Current Biology

Zombie-Ant Fungi Emerged from Non-manipulating, Beetle-Infecting Ancestors

Highlights

- Phylogenetic host association reconstructions of the fungal genus *Ophiocordyceps*

- Zombie-ant fungi evolved from fungal parasites of beetle larvae buried in soil/logs

- Niche overlap played crucial role for the fungal transition from beetles to ants

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In Brief

The ability of parasites to manipulate animal behavior is a complex adaptation that evolved multiple times to increase parasite fitness. Araújo and Hughes show that the zombie-ant fungi evolved from a fungal ancestor that infected beetle larvae buried in soil and trunks.

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Zombie-Ant Fungi Emerged from Non-manipulating, Beetle-Infecting Ancestors

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SUMMARY

The manipulation of animal behavior by parasitic organisms is one of the most complex adaptations to have arisen via natural selection. Among the most impressive examples of behavioral manipulation are the zombie-ant fungi [1]. In this association, ants are controlled to leave the colony and perform a stereotyped death grip behavior, where they bite onto vegetation over foraging trails, before being killed for the *post mortem* fungal growth. Manipulation functions to provide a platform outside the nest, from which fungal parasites actively shoot out spores, targeting foraging ants because within colony transmission is prevented by strong social immunity exhibited by social insect societies [2, 3]. It is not clear how such complex examples of host manipulation arose. To address this, we performed a broad-scale phylogenetic reconstruction of the order Hypocreales, to which the zombie-ant fungi, *Ophiocordyceps*, belong. In order to understand the patterns of host association and host switching along the evolution of *Ophiocordyceps*, we performed ancestral character state reconstruction analysis. We found that zombie-ant fungi likely arose from an ancestor that infected beetle larvae residing in soil or decaying wood, similar to extant beetle-infecting *Ophiocordyceps* species. Surprisingly, the jump led to an extensive species radiation observed after the development of behavioral manipulation. We suggest that the jump from solitary beetle larva to ants within a colony exposed the fungus to the robust social immunity of ant societies.

RESULTS

To understand the possible evolutionary and ecological processes that led to the emergence of the zombie-ant fungi and their ability to manipulate ant behavior, we built a comprehensive phylogeny of the order Hypocreales, with 629 putative species, to provide a broader phylogenetic context to our target genus,

Ophiocordyceps. Our phylogenetic reconstruction is in agreement with previous studies [4–7] but differs in having a broader taxon sampling and the mapping of behavioral manipulation and host identity traits made possible by the construction of a recent database based on 1,601 publications [8]. The zombie-ant fungi, *O. unilateralis s.l.*, belong to the genus *Ophiocordyceps*, within the Ophiocordycipitaceae family. Here, we present for the first time the evolution of host affiliation at host order level for the genus *Ophiocordyceps* (Figure 1A). We found that, among the 129 species of *Ophiocordyceps* we examined, 31 species are associated with Hemiptera, 49 with Hymenoptera, 22 with Coleoptera, and 19 with Lepidoptera (Figure 1). Other insect orders are also infected but with few species recorded on them: Megaloptera (1 sp. of fungi infecting them—*O. tiputini*); Odonata (1 sp.—*O. odonatae*); Orthoptera (1 sp.—*O. amazonica*); Blattaria (3 sp.—*O. blattae*, *O. blattarioides*, and *O. communis*); and Diptera (3 spp.—*O. dipterigena*, *O. forquignonii*, and *O. variabilis*). In its original description, the host of *Ophiocordyceps variabilis* was misidentified by Petch [9], probably because these dipteran larvae are unusually large, i.e., Xylophagidae (Diptera) [10]. The host association is heterogeneous across the genus, but host orders tend to form clusters (Figure 1). In Figure 1, node 1 indicates the ancestral state as parasitic of beetle larva (99.94% for Coleoptera), node 2 indicates the transition from Coleoptera to social hymenopteran insects (91.83% for Coleoptera, 4.73% for Lepidoptera, and 3.08% for Hymenoptera), node 3 indicates the first ancestral associated with hymenopterans (78% for Hymenoptera, 13% for Coleoptera, 6% for Lepidoptera, and 1.5% for Megaloptera), and node 4 indicates the first origin of myrmecophily (ability to infect ants) within *Ophiocordyceps* (99.91% for Hymenoptera).

The zombie-ant fungi clade—together with *O. kniphofioides* clade and *O. humbertii*—appears to have originated from an ancestral beetle parasite (Figure 1, node 2). There may have been an intermediate step via social wasps (Figure 1, node 3). *Ophiocordyceps humbertii* infects social wasps and induces to an identical mode of behavioral manipulation where adult wasps bite leaves, but the taxon sampling of wasp pathogens is poor and future work will resolve this. It is also possible the step from beetle to ant was via some other insect association that has since gone extinct. But based on our work, it seems that the ancestor of this clade was a pathogen of beetles.

All the 46 extant *Ophiocordyceps* species that are parasites of Coleoptera attack and kill immature beetle larvae in wood or

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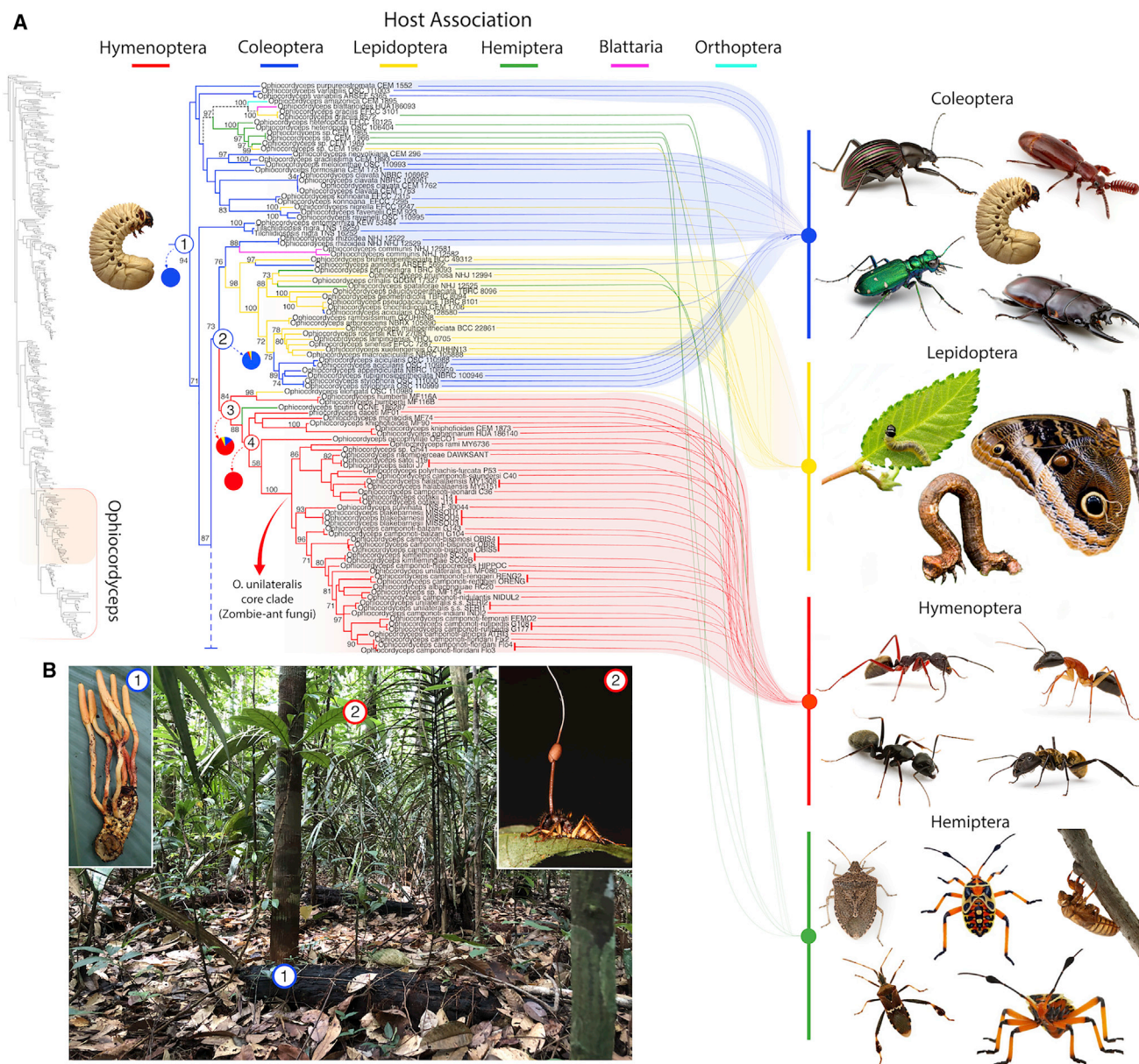
Figure 1. Evolution of Host Association in *Ophiocordyceps*

(A) Maximum likelihood tree obtained from RAxML analyses with a concatenated 5-loci dataset (SSU, LSU, tef, RPB1, and RPB2) and ancestral character state reconstruction (ACSR) analyses based on host association of the genus *Ophiocordyceps* (Data S1). Branches are color coded to reflect host associations from ACSR analysis, and pie charts represent the probability for each host order. Node 1 indicates the ancestral state as parasitic of beetle larva, node 2 indicates the transition from Coleoptera to social hymenopteran insects, node 3 indicates the first ancestral associated with hymenopterans, and node 4 indicates the first origin of myrmecophily (ability to infect ants) within *Ophiocordyceps*. Dashed line pointing downward indicates hidden clades that, although included in the analyses, are not displayed in this figure.

(B) Picture at the bottom illustrates a typical tropical environment (Reserve Ducke, Brazilian Amazon) where both ecological groups occur: pathogens of beetle larva (A; e.g., *O. melolonthae*) and zombie-ant fungi (B; *O. camponoti-atricipis*). Killing hosts and shooting spores from the ground level is a basal feature (blue branches, Coleoptera), whereas "summit disease" and shooting spores from an elevated position led by the fungus (behavior manipulation) is derived within the genus *Ophiocordyceps* (red branches, Hymenoptera). Beetle larva photograph by Danny Newman, forest and infected ant by João Araújo, and insect hosts (right side) by Alex Wild.

See also Figure S1.

soil, except three (*O. curculionum*, *O. entomorrhiza*, and *O. salebrosa*) that infect adult beetles [11, 12]. Our results also suggest the switch from Coleoptera to social Hymenoptera (ants/wasps), in addition to leading to an example of complex behavioral manipulation, also promoted a diversification of *Ophiocordyceps* fungi associated with ants (Figure 1).

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DISCUSSION

Our results suggest the ancestor of the zombie-ant fungi, *O. unilateralis s.l.*, was likely a species infecting beetle hosts (Figure 1, node 1). The "host habitat hypothesis" [13] suggests that host jumps by fungal pathogens tend to occur between species sharing the same microhabitat and niche overlap can be a dominant factor for host-switching events. This has been suggested to have facilitated another major jump among fungal pathogens, from cicadas (kingdom Animalia) to truffle fungi (kingdom Fungi), which both share the same subterranean habitat [13]. Spatafora et al. [14] showed that unidirectional interkingdom host jumps occurred since the origins of hypocrealean fungi, dated from approximately 158–232 mya [15].

Our results (Figure 1) imply the zombie-ant fungal lineage arose from beetle ancestors. Among the 46 species of *Ophiocordyceps* recorded on Coleoptera [8, 12, 16, 17], the two major families recorded as hosts are Scarabaeidae and Elateridae, although there are records of infections in Erotylidae, Geotrupidae, Staphylinidae, Curculionidae, Tenebrionidae, Carabidae, Cerambycidae, Chrysomelidae, and Lecanidae [12]. Those infecting larvae are found inside rotten logs, buried in soil or underneath leaf litter (Figure 1B).

Carpenter ants commonly occur in dead wood or soil [18], and the character state of the ancestral ant is suggested to be soil nesting [19]. It is reasonable to suggest, based on the evolutionary history of the order Hypocreales in which host jumps are common [13, 14] and sometimes associated with habitat overlap, that the co-occurrence of beetle larvae in wood/soil and ant societies in the same wood or in soil led to the host jump from beetles (Coleoptera) to ants (Hymenoptera). But what then was the evolutionary pressure for the lineages in ants to have evolved behavioral manipulation?

The high density of ants living within the confined nests together with the high degree of relatedness between workers makes them prone to parasitic infection [2]. However, we know that all extant parasitic *Ophiocordyceps* can only develop and transmit from dead insects [8]. An experimental placement of *O. unilateralis s.l.*-killed hosts into carpenter ant nests resulted in no fungus growth and rapid destruction of the host cadaver [3]. This is due to the robust social immune systems, which are defining characters of social insect societies, including ants [2].

We suggest that the transition to ant pathogenesis, due to niche overlap, stimulated a selection pressure that led to the evolution of behavioral manipulation. As ants are central place foragers, there was likely strong pressure to have the host to die outside the nest to avoid the social immunity of the colony but still sufficiently close to infect foraging ants. In the case of zombie-ant fungi group (*O. unilateralis s.l.*), the manipulation is stereotyped with worker ants biting vegetation above foraging trails [3]. Recent work presented evidence for convergent evolution of host parasitic manipulation by *O. unilateralis s.l.* in response to environmental conditions [20]. In that case, the biting behavior of manipulated ants occurring in temperate habitats was on twigs rather than leaves to avoid the loss of the necessary spore-release platform due to leaf fall in deciduous forests. That work showed behavioral manipulation is a trait that responds to selection associated with environmental conditions.

We suggest then that behavioral manipulation of ants by *Ophiocordyceps* was a key innovation that arose after the host jump from beetle larvae to adult ants. The strong social immunity present in ant societies likely necessitated the evolution of nest departure and leaf/twig biting. Somewhat surprisingly, we note that despite the obvious specialized nature of behavioral manipulation [21–23], the jump to ants and subsequent behavioral manipulation led a radiation with diversification of species within *O. unilateralis* clade [24] (Figure 1, node 2).

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.09.004.

AUTHOR CONTRIBUTIONS

J.P.M.A. and D.P.H. conceived this study and wrote the manuscript; J.P.M.A. conducted analyses and created figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
DNA sequences used in this study to create phylogenetic tree of the order Hypocreales and ACSR of the genus *Ophiocordyceps*	[24]	https://www.sciencedirect.com/science/article/pii/S0166061617300593
Additional sequences added to [24] to create a broad scale phylogeny of Hypocreales, including plant pathogens	Data S1	N/A
Software and Algorithms		
Geneious 11.1.5	[25]	https://www.ncbi.nlm.nih.gov/pubmed/22543367
MAFFT 1.3.7	[26]	https://www.ncbi.nlm.nih.gov/pubmed/23329690
RAxML 8.2.4	[27]	https://www.ncbi.nlm.nih.gov/pubmed/16928733
Mesquite 3.51	[28]	https://www.mesquiteproject.org/
Dendroscope 3	[29]	https://www.ncbi.nlm.nih.gov/pubmed/22780991

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources including alignments, sequences, additional figures and any other inquiries should be directed to and will be fulfilled by João Araújo (joaofungo@gmail.com).

METHOD DETAILS

Taxa sampling and sources
Our data matrix consisted of 629 sequences across 10 families of the order Hypocreales. We aimed to construct the most inclusive phylogenetic tree of hypocrealean fungi, covering a wide variety of ecologies, morphologies and life-styles. The dataset used in this study was primarily based on the phylogeny presented by [24], in addition to other sequences from [30] (Data S1A) and *Ophiocordyceps* species from [5], [31] and [32], in addition to new sequences deposited after [24] that we downloaded from GenBank (Data S1B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Phylogenetic analyses
Five loci were used in this study comprising the nuclear small (SSU rRNA) and large (LSU rDNA) ribosomal RNAs, translation elongation factor 1-alpha – (tef1), the largest (RPB1) and second largest (RPB2) subunits of RNA polymerase II genes. DNA sequences were aligned with MAFFT [26]. The alignment of every gene was improved manually, annotated and concatenated into a single combined dataset using Geneious version 11.1.5 [25]. Ambiguously aligned regions were excluded from phylogenetic analysis and gaps were treated as missing data. The final alignment length was 4,772 bp: 1,178 bp for SSU, 909 bp for LSU, 982 bp for tef, 711 bp for RPB1, and 992 bp for RPB2. Maximum likelihood (ML) analysis was performed with RAxML version 8.2.4 [27] on a concatenated dataset containing all five loci. The dataset consisted of 11 data partitions, these included one each for SSU and LSU, and three for each of the three codon positions of the protein coding genes, tef, RPB1 and RPB2. The GTRGAMMA model of nucleotide substitution was employed during the generation of 1,000 bootstrap replicates.

Ancestral character state reconstruction
To understand the evolutionary pathways of host association of *Ophiocordyceps*, we conducted ancestral character reconstruction in Mesquite [28] of the whole genus, using the best-scoring ML tree produced in RAxML. We coded each taxon based on host association (8 categories: Coleoptera, Hymenoptera, Lepidoptera, Hemiptera, Blattaria, Orthoptera, Odonata, Megaloptera and Diptera – see Figure S1A). Additionally, we performed a second analyses, distinguishing adults and larval stages of Coleoptera (9 categories in total, see Figure S1B) in order to clarify the developmental stage of the *Ophiocordyceps* ancestral host. We used maximum likelihood model MK1, as implemented in Mesquite 3.51 [28]. Only nodes presenting > 75% of probability were displayed and used to color-code the branches on the figures. Nodes below this limit were displayed as dashed lines.

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Post-analyses treatments

Following the phylogenetic and ancestral character state reconstruction analyses, we used tools available in Geneious 11.1.5 [25] and Dendroscope [29] in order to optimize the tree layout.

DATA AND CODE AVAILABILITY

The raw tree generated in this study and also used as a template for the Ancestral Character State Reconstruction are available at: https://zenodo.org/record/3361137/files/ACSR_Current_Biology_adult_larva_FINAL.newick?download=1. Any additional information and files are available from the Lead Contact on request.
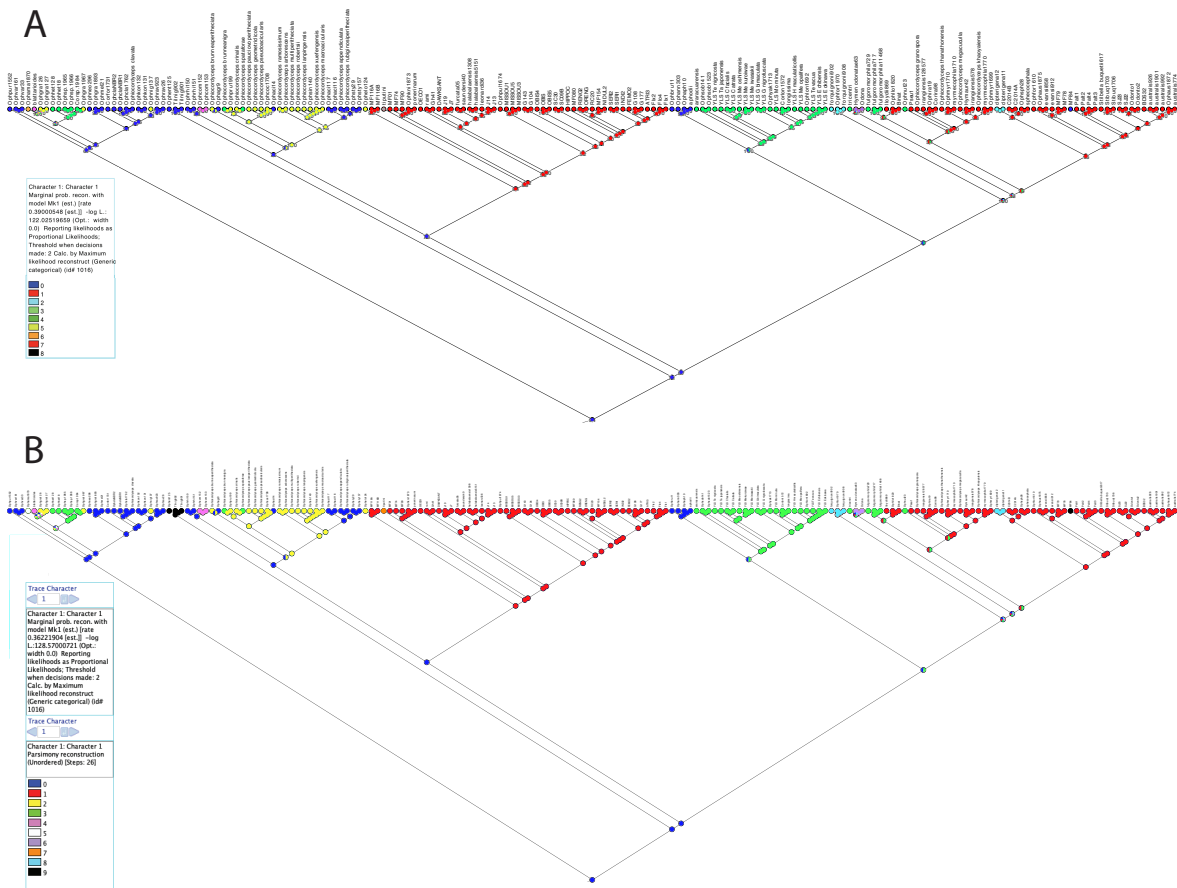
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Figure S1. Ancestral Character State Reconstruction of the genus *Ophiocordyceps*. Related to Figure 1. A) Dataset divided into 8 categories (Coleoptera, Hymenoptera, Lepidoptera, Hemiptera, Diptera, Megaloptera, Odonata, Blattaria). B) Additional analyses using 9 categories (Coleoptera adult, Coleoptera larva, Hymenoptera, Lepidoptera, Hemiptera, Diptera, Megaloptera, Odonata, Blattaria). Since the differentiation into the Coleoptera stage (adults and larvae) did not impact in our results, we chose to use Coleoptera as a single category (A) which was used in figure in the main text.

Supplemental References

S1.   Araújo, J.P.M., Evans, H.C., Kepler, R., and Hughes, D.P. (2018). Zombie-ant fungi across continents: 15 new species and new combinations within *Ophiocordyceps*. I. Myrmecophilous hirsutelloid species. Stud Mycol 90, 1–42.

S2.   Lombard, L., van der Merwe, N.A., Groenewald, J.Z., and Crous, P.W. (2015). Generic concepts in Nectriaceae. Stud Mycol. 80, 189–245.

S3.   Quandt, C.A., Kepler, R.M., Gams, W., Araújo, J.P.M., Ban, S., Evans, H.C., Hughes, D., Humber, R., Hywel-Jones, N.L., Li, Z., et al. (2014). Phylogenetic-based nomenclatural proposals for Ophiocordycipitaceae (Hypocreales) with new combinations in *Tolypocladium*. IMA Fungus. 5, 121–134.

S4.   Kepler, R.M., Sung, G.-H., Harada, Y., Tanaka, K., Tanaka, E., Hosoya, T., Bischoff, J.F. and Spatafora, J.W. (2012). Host jumping onto close relatives and across kingdom by *Tyrannicordyceps* (Clavicipitaceae) gen. nov. and Ustilaginoidea (Clavicipitaceae). Am. J. Bot. 99, 552–561.

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