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Review Article

A Review on Fungal Isolates Reported as Anamorphs of *Ophiocordyceps sinensis*

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This brief review presents current developments on *Ophiocordyceps sinensis* and fungal strains which have been reported as its anamorphs. A survey of literature has shown that *Hirsutella sinensis* is currently receiving general acceptance as a true anamorph of *O. sinensis*. This isolate has been confirmed as the true anamorph by both morphological and molecular methods. The other isolates such as *Paecilomyces sinensis, Scytalidium hepiali, Tolypocladium sinensis, Chrysosporium sinensis, Synnematium sinensis, Paecilomyces hepiali, Mortierella hepiali,* and *Scytalidium hepiali* have been discarded as anamorphs of *O. sinensis*. The review also discusses various methods used to determine or confirm anamorphs of *O. sinensis*. Considering that the methods have strengths and weaknesses of varying magnitudes, a collective use of various methods is recommended for more reliable conclusions.

1. Introduction

Jennings and Lysek [1] define anamorph as a term for the vegetative form of reproduction (giving the conidial or imperfect state) and also the taxonomic name of the species with this form of reproduction. They describe the term teleomorph as a sexual form of reproduction and also the taxonomic state of the species with this form of reproduction. *Ophiocordyceps sinensis* has a sexual stage (teleomorph) and an asexual stage (anamorph). It is generally seen in the sexual stage with a stalked fruit body [2]. *Ophiocordyceps sinensis* is most probably a macro fungus with, by far, the highest number of isolates reported as its possible anamorphs. The question would be "how come a single species could have so many associated anamorphs with significantly varying genetic makeup, chemical profile, morphology, and growth characteristics?" Certainly, this is an indication that there is something wrong. Some mycologists have suggested that the remarkable variations within isolates from this mushroom could mean that it is not a single species but rather an association of a number of fungi.

> *There is debate among many scientists at present whether the species of the genus Cordyceps are in fact single organisms or if they are symbiotic colonies of more than one organism. Perhaps what we are calling Cordyceps sinensis today will one day be known as a fungal/bacterial symbiosis. (Holliday et al. [3])*

Zhu et al. [4] demonstrated that *Paecilomyces hepiali* and *Hirsutella sinensis* do coexist in *O. sinensis*. They found DNA of these fungi in both the caterpillar and fruiting bodies of natural *O. sinensis*. They, therefore, concluded that their results provided strong support on the multifungi hypothesis for wild *O. sinensis*. However, other researchers such as Chen et al. [5] and Yang et al. [6] have reported that, apart from *H. sinensis*, the other isolates are different species of fungi which commonly exist in natural *O. sinensis* as a result of being endoparasites or epiphytes of the host insect. Various morphological as well as molecular methods have been developed and reported to confirm anamorph-teleomorph connection in *O. sinensis*. However, there seems to be none that is universally accepted. Some isolates which have been proven not to be real anamorphs of *O. sinensis* are still being used to make mycelial products labelled and marketed as *O. sinensis* or *C. sinensis*.

This brief review is aimed at highlighting recent developments on *O. sinensis* and species reported as its anamorphs. Methods which are used to determine or confirm anamorphs of *O. sinensis* are discussed. The review also presents the true anamorph of this fungus based on current research articles.

2. Collection of Information

The information presented in this review is based on published articles in English. That means leaving out a great deal of important information written in other languages especially Chinese. There is so much documented information about this fungus in Chinese because most of research work on it has been done by Chinese scientists. In 2006 Li et al. [7] indicated that, since 1980, about 2000 scientific articles on *C. sinensis* had been published. Among these, approximately 1500 were in Chinese. However, we believe that the English publications are representative enough to provide a true overview of the present situation.

Data on popularity of reported anamorphs was gathered using Google Scholar (http://scholar.google.com). Full scientific names of isolates were used as search terms. For isolates without full scientific names (i.e., those identified only to generic level) such as *Stachybotrys* sp., the search was filtered to cover only articles related to the terms "*Cordyceps sinensis*" and "*Ophiocordyceps sinensis*." Google Scholar was selected because it has a wider coverage than other search databases such as PubMed, ScienceDirect, Scopus, and Web of Science.

3. Anamorphs Reported for *Ophiocordyceps sinensis*

A number of fungi have been described as anamorph of *O. sinensis*. Since the 1980s 22 species in 13 genera have been attributed to the anamorph of *O. sinensis* [8]. The first fungal culture which was considered to be the anamorph of *O. sinensis* was reported by a Japanese scientist, Kobayasi [9]. The isolate was identified only up to generic level and he named it *Stachybotrys* sp. Thereafter, several isolates have been reported as possible anamorphs of *O. sinensis*. These include *Paecilomyces sinensis* [10], *Scytalidium hepiali* [11], *Tolypocladium sinensis* [12], *Chrysosporium sinensis* [13], *Hirsutella sinensis* [14–16], *Synnematium sinensis* [17], *Cephalosporium* sp. [18], *Paecilomyces hepiali*, *Mortierella hepiali*, and *Scytalidium hepiali* [18]. A full list of the 22 names related to *O. sinensis* anamorph is included in a publication by Jiang and Yao [8].

4. Popularity of Names Related to *Ophiocordyceps sinensis* Anamorph

Figure 1 shows popularity of some of the isolates which have been reported as anamorphs of *O. sinensis*, based on the number of published articles. *Hirsutella sinensis* has, by far, the highest number of published reports than other reported anamorphs. This is a reflection of many studies which have confirmed *H. sinensis* as the true anamorph of *O. sinensis* [5, 6, 15, 19, 20]. This is also a clear indication that there is now much more research interest on *H. sinensis* than the other isolates. It is not surprising to see *Paecilomyces hepiali* and *Paecilomyces sinensis* having much more published articles than the other remaining isolates, because these two had previously been regarded as true anamorphs of *O. sinensis*.

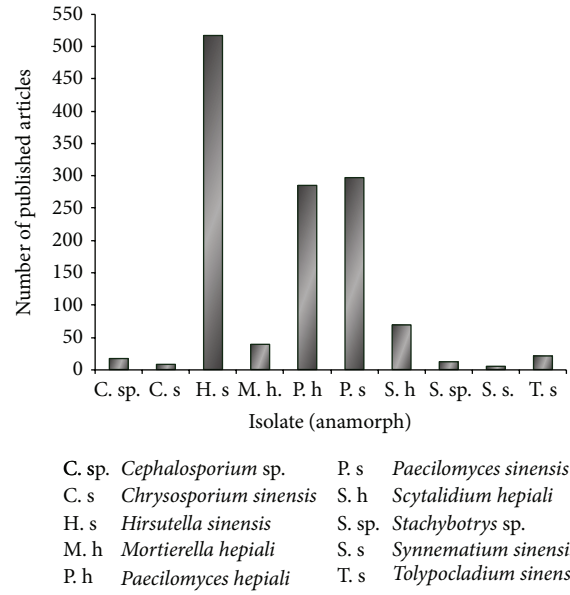

Figure 1: Number of publications on some of the isolates which have been reported as anamorphs of *Ophiocordyceps sinensis*. Data was generated on June 13, 2014, using Google Scholar (http://scholar.google.com).

5. Methods Used to Determine or Confirm Anamorphs of *Ophiocordyceps sinensis*

Several methods to determine or confirm an isolate as a true anamorph of *O. sinensis* have been developed, tested, and reported by many researchers. Here some of the techniques are presented and discussed.

5.1. Chemical Composition/Profiles. In this method the HPLC chemical profile of the tested strain is overlaid on a plot of wild *O. sinensis*. If you get identical or nearly identical profiles the strain may possibly be genuine. In this method special target compounds which are unique to wild *O. sinensis* are used. Holliday et al. [3] suggested this method for determining the quality of cultivated strains of *O. sinensis*. They recommended the use of adenosine class of compounds which they referred to as HEAA (hydroxylethyl adenosine analogs). These hydroxyethyl adenosine analogs include cordycepin, adenosine, and hydroxyethyladenosine. Hsu et al. [21] also suggested the use of adenosine and cordycepin as indexing ingredients for differentiating *O. sinensis* from the counterfeit and the mimic. The problem with this method, however, is that it does not give direct evidence to determine whether the tested strain is a true anamorph of wild *O. sinensis* or not. Therefore, there are high possibilities of making wrong conclusions.

5.2. Macro- and Micromorphological Characteristics of Mycelia. Barseghyan et al. [22] suggested the use of macro- and micromorphological characteristics of mycelia. In this method, colony morphology, growth characteristics, surface color, reverse colony surface, and microscopic characteristics of mycelium are used as indictors to determine the anamorph. A comparison between the tested strain and a known true anamorph is made based on the aforementioned indicators. If morphological similarities are observed, the tested strain may be confirmed to be a true anamorph. This method cannot apply where one does not have a true known anamorph to act as a reference point. If the strain being used as the known anamorph is wrong or misidentified then conclusions to be made will be incorrect. Another challenge with this method is that the phenotype of the fungus can be affected by so many factors which include the growth media and environmental parameters. This can easily lead to erroneous conclusions.

5.3. Microcyclic Conidiation. Microcycle conidiation is defined as the germination of spores by the direct formation of conidia, without the intervention of mycelial growth. The term includes the formation of secondary conidia directly on both the spore and a greatly abbreviated germ tube. It is a method of asexual spore formation in mycelial fungi in which normal cycle of the fungus is bypassed [23].

Liu et al. [20] described microcyclic conidiation as another effective method of determining teleomorph-anamorph connections. In this method conidia and conidiogenous cells produced from ascospores are observed under the microscope. However, ascospores of some species do not directly produce conidiogenous cells and conidia. Single ascospore isolation is also not possible from immature specimens. Microcyclic conidiation (short life cycle) is difficult to observe, and its use is very limited for other species [2].

5.4. Molecular Methods. The application of molecular methods to phylogenetic analysis has enhanced ability to understand the genetic variability in groups of entomopathogenic fungi [24, 25] and has made it possible to confirm anamorph-teleomorph connections from the similarity of gene sequences. Internal transcribed spacer (ITS) regions are rapidly evolving regions of the nuclear ribosomal DNA, which have been widely used for species or population analysis of various organisms such as animals [26] and fungi [27, 28]. The rDNA-ITS region is thought to be unaffected by the selection that operates for the coding regions. Variation within the ITS region at the interspecies or intraspecies level is useful for species identification because of the high diversity of this region between species as well as the homogeneities of this region within a species [5]. In fungus, the distance value (K value) of rDNA-ITS sequences within a species is generally from 0.00 to 0.05. Values larger than 0.05 may represent different species [29, 30].

The use of ITS and 5.8S rDNA sequence data to determine the relationship between the anamorph and teleomorph of several *Cordyceps* species has been reported by a number of researchers, including Liu et al. [20], Nikoh and Fukatsu [31], and Chen and Hseu [32].

This method provides direct evidence and is gaining so much popularity and acceptance. However, Holliday and Cleaver [33] pointed out that the DNA sequence in *Cordyceps* is apparently rather variable. They indicated that *Cordyceps* may even incorporate some of the insects' own DNA in order to fruit and then loses this insect DNA when grown from the spores produced or from tissue specimens of the fruit body. If this argument is still valid today, one would say that this method is also not completely dependable.

Which of the methods discussed above is the best to use then? Considering that each of the methods has some strengths and weaknesses (of course, of varying degrees), it would sound logical to recommend that a combination of these methods should be used. Any conclusion made based on collective use of these methods would be more reliable than using each method alone.

6. The True Anamorph

Paecilomyces hepiali Chen (strain CS-4) is probably the most popular commercial strain used to produce most of the mycelial products sold in the world marketplace as *O. sinensis*. It used to be called *Cordyceps sinensis* (strain CS-4). The strain CS-4 was isolated by the Chinese Academy of Sciences in 1972 from specimens found in Qinghai province, central China [34]. It was tested for its medical potential, and by 1988 it had been approved in China as a medicine under the name Jin Shui Bao [34]. At present there is a lot of reported evidence that CS-4 is not a correct anamorph of *O. sinensis* but belongs to a different fungus, *Paecilomyces hepiali* Chen.

Currently *Hirsutella sinensis* is generally being reported and accepted by many mycologists as the true anamorph of *O. sinensis*. *Paecilomyces hepiali* and other associated anamorphs have been discarded as anamorphs of this fungus. Liang [19] and Liu et al. [15] confirmed *H. sinensis* as a true anamorph of *O. sinensis* based on microcyclic conidiation (short life cycle) observation. Liu et al. [15] also made this confirmation based on DNA sequences. They also found that the species *C. multiaxialis* and *C. nepalensis* were sharing identical or almost identical ITS sequences with *O. sinensis* and they, therefore, concluded that these two species and *H. sinensis* should be regarded as synonyms. Liu et al. [20] also confirmed *H. sinensis* as anamorph of *O. sinensis* based on ITS-5.8S rDNA sequences. Chen et al. [5] also determined *H. sinensis* as the anamorph of this fungus based on the sequences of the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal RNA. They discarded *Paecilomyces sinensis*, *Stachybotrys* sp., and *Tolypocladium* sp. as anamorphs of *O. sinensis*.

Yang et al. [6] conducted a study on phylogenetic relationship between *P. hepiali* and wild *O. sinensis* by analysing the sequences of rDNA-ITS. Their results showed that all samples of wild *O. sinensis* shared identical or almost identical rDNA-ITS regions and had over 99% identity with some rDNA-ITS sequences of *H. sinensis* and *O. sinensis* registered in the NCBI GenBank, but all of them had only about 72% shared identity with that of *P. hepiali*. Their results confirmed that *P. hepiali* is not a true anamorph of natural *O. sinensis*. However, it should be pointed out here that the use of DNA sequences in the International Nucleotide Sequence Databases (INSD)

to confirm anamorphs of *O. sinensis* has the possibility of leading to wrong conclusions because some of the DNA sequences are erroneously annotated as reported by Zhang et al. [35].

Starting from January 1, 2013, one fungus can only have one name; that means an end to the system of permitting separate names to be used for anamorphs [36]. Therefore, as reported by Lo et al. [37], the anamorph name *H. sinensis* will be changed by the *International Code of Nomenclature for algae, fungi, and plants* (formerly called the *International Code of Botanical Nomenclature*) to *O. sinensis*. This follows its confirmation as the true anamorph of *O. sinensis*.

7. Conclusion

Hirsutella sinensis is currently being accepted as the true anamorph of *O. sinensis* based on both molecular and morphological methods. This has also been reflected in the current higher volume of published articles on *H. sinensis* than the other isolates of *O. sinensis* (Figure 1). At present, the methods which are being used to determine or confirm anamorphs of *O. sinensis* have some strengths and weaknesses of varying magnitudes. Of course, molecular methods seem to be more reliable than the other techniques. However, we think that a collective use of various methods would be more reliable than using each method alone. Research efforts aimed at improving existing methods and/or finding other simpler and more accurate methods need to be continued.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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