gene trees from sequence data. *Methods in Enzymology,* **266:**427-449.

Sanderson, M.J. and Donoghue, M.J. 1989. Patterns of variation in levels of homoplasy. *Evolution,* **43:**1781-1795.

Sandri, G.R.M., Goldin, A.L., Levine, M., *et al*., 1983. High efficiency transfer of DNA into eukaryotic cells by protoplast fusion. *Methods in Enzymology,* **101:**402-411.

Santos, T.M.C. and Demelo, I.S. 1991. Preparation and regeneration of protoplasts of *Talaromyces flavus*. *Revista Brasileira de Genética*, **14:**335-340.

Scupham, A.J., Presley, L.L., Wei, B., *et al*., 2006. Abundant and diverse fungal microbiota in the murine intestine. *Applied and Environmental Microbiology,* **72:**793-801.

Selvakumar, P., Rajasekar, S., Anam, G.B., *et al.,* 2015. Improving biological efficiency of *Pleurotus* strain through protoplast fusion between *P. ostreatus* var. *florida* and *P. djamor* var. *roseus. Food Science and Biotechnology,* **24(5**):1741-1748; doi: 10.1007/s10068-015-022 6-5.

Singh, R.I., Aarti, K., Singh, S.S. 2007. Formation of interspecies fusants of *Agaricus bisporus* and *Agaricus bitorquis* mushroom by protoplast fusion. *Indian Journal of Microbiology,* **47:**369-372.

Sivan, A., Harman, G.E., Stasz, T.E. 1990. Transfer of isolated nuclei into protoplasts of *Trichoderma harzianum. Applied and Environmental Microbiology*, **56:**2404-2409.

Smith, B.J. and Sivasithamparam, K. 2000a. Internal transcribed spacer ribosomal DNA sequence of five species of *Ganoderma* from Australia. *Mycological Research,* **104:**943-951.

Smith, B.J. and Sivasithamparam, K. 2000b. Isozymes of *Ganoderma* species in Australia. *Mycological Research,* **104:**952-961.

Sonnenberg, A.S.M., Wessels, J.G., Van Griensven, L.J. 1988. An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis*. *Current Microbiology,* **17:**285-291.

Stasz, T.E. and Harman, G.E. 1990. Nonparental progeny resulting from protoplasts fusion in *Trichoderma* in the absence of parasexuality. *Experimental Mycology,* **14:**145-159.

Stewart, G.G. 1981. The genetic manipulation of industrial yeast strains. *Canadian Journal of Microbiology*, **27:**973-990.

Sun, L., Cai, H., Xu, W., *et al.,* 2001. Efficient Transformation of the Medicinal Mushroom *Ganoderma lucidum*. *Plant Molecular Biology Reports,* **19:**383a-383j.

Sunagawa, M. 1992. Interspecific Heterokaryon Formation between *Auricularia auricula-judae* and *Auricularia polytricha* by Electrical Protoplast Fusion. *Research Bulletins of College Experimental Forests,* **49:**219-260.

Sunagawa, M. and Miura, K. 1992. Interspecific heterokaryon formation between *Auricularia auricular-judae* and *A. polytricha* by electrical protoplast fusion using two metabolic inhibitors. *Transactions of the Mycological Society of Japan,* **33:**375-383.

Sunagawa, M., Miura, K. Ohmasa, M., *et al*., 1992. Intraspecific heterokaryon formation by protoplast fusion of auxotrophic mutants of *Auricularia polytricha*. *Mokuzai Gakkaishi*, **38:**386-392.

Sunagawa, M., Ohmasa, M., Shino, Y., *et al*., 1989. Isolation and reversion of protoplasts of *Auricularia auricula-judae* and *Auricularia polytricha*. *Mokuzai Gakkaishi*, **35:**1131-1138.

Sunagawa, M., Tamai, Y. Neda, H., *et al*., 1995. Application of random amplified polymorphic DNA (RAPD) markers I. Analyses of fusants in edible mushrooms. *Mokuzai Gakkaishi,* **41:**945-948.

Swafford, D.L., Olsen, G.J., Waddell, P.J. *et al*., 1996. Phylogentic inference. In: *Molecular Systematics* (Eds.: Hillis, D.M., Moritz, C., Mable B.K.). Sinauer Associates, Sunderland, Massachusetts, pp.407-514.

Swann, E.C. and Taylor, J.W. 1993. Higher taxa of basidiomycetes: an 18S rRNA gene perspective. *Mycologia,* **85:**923-936.

Swann, E.C. and Taylor, J.W. 1995. Phylogenetic perspectives on basidiomycete systematics: evidence form the 18S rRNA gene. *Canadian Journal of Botany,* **73:**S862-S868.

Takamatsu, S., Hirata, T., Sato, Y., *et al.,* 1999. Phylogenetic relationships of *Microsphaera* and *Erysiphe* section *Erysiphe* (powdery mildews) inferred from the rDNA ITS sequences. *Mycoscience*, **40**:259-268.

Takehara, T., Kumata, A., Aono, S. 1993. Interspecific Protoplast Fusion between Some *Pleurotus* Species Using Auxotrophic Mutants. *Mokuzai Gakkaishi*, **39:**855-859.

Tashpulatov, Z.H., Shulman, T.S. Baibaev, B.G., *et al*., 1991. Protoplast formation and regeneration by cellulolytically active fungus *Trichoderma harizianum. Microbiologia,* **60:**541-545.

Tokimoto, K., Fukuda, M., Matsumoto, T., *et al*., 1998. Variation of fruiting body productivity in protoplast fusants between compatible monokaryons of *Lentinula edodes. Journal of Wood Science,* **44:**69-472.

Toyoda, H., Hirai, T., Sumiya, H., *et al*., 1984. Preparation and cell wall regeneration of protoplasts of *Lentinus edodes. Memoirs of the Faculty of Agriculture of Kinki University,* **17:**121-130.

Toyomatsu, T., Matsumoto, T., Mori, K. 1986. Interspecific protoplast fusion between *Pleurotus ostreatus* and *Pleurotus salmoneostramineus*. *Agricultural and Biological Chemistry,* **50:**223-225.

Toyomatsu, T. and Mori, K.I. 1987. Intra- and Interspecific Protoplast Fusion between Some *Pleurotus* Species. *Agricultural and Biological Chemistry,* **51:**935-937.

Tschen, J.S.M. and Li, I.F. 1994. Optimization of formation and regeneration of protoplasts from biocontrol agents of *Trichoderma* species. *Mycoscience,* **35:**257-263.

Verma, R.N., Yadav, M.C., Dhar B.L., *et al*., 2000. Strategies for genetic improvement of mushrooms future perspectives. *Mushroom Research,* **9:**1-10.

Vijaya Palani, P. and Lalithakumari, D. 1999. Inhibition of *Venturia inaequalis* by genetically improved *Trichoderma longibrachiatum* strains. *Journal of Plant Diseases and Protection*, **106(5):**460-465.

Wallin, A., Glimelius, K., Ericksson, T. 1974. The induction and aggregation of *Daucus carota* protoplasts by polyethylene glycol. *Zeitschrift für Pflanzenphysiologie,* **74:**64-80.

White, T.J., Bruns, T., Lee, S., *et al*., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (Eds.: Innis, M.A., Gelfand, D.H., Sninsky J.J., *et al*.,). Academic Press, Inc., New York, pp.315-322.

Wilmotte, A., Van de Peer, Y., Goris, A., *et al*., 1993. Evolutionary relationships among higher fungi inferred from small ribosomal subunit RNA sequence analysis. *Systematic and Applied Microbiology,* **16:**436-444.

Woestemeyer, A. and Woestemeyer, J. 1998. Fungal protoplasts: relics or modern objects of molecular research? *Microbiological Research,* **153:**97-104.

Worheide, G., Nichols, S.A., Goldberg, J. 2004. Intragenomic variation of the rDNA internal transcribed spacers in sponges (*Phylum Porifera*): implications for phylogenetic studies. *Molecular Phylogenetics and Evolution,* **33:**816-830.

Yamada, O., Magae, Y., Kashigana, Y., *et al*., 1983. Preparation and regeneration of mycelial protoplasts on *Collybia velutipes* and *Pleurotus ostreatus. European Journal of Applied Microbiology and Biotechnology*, **17:**298-300.

Yan, P.S., Jiang, J.H., Cui, W.S. 2004. Characterization of protoplasts prepared from the edible fungus, *Stropharia rugoso-annulata*. *World Journal of Microbiology and Biotechnology,* **20:**173-177.

Yan, P.S., Li, G., Luo, X., *et al*., 2002. Introduction of foreign drug-resistant gene into *Lentinula edodes*. *Acta Edulis Fungi,* **9:**6-9.

Yan, Z.H., Rogers, S.O., Wang, C.J.K. 1995. Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. *Mycologia,* **87:**72-83.

Yoo, Y.B. and Cha, D.Y. 1993. Gene transfer in edible fungi using protoplasts. In: Genetics and Breeding of Edible Mushrooms (Eds.: Chang, S.T., Buswell, J.A., Miles, P.G.). Gordon and Breach Science Publishers, Philadelphia, pp.157-191.

Yue, H. and Alessandra, Z. 2024. Status and prospects of research on edible fungi protoplast fusion technology. *Archives,* **53**; doi: 10.6092/ issn.2531-7342/19476.

Zhang, C.K., Liang, Y., Qin, H.M. 1994. Study on regeneration conditions of monokaryotic mycelium 9101(12) of *Lentinus edodes*. *Journal of Microbiology,* **5:**9-14.

Zang, J.M., Zheng, Y.P., Chen, M.Y. 1992. Study on intergenetic electrofusion of protoplasts between *Lentinus* and *Pleurotus*. *Acta Botanica Yunnanica,* **14:**283-288.

Zhao, J. and Chang, S.T. 1996. Intergeneric Hybridization between *Pleurotus ostreatus* and *Schizophyllum commune* by PEG-induced Protoplast Fusion and Electro-fusion. *World Journal of Microbiology and Biotechnology,* **12:**573-578.

Zhao, J. and Chang, S.T. 1997. Interspecific hybridization between *Volvariella volvacea* and *V. bombycina* by PEG-induced protoplast fusion. *World Journal of Microbiology and Biotechnology,* **13:**145-151