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COMMENT

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Glomeromycota rRNA genes—the diversity of myths?

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We are writing this to further elaborate on some aspects of published work on rRNA gene diversity among arbuscular mycorrhizal (AM) fungi (most of the members of the *Glomeromycota*), which is based in part on unfounded assumptions about their general genetic structure raised in a Comment by Clapp et al. (2002).

The first, least serious but perhaps most confusing aspect is the reference to the *Glomales* in the title of their Comment. The intended reference was to the AM fungi in general, which are now placed in the *Glomeromycota*. The term *Glomerales* (formerly orthographically incorrectly termed *Glomales*) now represents one of four orders within the phylum, and its use in a broad sense may result in lack of clarity. The taxonomic changes have been adopted also by the main sequence databases (EMBL, NCBI). The BEG culture collection has changed its name recently to 'International Bank for the *Glomeromycota*'.

We now address in more detail the main points discussed by Clapp et al. (2002) as errors in work on glomeromycotan molecular diversity and phylogeny.

The 'lack of appropriate outgroups to secure the origin of the sequences'

This criticism of Clapp et al. (2002) is correct and well-founded, and it is certainly worth strengthening the case that suitable outgroups are needed. Indeed, there exist several reports that draw conclusions based on contaminant sequences because of the lack of a comprehensive phylogenetic comparison. Some studies have been demonstrated previously to be based on contaminant sequences, and others were noted in Clapp et al. (2002), but the phylogenetic tree presented in their Comment as supplementary material does not conclusively resolve the problem. This tree is labelled as being based on 5.8S rDNA sequences but without any further data. However, such a tree alone, without any statistical and raw data, is inadequate for proving the origin of all sequences shown. Although in some journals it is not mandatory to provide the alignment and sites used for the analysis, it is helpful to the proof and for future comparisons to do so.

Doubtless the warning about using contaminant sequences is correct. Although some researchers may know which sequences are contaminants, there is a real danger that those mainly working in other disciplines may use them innocently. It is, therefore, of crucial importance that database entries are updated as soon as authors become aware that their sequences were derived from contaminants and not from the species named in the database. As a source of information, tables relating to the sequences cited by Clapp et al. (2002) and in other publications are shown on our website (see below). This should be scrutinised and updated by other researchers, and we invite them to do so.

Regarding the cited BLAST searches, one should also bear in mind that sequence homology values alone sometimes are not sufficient for conclusions about phylogenetic affiliation. In particular, this is true for the highly variable ITS sequences, especially from organisms whose close relatives have not yet been sequenced, or if BLAST searches match erroneously assigned sequences stemming from contaminants. A recent example of an error in the latter context can be found in Chen and Cairney (2002). Many of the shown sequences, determined by BLAST to be of AM fungal origin, are related to those of unidentified nonglomeromycotan fungi (AF461576–578; AF461596–600; AF461603; AF461615–619; AF461621–627; AF461646; AF461657–658), to fungi presumably belonging to a completely different clade of the *Glomeromycota* (*Paraglomerales*; AF461620; AF461601–602), or even arachnids (AF461578). All these errors are caused by mislabelled sequences in the databases (AF133780;

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AF133791; U15692). For some of the sequences noted above, unpublished ITS and 18S rDNA sequencing studies performed in collaboration with Jill Hoff and Ned Klopfenstein (USDA Forest Service, Moscow, USA) showed that they stem from organisms closely related to *Umbelopsis* and *Micromucor*, which form a basal clade within the *Mucorales* (O'Donnell et al. 2001); detailed information can be found on www.amf-phylogeny.com. This shows the risks of drawing erroneous conclusions from BLAST searches and the need to check at least questionable sequences by phylogenetic analyses.

The 'mislabelling of sequences' from glomeromycotan fungi

Clapp et al. (2002) state that another major problem for work on AM fungal phylogeny and diversity is the mislabelling of sequences or incorrect citation of cultures or species names. They show two supposed examples of this, both related to one of our publications (Schüßler et al. 2001a). The criticism here is almost totally unfounded, as can be concluded from a careful reading of our work. The sequence with the accession number AJ276083 was not related to '*Glomus clarum* BEG14', as indicated by Clapp et al. (2002). It was quoted for culture 'Att672-13/W3161 from Walker, clone pWD116-1-2', which was determined originally to be *Glomus clarum* (detailed specifically in Table 1 in Schüßler et al. 2001a). The reason for the misrepresentation by Clapp et al. (2002) is an orthographical error in the phylogenetic tree (Fig. 1) shown in our paper. It is indeed written there as '*Glomus clarum* BEG14 (AJ276083)'; however, this mistake (the 'BEG14' designation) occurred only once in the paper and was easy to interpret, since directly below *G. claroideum* BEG14 is shown in the tree. But the fungus was correctly referenced as *G. clarum* (as far as we could identify it at the time) twice, once in the narrative and once in Table 1 (listing all isolates used), both times without the erroneous BEG label.

Later, this fungus was re-classified as *G. lamellosum* (see below) and an appropriate correction was made in the database. The given accession number and references in the EMBL (and GenBank) database also were correct and all information is shown correctly (without our erroneous reference to BEG14) in the table in the paper and the other figures. The recognition that the sequence for the supposed *G. clarum* did not resolve into the clade containing that species stimulated further study of the organism. After characterising the full-length sequence and investigating fresh, living spores from an ex-type pot culture of *G. lamellosum*, we later discovered that 'Att672-13/W3161' was not *G. clarum*, but corresponded to *G. lamellosum*. Therefore, the species name was updated together with the full-length sequence in the database and used in further publications from our laboratory (e.g., Schwarzott et al. 2001; Schüßler et al. 2001b). All this information was available to Clapp et al. (2002). We apologise for our proof-reading error.

The comments of Clapp et al. (2002) about the sequence U36592 from isolate BR212—which in fact does not stem from our laboratory—are also misleading. In the work cited (Schüßler et al. 2001a), we used a species determination made by the originator of the culture himself (J. Morton, personal communication). This information is given in detail as a footnote to Table 2, showing the sequences from the database used in our publication. Therefore, both comments made by Clapp et al. (2002) that focus on our work are misleading and, moreover, we note that there are several errors in the 5.8S tree they themselves published in their Comment paper (Fig. 1, as supplementary electronic material) and also in the BEG database. Some examples are:

1. The species identification '*Glomus fistulosum*' given for sequence X96845 is wrong. The only *G. fistulosum* sequence in the database at that time was AJ239126; probably this is the correct number here. X96845 belongs to *Glomus coronatum* BEG28 (also shown in the tree with the same accession number). Moreover, *G. fistulosum* has been synonymised with *G. claroideum* (Walker and Vestberg 1998), and the epithet '*fistulosum*' should not be used as species name unless the authors have reasoned disagreement with the synonymy. The species identification for sequence AJ239126 has been updated recently, now correctly referring to *G. claroideum*. This change is also implemented in the BEG database, but with one exception where the name of the fungus registered as BEG93 in the BEG database is still given as *G. fistulosum*.
2. A similar comment can be made regarding the fungus registered as BEG139. This is listed as of questionable identity in the BEG culture database and yet is described without doubt as *G. coronatum* in the 'genetic archive' and in Clapp et al. (2001). The important taxonomic change transferring several *Glomus* species into a new genus, *Paraglomus*, is not implemented in the BEG database, where BEG120 and BEG37 are listed confusingly as being members of the genus '*Glomus*', but in the sequence-search form as belonging to *Paraglomus*. Such errors are present understandably in many databases, but they should be corrected as soon as possible after their discovery.
3. In the legend of Fig. 1 of the Clapp et al. Comment is written '... The red colour sequences correspond to sequences obtained by Helgason et al. (1999) ...'. This is incorrect; the sequences are from Pringle et al. (2000).

We wish to reiterate that updating of sequences or species identifications in the databases should be carried out as soon as possible after discrepancies come to light. This is particularly important for contaminant sequences and has, correctly, been performed for sequences already demonstrated by Redecker et al. (1999) and Schüßler (1999) to be of contaminant origin. The criticism of Clapp et al. (2002) has, thus, already been resolved in these

instances. In the case of the sequences published by Pringle et al. (2000) and Millner et al. (2001), we contacted these authors and at least the sequences published by Pringle et al. (AF133777–791) are now labelled in the databases as 'uncultured fungus from *Acaulospora colossica* spore'.

Delay in updating the species affiliation also resulted in earlier, inadvertent 'mislabelling' in trees we have published. We used a sequence (X58726) presumed to represent '*Gigaspora margarita* DAOM194757', although the culture has since been identified as *Gi. rosea* (rDNA sequences AJ410746–AJ410747). The authors have also been contacted and the entry was recently updated.

As already noted, a list with comments about mislabelled sequences and tables of contaminant sequences can be found on the internet at www://amf-phylogeny.com and we request that scientists with relevant information send us comments, discussions and updates. Where necessary, we and others should also request that sequence database curators implement these changes to validate the database, following the EMBL rules: 'If you spot errors or inconsistencies in database entries not owned by yourself, first try contacting the authors so that they can update their sequences directly. If you are unsuccessful, then please also use the following form indicating third party update.'

Another kind of problem to be noted here is the reference to sequences published by Simon et al. (1992). Parts of these were never sequenced but were submitted as 'consensus sequence derived from fungal SSU comparison'. These positions must be replaced manually by 'unknown' before analysis. Since this information is not given in some sequence-viewing options, there is the risk that some researchers may include the consensus part in their analyses. The relevant sequences are those for *G. intraradices* X58725, *Gi. rosea* (formely *Gi. margarita*, see above) X58726, and *Endogone pisiformis* X58724.

The statement that AM fungi are 'a fundamentally different group of *Eukaryota*'

We now wish to draw attention to a serious problem and to draw our own conclusions about the statement that AM fungi are 'a fundamentally different group of *Eukaryota*'. As noted above, this conclusion is based on work by the authors themselves, mainly that published in Rodriguez et al. (2001). This conclusion is probably erroneous because it is based on contaminant sequences—precisely the error criticised by the same authors (Clapp et al. 2002). It is stated that an AM fungus, *Entrophospora infrequens*, contains LSU ribosomal genes within its genome that are related to distinct lineages ('families') within the AM fungi. Note that these lineages diverged hundreds of millions of years ago and if judged by the molecular distances would correspond to different classes within the *Ascomycota* (Schüßler et al. 2001b). Can this statement be true? Although much is possible in biology, there is no evidence, real or speculative, supporting such an outcome. Rodriguez et al. (2001) used single-spore DNA isolation protocols, resulting in sequences from '*Glomus*' groups A and B, which represent different families (Schwarzott et al. 2001), and *Gigasporaceae* (about one third of the sequences). However, *E. infrequens* is known only from spores produced in field conditions or in mixed pot culture with known AM fungi. Moreover, there is no corroborated evidence that *E. infrequens* is an AM fungus. The majority of *Glomeromycota* members are thought to be mycorrhizal symbionts only by analogy with the relatively few that have been so proven. The field trap-cultures used by Rodriguez et al. (2001) were admitted to be such mixed cultures. 'Contaminating' species are noted in the study and, interestingly, many of these fungi appeared to be represented by the sequences obtained from the single-spore DNA. The authors themselves write that the *Glomus* sequences obtained reflect the species community identified in the trap culture used. It is perhaps worth stressing the fact that the process of spore extraction from the substrate can cause severe damage to spores and hyphae of glomeromycotan (and other) fungi. Consequently, the individual spores might be bathed in a mixture containing DNA from several species. Spores of *E. infrequens* are both highly ornamented and encrusted with the deteriorating remnants of the sporiferous saccule wall. It is quite possible that the cleaning process is inadequate for removing traces of such extraneous DNA.

In fact, the most obvious contaminants are from a quite different group of fungi: six non-glomeromycotan sequences (AF378518–AF378523), related to extremely common soil fungi belonging to the genus *Rhizopus*, were obtained from the single-spore DNA isolations. They were shown in Rodriguez et al. (2001) but were not discussed at all. The different '*Glomus* sequences' obtained from the single-spore DNA isolations, but not the *Rhizopus*-related sequences, were referred to as belonging to the *E. infrequens* genome, though there seems to be no overwhelming reason for choosing one rather than the other. The following possible conclusions may be drawn:

1 The sequences obtained—including the AM fungal ones—are derived from contaminants.
2 The *E. infrequens* genome in addition to the AM fungal sequences also contains *Rhizopus*-related sequences.
3 The *E. infrequens* spores are not typical AM fungal spores, but are formed by, for example, a mycoparasitic fungus.

As to which of these is the most probable, the application of Ockham's razor would lead to the conclusion that contamination is the simplest and most likely solution, though the third possibility was mooted in the protologue of the genus *Entrophospora* (Ames and Schneider 1979). Another publication by the authors (Clapp et al. 2001), describing *G. coronatum* LSU rDNA sequence variation, is probably also erroneous in the same

way, but is used as evidence in support of their theories. For example, '... when BEG28 K was re-examined in March 2000 after sequencing studies, a *G. geosporum* morph was seen to be present along with the expected *G. coronatum* spores.' Moreover, sequences belonging to different families were found in the DNA extractions; it seems that the authors used mixed cultures for their work.

It is likely that the data reported in these publications are in fact the results of contamination, which may be caused by the method of fungus production or spore isolation, or by parasitism. Sequencing large numbers of DNA clones produces no guaranteed proof for the origin of a sequence: the source and handling of the experimental organism, in this case the open pot culture material used, is crucial. In our own work, we use protected microcosms (Walker and Vestberg 1994) to ensure that cultures are free at least of contamination by other glomeromycotan fungi.

Therefore, the comments made and conclusions drawn by Clapp et al. (2001, 2002) and Rodriguez et al. (2001) should be treated with scepticism. No hints for such extraordinarily large genetic distances in ribosomal genes of AM fungi have been found in other detailed studies based on well-defined cultures established from single-spore isolates (Jansa et al. 2002; de Souza et al., unpublished data).

References

Ames RN, Schneider RW (1979) *Entrophospora*, a new genus in the Endogonaceae. Mycotaxon 8:347–352

Chen DM, Cairney WG (2002) Investigation of the influence of prescribed burning on ITS profiles of ectomycorrhizal and other soil fungi at three Australian sclerophyll forest sites. Mycol Res 106:532–540

Clapp JP, Rodriguez A, Dodd JC (2001) Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phytol 149:539–554

Clapp JP, Rodriguez A, Dodd JC (2002) Glomales rRNA gene diversity—all that glistens is not necessarily glomalean? Mycorrhiza 12:269–270

Jansa J, Mozafar A, Banke S, McDonald BA, Frossard E (2002) Intra- and intersporal diversity of ITS rDNA sequences in *Glomus intraradices* assessed by cloning and sequencing, and by SSCP analysis. Mycol Res 106:670–681

Millner PD, Mulbry WW, Reynolds SL (2001) Taxon-specific oligonucleotide primers for detection of *Glomus etunicatum*. Mycorrhiza 10:259–265

O'Donnell K, Lutzoni FM, Ward TJ, Benny GL (2001) Evolutionary relationships among mucoralean fungi (Zygomycota): evidence for family polyphyly on a large scale. Mycologia 93:286–296

Pringle A, Moncalvo J-M, Vilgalys R (2000) High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycologia 92:259–268

Redecker D, Hijri M, Dulieu H, Sanders IR (1999) Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of Internal Transcribed Spacers reported from *Scutellospora castanea* are of Ascomycete origin. Fungal Genet Biol 28:238–244

Rodriguez A, Dougall T, Dodd JC, Clapp JP (2001) The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomalean families. New Phytol 152:159–167

Schüßler A (1999) Glomales SSU rRNA gene diversity. New Phytol 144:205–207

Schüßler A, Gehrig H, Schwarzott D, Walker C (2001a) Analysis of partial Glomales SSU rRNA genes: implications for primer design and phylogeny. Mycol Res 105:5–15

Schüßler A, Schwarzott D, Walker C (2001b) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol Res 105:1413–1421

Schwarzott D, Walker C, Schüßler A (2001) *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is non-monophyletic. Mol Phylogenet Evol 21:190–197

Simon L, Lalonde M, Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Walker C, Vestberg M (1994) A simple and inexpensive method for producing and maintaining closed pot cultures of arbuscular mycorrhizal fungi. Agric Sci Finland 3:233–240

Walker C, Vestberg M (1998) Synonymy amongst the arbuscular mycorrhizal fungi: *Glomus claroideum*, *G. maculosum, G. multisubstensum* and *G. fistulosum*. Ann Bot 82:601–624