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Construction of genomic phage libraries of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Scutellospora castanea* and isolation of ribosomal RNA genes

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Abstract Genomic phage libraries of arbuscular mycorrhizal fungi were constructed for the first time, and clones containing ribosomal RNA (rRNA) genes isolated for *Glomus mosseae* and *Scutellospora castanea*. The number of rDNA clones per library indicates that these libraries can be also used to isolate genes with low copy numbers. Sequences of the 18S rRNA gene, of the internal transcribed spacer and of the 5.8S rRNA gene were analysed and compared. Differences between the 18S and the 5.8S rRNA genes were few and in the range of variation found for other fungi. In contrast, the internal transcribed spacers of *G. mosseae* and *S. castanea* were highly variable, showing the potential of this region for the identification of different species or isolates. Interestingly, nucleotide exchanges were found in this region when the sequence for *G. mosseae* was compared to those of two other clones of the same isolate.

Key words Glomales · rRNA genes · ITS region · Genomic libraries · PCR

Introduction

Arbuscular mycorrhizal fungi (AMF) belonging to the Glomales represent a phylogenetic group that probably became specialized very early in evolution. It has been deduced from fossil and molecular data that these organisms originated in the Devonian together with the first land plants, about 415 million years ago (Pirozynski and Dalpé 1989; Simon et al. 1993a; Remy et al. 1994). The ancestral nature of these fungi and their evolution within a specific ecological niche, the root, makes the study of the organization and structure of their genome particularly interesting in comparison with other organisms. However, this requires the establishment of large genomic libraries, and this has posed problems because AMF are biotrophic obligate symbionts and cannot be grown in pure culture. Therefore, DNA must be extracted from spores in order to exclude contamination by plant DNA. This has yielded DNA that can be used for PCR analysis (Burggraaf and Beringer 1989; Simon et al. 1992; Waterman and Dales 1993; Wyss and Bonfante 1993; Lanfranco et al. 1995) but is not suitable for digestion by restriction enzymes. It was only recently that Zézé et al. (1994) developed a method for extracting restrictable DNA from AMF spores and obtained the first AMF plasmid library. Whilst such plasmid libraries are useful for the nontargeted isolation of specific and nonspecific DNA fragments that can be used to generate probes for analysing AMF genomes (Zézé et al. 1994), their size limits their use for targeted isolation of complete genes.

In this paper, we describe for the first time the establishment of phage genomic libraries for *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *Scutellospora castanea* Walker. Clones containing rRNA genes were isolated and analysed in order to show that these libraries are sufficiently large for the isolation of low copy genes and are free of DNA from contaminating organisms. Sequencing of the rRNA clones revealed a high similarity between the 18S and 5.8S rRNA genes and a high variability in the internal transcribed spacers of different AM fungal species.

Material and methods

Spores of *G. mosseae* (isolate BEG 12) and *S. castanea* (isolate BEG 1) were collected from pot cultures grown on *Allium porrum* L. Genomic DNA was isolated following the method of Zézé et al. (1994) except that instead of normal phenol/chloroform ex-

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traction after RNase treatment, samples were boiled with one volume of phenol followed by chloroform extraction. The amount of extracted DNA was estimated by gel electrophoresis using λ DNA as standard. The quality was tested by restriction enzyme digestion of 200 ng DNA by *Bam*HI, *Hin*dIII, *Eco*RI and *Sau*3AI and subsequent electrophoresis in minigels (slot size 4 mm).

The expression vector λZAP (Stratagene) was used for cloning. Genomic DNA was ligated with the phage arms and packaged (Gigapack II, Stratagene). The *Escherichia coli* strain XL1Blue was infected with the phages and plated onto NZY medium (Stratagene protocol). Phage clones were transferred onto Nylon membranes (NY 13 N, Schleicher and Schüll) and screened with digoxygenin-labelled probes (Boehringer protocol). Positive clones were prepared for further analysis by isolating the pSK plasmid with the cloned insert from the phage (Stratagene protocol). Clones were mapped by restriction enzyme digestion and Southern blot hybridization with digoxygenin-labelled probes (Boehringer protocol).

PCR was conducted in a 50 μl volume using 0.5 units Taq polymerase (Appligene), 1 ng plasmid DNA as template, 200 μM dNTPs, 1 μM of each primer and the buffer of the enzyme supplier. For PCR labelling, 2 μM digoxygenin-dUTP was included in the reaction. After incubation for 2 min at 95 °C, 30 cycles were performed with 1 min at 93 °C, 45 s at 56 °C and 1 min at 72 °C, followed by 5 min extension at 72 °C. Primers were synthesized by Eurogen.

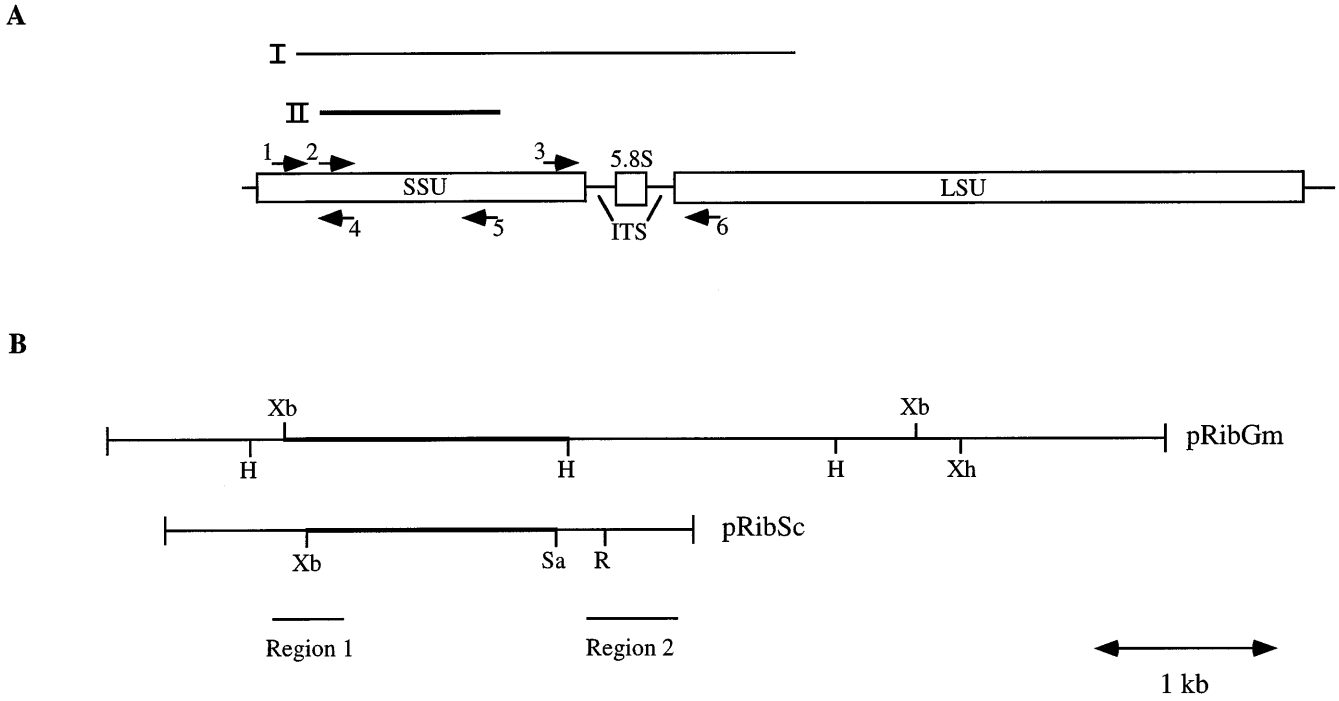
Plasmid DNA was isolated (Birnboim and Doly 1979) and cleaned on a Quiagen column. Sequence analysis (one strand for region 1 and both strands in region 2 in Fig. 1B) was conducted by the Euro Séquences Gènes Services (Montigny le Bretonneux, France). Complete sequences will appear in the EMBL/Genebank nucleotide sequence database under the accession numbers U31995 (18S rRNA gene of pRibGm), U31997 (18S rRNA gene of pRibSc), U31996 (ITS of pRibGm) and U31998 (ITS of pRibSc).

Fig. 1 **A** A schematic representation of the rRNA genes. The large subunit (LSU) and the small subunit (SSU) of the rRNA gene cluster are shown as well as the internal transcribed spacer (ITS) with the 5.8S RNA gene. The fragments are indicated that were used for screening of the genomic libraries (probe I, normal line) or for the analysis of the isolated clones shown in Fig. 1B (probe II, bold line). In addition, the positions of the primers used for PCR or for sequencing are marked as *arrows*. (1:VANS1; 2:NS3; 3:ITS1; 4:NS2; 5:NS6; 6:ITS4). **B** Restriction maps of the isolated *Eco*RI clones. Plasmid clones isolated from the genomic libraries of *Glomus mosseae* (pRibGm) or *Scutellospora castanea* (pRibSc) were digested with *Eco*RI (R), *Hin*dIII (H), *Sal*I (Sa), *Xba*I (Xb) and *Xho*I (Xh). Restriction fragments which hybridize to probe II are drawn as a *bold line*. The position of the clones in the scheme corresponds to the position of the rRNA genes in **A**, according to the PCR experiments with rDNA- and vector-specific primers. In addition, the regions 1 and 2 which were sequenced are shown

Results

Genomic DNA extraction from 1000 spores of *G. mosseae* and *S. castanea* yielded on average 600 ng. DNA of each species was of good quality, since it could be easily digested with various restriction enzymes. Aliquots of 100 ng *Eco*RI-digested DNA of each fungal species were used for cloning, and 500 000 plaque-forming units were obtained. Random analysis of 10 clones revealed a range of insert sizes of 2–7 kb.

Sixty thousand clones were screened with an rDNA probe of *Populus fremonte* L. (Faivre Rampant 1992) to isolate ribosomal RNA genes. This DNA fragment contains part of the large and the small subunit (LSU and SSU) as well as the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster (probe I; Fig. 1A). Positive signals were detected in 20 clones of each library, of which five were further analysed and found to be all the same size (6 kb for *G. mosseae* and 3 kb for *S. castanea*). PCR with the AMF specific primer VANS1 (Simon et al. 1992) and the primer NS2 (White et al. 1990), using *Aspergillus nidulans* genomic



DNA as a negative control, confirmed that these clones were of AMF origin (data not shown).

One clone of each library (pRibGm for *G. mosseae* and pRibSc for *S. castanea*) was characterized in more detail by cutting with different restriction enzymes and Southern blot analysis. Hybridization of the restriction fragments was conducted with the rDNA probe used for screening the library, and a new probe synthesized by PCR with the primer pair NS3/NS6 (White et al. 1990; probe II, Fig. 1A). To determine the orientation of the two clones corresponding to the different regions of the rRNA genes, PCR analyses were conducted using the rDNA-specific primers NS2 and ITS1 in combination with universal and reversal sequencing primers. The restriction maps for both clones deduced from these experiments are given in Fig. 1B.

Two regions of the 18S rRNA gene in clones pRibGm and pRibSc were sequenced. Primer NS2 was used for region 1 (Fig. 1B), and the primers ITS1 and ITS4 (White et al. 1990) for region 2 (Fig. 1B). When compared with sequences of other 18S rRNA genes in the EMBL database, region 1 of the analysed clones showed the highest similarity to those of AMF. The *G. mosseae* sequence (isolate BEG 12) was identical to that of *G. mosseae* (isolate FL156B of the INVAM collection) analysed by Simon et al. (1993a; accession number Z14007), although the isolates are of totally different origin. In contrast, the *S. castanea* sequence showed small differences to those of other *Scutellospora* species or isolates (98% similarity) (accession numbers Z14012 and Z14013; Clapp et al. 1995), as shown in Fig. 2A. The *S. castanea* sequence was as similar to *Glomus* spp. sequences (about 92%, not shown) as to those of other fungi such as *Taphrina deformans* (M. L. Berbee and J. W. Taylor; accession number U00971), *Schizosaccharomyces pombe* (B. Lapeyre and J. Feliu; accession number Z19578) and *Ambrosiozyma platypodis* (Spatafora and Blackwell 1993). Region 2 includes the ITS with the 5.8S RNA gene (Fig. 2B). Alignment of the sequences of the two AMF species outside the 5.8S RNA was very difficult because of the high degree of variability between them, whilst the 5.8S RNA sequence itself showed 85% identical nucleotides. This was in the same range of similarity (80–90%) as found with 5.8S RNA from other fungi such as *Candida albicans* (Mercure et al. 1993), *Nematospora coryli* (R. Messner, unpublished; accession number U09326) or *Saccharomyces cerevisiae* (Rubin 1973). Comparison of the 5.8S RNA region with organisms other than fungi revealed 60–70% identical nucleotides: those of *Saccharomyces cerevisiae*, *Arabidopsis thaliana* (Unfried and Gruendler 1990) and *Homo sapiens* (Nazar et al. 1976) are given as examples in Fig. 2B. Comparison of the ITS region cloned from *G. mosseae* (BEG 12) with the the two ITS sequences of the same isolate published by Sanders et al. (1995) showed some small differences (Fig. 2B).

Discussion

This is the first report of the cloning of AMF genomic DNA into phages. A similar approach has since been used to establish a genomic library for the AMF *Gigaspora rosea* Nicolson and Schenck (isolate BEG 9) (unpublished). The strategy (cloning into λZAP) does not need a large quantity of DNA, which is of particular advantage for cloning AMF DNA. In addition, further analysis is facilitated since purification of phage DNA and subcloning into plasmids is not required. However, the disadvantage of λZAP is that it cannot take up inserts larger than 10 kb; therefore, certain *Eco*RI fragments may not be cloned using this approach.

The number of rDNA clones in both the *G. mosseae* and *S. castanea* libraries can be estimated from the number of positive hybridization signals obtained using the rDNA-specific probe (20 out of 60 000); since the total number of clones was 500 000, the libraries probably contain about 160–170 rRNA gene copies. rDNA is moderately repetitive in fungi (100–400 copies; Long and Dawid 1980), and preliminary results (Passerieux 1994) indicate that there could be at least 90 copies for *S. castanea*. Thus both libraries may be useful for the isolation of genes occurring in lower copy numbers. They are presently being further screened using other heterologous probes and antisera to isolate different genes encoding actin, tubulin and glyceraldehyde phosphate dehydrogenase.

Phage clones with rRNA genes of the two AMF *G. mosseae* and *S. castanea* have been isolated, and in the case of *G. mosseae* the clone contains the complete 18S rRNA gene, the ITS with the 5.8S RNA gene and a major portion (2.8 of about 3.6 kb) of the 25S rRNA gene. In the case of *S. castanea*, the 18S and the ITS regions have been isolated but not the 25S RNA region, which might be located on an *Eco*RI fragment too large for this library. It will be possible to overcome this problem by establishing a new library using a restriction enzyme giving fragments of this region small enough to be cloned into λZAP. The fact that there is an *Eco*RI site in the clone is probably due to partial digestion of the genomic DNA.

Analysis of the 18S sequence of the ribosomal gene cluster of the two AMF confirmed that the isolated clones are of AMF origin and not derived from contaminating organisms, such as bacteria (Scannerini and Bonfante 1991) or other fungi (Lee and Koske 1994) which can be associated with AMF spores. It seems likely that the library obtained for each fungus is free of such contamination, since all the isolated clones with ribosomal DNA gave a PCR amplification product with the AMF-specific primer VANS1. In addition, analysis of the 18S revealed that, although there are slight differences between the sequences of *S. castanea* and the other Gigasporineae, the taxon-specific primer VAGIGA designed by Simon et al. (1993b) for *Gigaspora* species can also be used for specific amplification of *S.*

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S. castanea     GCGAATGGCT CATTAAATCA GTTATAGTTT ATTTGATAGT ACAATTACTA  50
S. pellucida    .......... .......... .......... .......... ..........
S. dipapillosa  .......... .......... .......... .......... ..........
Scut 1          .......... .......... .......... .......... ..........
Scut 2          .......... .......... .......... .......... ..........

                CTTGGATAAC CGTGGTAATT CTAGAGCTAA TACATGCTAA AAGTCTCGAC 100
                .......... .......... .......... .......... ..........
                .......... .......... .......... .......... ..A..C....
                .......... .......... .......... .......... ..A..C....
                .......... .......... .......... .......... .....C....

                TTCAGGAAGG GACGTATTTA TTAGATAAAA ACCAATAACC TTCGGGTTTC 150
                ...T...... .....T.... .......... .......... ..........
                ...T...... ..T....... .......... .......... ..........
                ...T...... ..T....... .......... .......... ..........
                ...T...... ..T....... .......... .......... ..........

                ACTTGGTGAT TCATGATAAC TTTTCGAATC GTATGGCCTT GCGCTGACGA 200
                .......... .......... .......... .......... .T..C.....
                .......... .......... .......... .......... ....C.....
                C........
                C........

                TGAATCATTC AAATTTCTGC CCTATCAACT TTCGATGGTA GGATAGAGGC 250
                .......... .......... .......... .......... ..........
                ..T....... .......... .......... .......... ..........

                CTACCATGGT TTTAACGGGT AACGGGGAAT TAGGGTTCGA TTCCGGAGAG 300
                .......... .......... .......... .......... ..........
                .......... A..T...... .......... .......... ..........

                GGAGCCTGAG AAACGGCTAC CACATCCAAG GAAGGCAGCA GGCGCGCAAA 350
                .......... .......... .......... .......... ..........
                .......... .......... .......... .......... ..........

                TTACCCAATC CCGACACGGG GAGGTAGTGA CAATAAATAA CAATACAGGG 400
                .......... .......... .......... .......... ..........
                .......... .......... .......... .......... ..........

                CTCTTTTGGG TCTTGTAATT GGAATGAGTA CAATTTAAAT CCCCTAACGA 450
                ......A... .......... .......... ........N. ...T......
A               ......C... .......... .......... .......... ...T......
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Fig. 2 **A** Sequence comparison of 18S rRNA genes. The analysed part of *S. castanea* is compared with those of *S. pellucida* and *S. dipapillosa*, as well as two nondefined *Scutellospora* isolates (sequenced until nucleotide 159). Identical nucleotides are shown as *dots*. The region of the genus-specific primer VAGIGA is underlined. **B** Sequence comparison of the ITS including the 5.8S RNA gene. The ITS 1 and the ITS 2 regions (*lower-case*) of *G. mosseae* (1–109/267–491) and of *S. castanea* (1–82/240–420), as well as the 5.8S RNA gene sequences (*capital letters*) of *G. mosseae* (110–266), *S. castanea* (83–239), *Saccharomyces cerevisiae, Arabidopsis thaliana* and *Homo sapiens*, are compared. Nucleotides which are identical to the sequence of *G. mosseae* are represented as *dots*. *Dashes* indicate gaps in the sequences which where introduced for a better alignment. In addition, the differences from the two PCR clones from *G. mosseae* (same isolate) analysed by Sanders et al. (1995) are shown below the sequences

castanea, because the corresponding region is identical (Fig. 2A). It is also evident that the two *Scutellospora* sequences obtained from bluebell mycorrhiza (Clapp et al. 1995) suggest the presence of species or isolates that are different from *S. castanea* BEG 1, while the *G. mosseae* isolate BEG 12 is more closely related to the isolate FL156B of the INVAM collection analysed by Simon et al. (1993a).

Analysis and comparison of the ITS region showed the typical pattern observed in other organisms: the 5.8S DNA of the ITS region is similar in *G. mosseae* and *S. castanea*, especially in the 5' part, whilst the flanking regions show no alignment. This makes the ITS region extremely interesting for studies of biodiversity in AMF or for their identification, for example, by PCR-RFLP or PCR with species-specific primers, an

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G. mosseae      atgattttaa ggcgagtcga agcgttaagc gaggcttgcg aaaatattta   50
S. castanea     gaa.a..g.g .t--.t...t .c.tc.ttt- ---------- ---g......   34
clone 1                    a          -
clone 2                    a

                aaaccccact ctttataact ttaa-aaaat aaatcatgat acatgaattt   99
                .....tt... a.a..a...c .a..-a---- -------tt. .t..at.aaa   72
                                          g
                            c  t          g

                aaaaaaaaga TCACTTTCAA CAACGGATCT CTTGGCTCTC GCATCGATGA  149
                t...t...a. AA........ ...T...... .......... ..........  122
S. cerevisiae               AA........ .......... .....T.... ..........
A. thaliana                 CG...C..GG ........A. ..C....... ..........
H. sapiens                  CG...C.T.G .GGT.....A ..C.....GT ..G.......

                AGAACGCAGC GAAA--TGCG ATAAGTAGTG TGAATTGCAT AATTTTGTGA  197
                .......... ....--.... ......A... .........G ....CC.C..  170
                .......... ....--.... ..C...A... .........G ....CC....
                ......T... ....--.... ..CT.G.... .........G  ...CCC...
                .......... .CT.GC.... ...T..A... .........G G.CACAT...

                ATCATCGAAT CTTTGAACGC AAATTGCACT CCCTGGTATT CCGGGGAGTA  247
                ......A... T.....G... .......... T.T....... ..AA.A....  220
                .......... .......... .C.....G.C ..T....... ..A...G.C.
                ........G. .......... ..G....G.C ..AA.CCT.C TG.CC...GG
                T-......CA ...C...... .-C....GGC . .-..GT.C .TCCC.G.GC

                TGCCTGTTTG AGGGTCGTTa gaataaaaaa tcgag-gcgt cgctcttttt  296
                CA....C... ......AG.- t..a....t. ...ta-cat- --....ga.g  265
                .......... ..C...A..     clone 1      c t
                CA.G.C.GCC T....GTCA      clone 2      a c
                .A.GCC.G.C T.A.CGTCG

                tttaagggtg atcgcgtcgg aattg-agcc gtctttcaaa tgttaattca  345
                ..-ac..a.c tgg.ttatcc g...t----- t.....t.g. a-..gg..ac  308
                                        g   c      -
                                            agc g t-

                tgtcaaagtg gcttaaaatt catccatccg gtacggttta aagcgtattt  395
                ctaa...--- --........ gta--..gt. aag..tac.. .taa..cg..  351
                               g                          -
                                                          a  -


                aagatcaatt ttgattaaga acgcgcgatg acg-taccat ctcatgtagt  444
                ..cca.tt.a c.acccttt. tta..tt.ct ..taa.gtta t.t.gtat..  404

                             t c                   t

                acgttgacct gggtcgtcag gttcactcgt atactaaact cgaactt     491
                gg-------- ---------- ------.... ..aa.tttaa ..g        420

B                              a          t         g t    ta
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approach already applied to the analysis of ectomycorrhizal fungi (Gardes et al. 1991; Henrion et al. 1992; Gardes and Bruns 1993; Henrion et al. 1994). ITS regions seem to be highly variable. Sanders et al. (1995), using a different approach, showed that at least two sequences exist in one spore of *G. mosseae* BEG 12, and the ITS sequence published here for this fungus is again different. At least two different rRNA clusters have also been detected for *S. castanea* (Passerieux 1994). Whether these differences are due to a heterozygous genome in one nucleus or to heterogeneous nuclei in one spore is not clear.

Another interesting result comes from the comparison of sequences from the 18S regions of *G. mosseae* (Glomineae) and *S. castanea* (Gigasporineae). The similarity between the two is as high as in comparisons with other fungi. This is also true for other AMF 18S sequences deposited in the EMBL Genebank database (Simon et al. 1993a). Fungi with high similarity belong not only to the Zygomycetes, but also to other groups like the Ascomycetes (*Taphrina*). This is supported by the high phylogenetic divergency found between different Zygomycetes based on 18S rRNA data (Nagahama et al. 1995). The ITS region, on the contrary, could not be compared to other Zygomycetes, since other sequences from *G. mosseae* and *S. castanea* have not yet been deposited in the EMBL Genebank database. However, the wide variation between the two AMF observed here supports divergence of the two suborders in the Glomales as defined by Morton and Benny (1990) and as suggested on the basis of cell wall composition (Gianinazzi-Pearson et al. 1994). It also underlines the importance, in establishing phylogeny, of not only looking at one region of the genome, but also taking other sequences (e.g. coding regions of single copy genes), as well as isoenzyme data or morphological features, into account.

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