8 DISCUSSION

A dendrogram constructed from molecular data reveals diversity patterns within the culture collection studied (COLPOS), facilitating both management and genetic improvement. The clustering based on sequence analysis allowed the separation of 10 strains in three major groups with different geographic origins. This information will be essential for shiitake breeding programs aiming at high yields and fruit-body quality.

The dendrogram topology derived from the ITS sequences from international databases suggests that there are at least 5 major lineages of shiitake in several geographic locations. According to the genetic analysis with an expanded *Lentinula* rDNA database, the strains at the COLPOS culture collection belong to two of these worldwide groups: group I (*L. edodes* in Japan, Hong Kong, North Korea) and group II (*L. boryana* in America). These results showed a fundamental genetic base for further improvement.

ITS presents low levels of conservation so it is not surprising that substantial intraspecific heterogeneity was observed. High levels of similarity (e.g. CP-9, Japan; and CP-7, Hong Kong) indicate a common regional origin of the populations from which strains were isolated. Alternative explanations may be that the isolates sampled are the product of a rapid population expansion over a large geographic area or from breeding programs to develop shiitake cultivation [21].

8.1 COMPARISON WITH OTHER STUDIES

ITS-based phylogenetic trees obtained were compared with trees derived from different data sets. Analyses of ITS and other nuclear rDNA sequences have previously suggested that there are seven species of *Lentinula* worldwide, which occur in two main clades, one in

the New World and another one in the Old World (mainly Asia) [22]. The results shown here are consistent with previously observed polymorphisms (mtDNA, rRNA, etc.) that demonstrated distinct intraspecific groups which are genetically divergent at both mitochondrial and nuclear genome levels. Its geographical distribution ranges from eastern Asia to Oceania. Significant variation is detected in morphological characters of fruiting bodies among the strains from these geographically separated regions [22].

Other studies have also shown wide genetic variation numbers. The proportion of polymorphic ALLOZYME-encoding loci is relatively high in shiitake [23]. RFLP analyses of IGRs of the rDNA repeat have confirmed the results of allozyme analysis. Data derived from enzymatic amplification of the IGR of *Lentinula boryana* have shown high levels of genetic variation. Such high levels of variation may be indicative of a large population size, confirmed in this study (distribution ranged from North America to South America), with distinct clades in Mexico, Venezuela, Brasil, Costa Rica and USA.

In an analysis of ITS regions, Kwan *et al*. (1992) and Molina *et al*. (1992) found little or no variation in selected isolates of shiitake [23]. In this study, the ITS variability obtained can be explained by differing geographic origins. Strains were in fact selected on the basis of their origin, including both Asia and America.

Phylogenetic relationships among shiitake isolates based on analysis of ITS sequences carried out by Hibbett et al. indicated five groups (1. America / 2. Japan–China / 3. New Zealand / 4. Papua New Guinea–Australia / 5. Nepal–China). *L. edodes* dominated Japan, China and Nepal, *L. lateritia* dominated Papua New Guinea and Australia, and *L. boryana* dominated America [24]. The present study found similar results, with *L. lateritia* confined to Australia and Papua New Guinea, *L. edodes* occurring in Japan, China, Thailand, etc., and *L. boryana* circumscribed in America.

Fukuda and Ono's study with several rDNA regions (18S, 26S, and ITS) showed that shiitake strains could be divided into two major groups: Japan–Thailand group and Papua New Guinea–New Zealand group [25]. The present study divided *L. edodes* strains in different groups: Japan–Hong Kong–China and Japan–North Korea–China–Thailand–Nepal, elucidating for more genetic diversity.

Scientific classification remains controversial, a problem expressed in other studies. Sequence distance analysis results and reconstruction of phylogenetic relationships in this project advocate for the phylogenetic species concept since *L. edodes* strains clustered in different groups. Intercompatibility within different *L. edodes* strains around the world can be explained by their common origin or the homogeneity introduced by commercial farm workers. There are definitely separate lineages in shiitake, even though organisms in these lineages have retained the ability to interbreed.

8.2 GENETIC IMPROVEMENT PROPOSAL

The phylogenetic analysis described herein may prove useful in the breeding of new and improved strains of this commercially important mushroom species. Previous studies on genetics of this mushroom species have suggested that fruiting is controlled by multiple genes and that increased yields are obtained through hybrids produced by crossing strains that are genetically divergent. The most comprehensive mating study available in *Lentinula* to date is that of Shimomura *et al*. (1992), who found interstrain compatibility among isolates from Japan, Thailand, Borneo, Nepal, Papua New Guinea, and New Zealand [26]. Therefore, natural isolates from geographically different origins are potential genetic resources for improving new cultivars.

Conventional methods of strain improvement are an efficient way to achieve strain improvement through controlled crossing, and progeny selection. The desired result should be clearly defined in terms of desired traits expressed within a chosen set of conditions employed consistently throughout the breeding programme. The primary requisite is knowledge of the life cycle, knowledge of the pattern of inheritance, mating type specificities and nutritional requirements or enzyme variants. In second place, monokaryons must be distinguished from heterokaryons (e.g. clam connections). Finally, there must exist a means of separating the nuclear types to regenerate the two component monokaryons [27]. Most of these information and techniques are available for shiitake.

Improvement of heterothallic species, such as *Lentinula*, would best be achieved by making crosses between strains with contrasting characteristics or by selection within a given strain. Strains from geographically diverse regions can be involved in an improvement program since a wide range of DNA variation and/or mating type polymorphisms are present in isolates collected from nature. It would obviously be advantageous if characteristics could first be selected in monokaryons. Selection could also be readily practised among dikaryons. Progeny from the best dikaryons would be inter-mated and the resulting dikaryons again assessed for agronomic potential. Important simply inherited characteristics could be introduced into existing cultivars by back-crossing and recurrent selection [16].

As in the breeding of other organisms such as maize, one might expect reduced vigour with continued back-crossing or selfing; but the mating of the final product selected at the end of two different series of crosses should re-establish hybrid vigour. The use of a variety

of *Lentinula* strains, bred separately for yield, would reduce the risk of increased susceptibility to certain factors such as pathogens [27].

Since only the mating type genes are involved in control of incompatibility reactions in *L. edodes*, CROSS-BREEDING using different ecotypes would be a practical way to introduce commercially desirable characteristics into cultivated strains. Taking into consideration the average production and biological efficiency of the strains already studied by Pallach at the COLPOS and the dendrogram produced in this study, several crossings are proposed: CP-188xCP-95; CP-188xCP-13, CP-7xCP-189, CP-188xCP-7, CP-13xCP-7, among others. If closely related strains have been used for fusion (eg. CP-9XCP-7), the hybrid will frequently be fertile, and further breeding can be carried out from single-spore cultures. The classical methods for the genetic improvement of the cultivated edible mushrooms involve one or several cycles of crosses and fruiting trials [28].

During the last decade several major breakthroughs have been achieved in mushroom biotechnology, which greatly enhanced classical mushroom breeding. Following the measure of genetic diversity, DNA-based technologies such as RFLPs and RAPDs have allowed the isolation of monokaryons, the determination of inheritance of nuclear and mitochondrial markers, and the production of genetic linkage maps [29]. The evolution from classical mushroom breeding to MOLECULAR BREEDING has already accelerated breeding activities and will greatly benefit the mushroom breeding programs. Mushroom breeding companies have currently invested in these DNA-based technologies and are adapting their breeding strategies with success.

The molecular techniques reported in *Agaricus* breeding can be applied to *Lentinula*: genetic markers (ISO-ENZYME analysis, RFLPs, RAPDs), protoplasting technology already

used in *Pleurotus* [30], and methods for verifying hybrids. Current breeding strategies in *A. bisporus* include strain hybridisation in which biological diversity can be used in strain improvement programs that attempt to introgress novel traits into the cultivated genepool. In *A. bisporus* new lines with hybrid vigor consisting of faster vegetative growth over a broader temperature range have been obtained. The same could be done with *Lentinula* [31]. Hybridisation is certainly possible in Shiitake because of the demonstrated ability of strains throughout Asia-Australasia to interbreed [26].

Hybridisation can be achieved apart from the classical sexual procedures and molecular methods by so-called parasexual procedures and by GENETIC ENGINEERING also. A joint application of these three parameters – selection, mutation, and recombination – is a successful method for strain improvement. It is important to point out that no strain improvement is successful unless stability of the genetic material is achieved [32]. Due to *Lentinula*'s tetrapolar incompatibility, it has an INBREEDING POTENTIAL of 25% and an OUTBREEDING POTENTIAL of 50% [1]. Therefore, a larger progeny sample size would be required to assure the inclusion of the desired genotype when breeding tetrapolar heterothallic fungi such as *Lentinula* [27].

Previous studies with shiitake have shown that crosses were possible and that F_1 hybrids were fertile. The ability to manipulate phenotypic characteristics, nevertheless, also demands an understanding of their genotypic determinants. LINKAGE ANALYSIS and KARYOTYPING are useful tools in this regard [33]. The future for strain improvement lies in successful breeding approaches: breeding programs that combine linkage analysis with the introgression of novel gene sources such as GENETIC MAPS. AFLP markers can be used to construct genetic maps, map quantitative trait loci, and monitor the genome during the breeding process [34].

Besides the use of molecular markers to target breeding, genetic transformation constitutes an alternative to improvement programs and should allow the rapid acquisition of a cloned dominant character without other changes in the genetic context of the recipient strain [35]. Genetic manipulation offers advantages over breeding, the main one being that undesirable charactersitics are avoided. Genetic manipulation is characterised by the introduction or transfer of characteristics into a strain without making use of sexual interaction. The two most important techniques of genetic manipualtion are genetic transformation (through vectors) and SOMATIC HYBRIDISATION [36].

A close relationship between *L. boryana* and *L. lateritia* just as the one found here, also supported by an analysis of mt small subunit rDNA sequences [26], encourages genetic breeding. Notwithstanding, when strains to be crossed are too dissimilar to each other for crossing to be possible using conventional techniques (eg. *L. edodes* + *L. boryana*), cells of the two strains must be fused artificially in order to produce the hybrid. Given that somatic hybridisation involves the fusion of complete cells, it is possible for undesirable charactersitics to be transmitted to the hybrid. To produce a strain from the heterokaryon thus formed, that has as many desirable characteristics as possible, requires a return to conventional breeding techniques.

8.3 ADVANTAGES OF CURRENT TECHNIQUES

Selection of appropriate regions to sequence is based on several factors. First of all, the region should be evolving at an appropriate rate. Ideally, this means that the region supplies enough consistent differences to separate the taxa into statistically supported monophyletic groups. Secondly, the region should be present as a single copy or should at

least evolve like a single copy region (eg. rDNA). Finally, the region should have the same function in all taxa [37]. The ITS region chosen here includes all these characteristics, making it ideal for phylogenetic analyses. In addition, the ITS region is present at a very high copy number in the genome of fungi, as part of the tandemly repeated nuclear rDNA; this, coupled with PCR amplification, makes it feasible to produce a highly sensitive assay [38].

The accumulated differences in a given sequence over a period of time have been used to discriminate between genetically related individuals [41]. Sequence studies in fungi have focused on rRNA genes. Ribosomal RNA genes of fungi are located on a single chromosome and are present as repeated subunits of a tandem array of transcribed and nontranscribed stretches of DNA [39]. Their popularity is caused primarily by the presence of universally conserved regions that serve as ideal primer sites. ITS primers make use of conserved regions of the 18S, 5.8S, and 28S rRNA genes to amplify the noncoding regions between them [37]. With the exception of some variable regions from rRNA genes, coding regions are highly conserved between organisms. Spacing regions, nevertheless, are less conserved (ITS) and can be partially or totally amplified using universal primers described by White *et al* [40]. Furthermore, since they do not codify for rRNA their apparent lack of function can make their mutations neutral.

The polymerase chain reaction (PCR) and direct sequencing offered several advantages: the method utilizes relatively crude preparations of total DNA, and only small amounts of DNA are required. The accuracy of the PCR sequencing results could be improved with the analysis and sequencing of the complementary strand (ITS1 – ITS4). In this way the sequence analysis of the strand used in this research (ITS4 – ITS1) could be confirmed. A major concern with PCR amplification of genes for subsequent sequence

analysis, however, is the introduction of errors during the amplification process. Nonetheless, the error rate appears to be sufficiently low so as not to confuse interpretations of phylogenetic relationships on the basis of sequence comparisons of PCR amplification products [18].

8.4 FUTURE RESEARCH STUDIES

Only through MOLECULAR PHYLOGENETIC studies can we understand fully the rapidly accumulating genomic sequence data and information regarding proteins' structure and function [42], making it possible to determine potential for genetic improvement. Although this mushroom variety is of significant commercial importance, there are very few reports in the literature with regard to its genetics and breeding [43]. It is imperative that fieldwork be undertaken to document the remaining biodiversity of shiitake, starting with *L. boryana* in Mexico, and to establish culture collections for conservation of germplasm.

The results obtained in this project should assist future studies where other molecular markers can be combined to facilitate complete mapping of *L. edodes* genome. Further characterization can be made using arbitrarily-primed polymerase chain reaction (AP-PCR) profiles, amplified polymorphic DNA marker (RAPD) profiles and restriction patterns (RFLP) [39]. RFLPs generated by restriction digestion of the PCR-amplified ITS region have been used successfully to study intraspecific variation [38]. Moreover, previous *Lentinula* RFLP analysis of mtDNA have indicated that there is considerable genetic divergence between geographically distinct populations [44].

An underlying assumption of sequence analysis is that the phylogeny of the region is a good indicator of the phylogeny of the organism. A good test of this assumption is to

compare the results from regions that are physically and functionally unlinked. Mitochondrial diversity should be studied as well, in order to assay the effects it has on strain performance. Research could also include AFLP analysis which estimates genetic diversity with the help of a large number of genetic markers.

Molecular markers may be used to "fingerprint" commercially important strains. Ideally, data for multiple, unlinked nuclear genes should have been considered as well. By comparing and combining evidence and trees from multiple loci a better understanding of the evolutionary history of population in *Lentinula* should be achieved. Such techniques reproducibly survey the widest range of the genome and could potentially differentiate strains with slightly different genotypes (similarity coefficients equal to zero) such as CP-9 and CP-7 used in this project.

So far, there have been some genetic studies on several mutations of vegetative and generative phenotype controlled by nuclear genes [45]. Further research, nonetheless, should offer a particular opportunity to advance our understanding of their biology and diversity, and potentially give the prospect of improvement [46].

8.5 PROSPECTS FOR THE COMMERCIAL CULTIVATION OF SHIITAKE IN MEXICO

Commercial strains of shiitake are derived entirely from northeast Asian stocks, suggesting that breeders have drawn on only a small fraction of the genetic diversity present across the range of shiitake. Because all strains of shiitake tested to date have been intercompatible, however, the genes resident in the various lineages of shiitake are readily accesible to mushroom breeders through standard crossing methods [47]. Strains would be selected and

the ones that could grow under local conditions and fruit without the need of a dramatic temperature change (as occurs with wild *L. boryana*) would be chosen.

Strains from the other world groups not found in the COLPOS collection should be obtained to assess their potential for genetic improvement. Furthermore, wild strains of *L. boryana* throughout America should be collected and studied to include them in the crosses proposed herein and to compare their behaviour in their natural habitat and in the laboratory cultures.

The commercial biotechnological process for cultivating shiitake in Mexico is still an ongoing process that requires more research as well as field work to become a reality. Nevertheless, the present study represents one of the first steps in opening that opportunity, enabling a genetic transformation programme to proceed. Due to the fact that transformation in shiitake has already been reported, the possibility of combining desirable *L. edodes* genes with native strains of *L. boryana* is high. In fact, shiitake's cultivation in Mexico will probably rely on local genetically engineered germplasm.