

## The generic placement of a morphologically enigmatic species in Asteraceae: evidence from ITS sequences

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**Abstract.** *Bidens cordylocarpa* is a high polyploid species restricted in distribution to stream sides in the mountains of Jalisco, Mexico. The morphologically enigmatic species was originally described as a member of the genus *Coreopsis*, but later transferred to *Bidens*, largely because the involucre bracts appear most similar to *Bidens*. Characters of the cypselae, often useful in generic placement, are of no value for this species because the fruits have features not detected in either *Bidens* or *Coreopsis*. Sequences from the internal transcribed spacer region of nuclear ribosomal DNA (ITS) were used to assess the relationships of *Bidens cordylocarpa*. The molecular phylogeny places *B. cordylocarpa* in a strongly supported clade of Mexican and South American *Bidens*, and provides more definitive evidence of relationships than morphology, chromosome number, or secondary chemistry. Molecular, morphological, and chromosomal data suggest that *B. cordylocarpa* is an ancient polyploid, perhaps the remnant of a polyploid complex.

**Key words:** *Bidens cordylocarpa*, *Coreopsis*, Asteraceae, ITS sequences, phylogeny.

In addition to providing extensive phylogenetic insights at the highest levels of flowering plants (e.g. P. S. Soltis et al. 1999), DNA sequence data may be used to address more narrowly focused questions, including elucidating the relationships for those taxa of

uncertain placement due to lack of or conflicting morphological characters. Recent examples come from both family (D. E. Soltis et al. 1999) and generic (Kim et al. 1998) levels. In the present study we report the utility of sequences from the internal transcribed spacer region of nuclear ribosomal DNA (ITS) to resolve the generic affinities of a relatively rare species in the Asteraceae (Heliantheae: Coreopsidinae). It is not surprising that sequences from this region might prove informative given their proven wide application in molecular phylogenetic studies at the specific and generic levels (Baldwin 1992, Baldwin et al. 1995).

The enigmatic species in question in the present study is endemic to stream sides and running water in the mountains of the Mexican state of Jalisco. The taxonomic history of the species was presented earlier (Crawford 1971), and thus a brief summary will suffice. *Coreopsis cordylocarpa* A. Gray ex S. Wats was described by Asa Gray in 1887 (Watson 1887). In two comprehensive treatments of *Coreopsis*, Sherff (1936) and Sherff and Alexander (1955) placed *C. cordylocarpa* in sect. *Coreopsis*, where it seems out of place with the largely North American annuals and herbaceous perennials, but did not comment on the species. However, later Sherff indicated on a specimen of

*C. cordylocarpa* in the Field Museum collected by Arthur Cronquist (Cronquist 9779) that it was the type of a new species of *Bidens* that he intended to name in Cronquist's honor. The name was never published, and still later Sherff annotated the specimen as *C. cordylocarpa*. This suggests that Sherff had doubts about the generic placement of the species.

Morphological distinctions between the two large, variable genera *Bidens* and *Coreopsis* are tenuous at best. Although a number of characters in combination are often useful for telling the genera apart, no single character serves to distinguish them (Sherff and Alexander 1955; Mesfin 1984, 1986, 1993; Mesfin et al. 1995). Sherff and Alexander (1955), in a footnote on the first page of their treatment of Heliantheae-Coreopsidinae for North America, commented that *Bidens* and *Coreopsis*, together with the genus *Cosmos*, "constitute fairly natural and easily distinguishable groups but cannot be separated definitely by any one character." A cladistic study of morphological characters by Ryding and Bremer (1992) provided no support for *Bidens* and *Coreopsis* as sister taxa, and Karis and Ryding (1994) questioned whether either genus as now recognized is monophyletic. Mesfin et al. (in manuscript) have carried out a more extensive cladistic analysis of Coreopsidinae, and the results are similar to those found in the other two investigations, i.e., the two genera are not monophyletic. A recent study using ITS sequence data provides compelling evidence that neither genus as now circumscribed is monophyletic (Kim et al. 1999). Kim et al. (1999) did, however, identify strongly supported monophyletic groups restricted to specific geographical areas.

With the lack of a defining character for *Bidens* or *Coreopsis*, placing a species in either genus as now defined can be problematic if that species lacks one or more of the "important" characters making up the suite of features normally employed in generic placement. The generic assignment of *Bidens cordylocarpa* (A. Gray ex S. Wats) Crawford is particularly challenging because characters

often employed to delimit *Bidens* and *Coreopsis*, imperfect as they are, are of little value for placing this species. This problem is particularly true for the cypselae because in *B. cordylocarpa* they are elongate, club-shaped, very obscurely quadrangular (essentially terete), obscurely lengthwise-striate, glabrous, wingless, contracted at the apex and with a small, bald disk. Crawford (1971) emphasized that this combination of features distinguishes the cypselae from the typically flat, winged fruits often found in *Coreopsis*. Crawford (1971) suggested that the cypselae of *B. cordylocarpa* are most similar to *Bidens* in general shape. However, the combination of features including nearly terete in cross section, lack of definite ribs, and almost obscure longitudinal striations distinguish *B. cordylocarpa* from most other species of *Bidens*. General cypselae shape, and particularly features of the involucre bracts, prompted Crawford (1971) to transfer the species to *Bidens*. While admittedly preliminary, comparative flavonoid chemistry of *B. cordylocarpa* suggested a closer relationship to *Bidens* than to *Coreopsis*. The chromosome number of  $2n = ca. 146$ , determined from four different populations, suggests that the species may be a dodecaploid (12x) on a base number of 12, a base number known for both *Bidens* and *Coreopsis* (Smith 1975, 1989; Robinson et al. 1981).

Given the equivocal nature of other data for placement of *B. cordylocarpa*, a molecular phylogenetic study utilizing ITS sequences was initiated in order to provide refined insights into the relationships of the species. Sequences from the ITS region have proven useful for resolving relationships in *Bidens* and *Coreopsis* (Kim et al. 1999), and sequences from a diverse array of taxa in the two genera were available from earlier studies (Kim et al. 1999, Ganders et al. 2000) for analyses with sequences from *B. cordylocarpa*.

## Materials and methods

Total DNA was isolated from dried material (Melchert, Sorensen & Crawford 6347 & 6371,

vouchers in IA, OS and RM) using CTAB (Doyle and Doyle 1987). PCR amplification of the entire ITS region was obtained using primer ITS4 (White et al. 1990) and a modified version of the White et al. (1990) primer ITS5 (Kim et al. 1999). PCR amplifications were performed using a hot start followed by standard cycling conditions. Negative controls were run to detect contamination. PCR products were purified using QIAquick PCR clean-up columns (Qiagen, Inc.).

Sequencing reactions were performed using the Big Dye™ Terminator Kit (PE Applied Biosystems). Manufacturers recommendations were followed except that reaction volumes were reduced. Sequence reactions were performed using the amplification primers described above, as well as two internal primers (ITS2 and ITS3; White et al. 1990). Sequences were analyzed using an ABI Prism™ 310 genetic analyzer. Chromatographs were examined individually, then assembled into double-stranded contigs.

Sequences for comparison were taken from recently published ITS phylogenies (Kim et al. 1999, Ganders et al. 2000). ITS sequences were initially aligned in ClustalW (Thompson et al. 1994), then manually optimized by eye. The alignment was trimmed and the 5.8S region deleted to match outgroup sequences (Kim et al. 1999). Since homology of some indels was not certain, all sites with gaps, and any neighboring sites where gaps could be placed, were removed from one data set to examine the effects of gap placement and treatment on the results.

Phylogenetic analyses were performed using PAUP\* 4.0b2a (Swofford 1999). Trees were rooted with *Coreopsis cyclocarpa* (see Kim et al. 1999 for justification). To obtain the most parsimonious (MP) tree using equal weighting of characters and character-state changes, a heuristic search with 100 random sequence additions and TBR branch swapping was performed. The reliability of specific groupings in parsimony was examined using 1000 bootstrap replicates and 10 random sequence additions per bootstrap replicate. Decay indices were calculated using AutoDecay 4.0 (Eriksson 1998) with 100 random addition sequence replicates. Gaps were treated as missing data.

Maximum likelihood (ML) analyses were performed using the HKY85 model of sequence evolution (Hasegawa et al. 1985). The MP tree obtained in the search above was used for maxi-

mum likelihood estimation of the transition-transversion ratio. For maximum likelihood analysis, the estimated transition-transversion ratio was used in a heuristic search with 10 random sequence additions and TBR branch swapping.

The relative rates test was performed using a modification of the method proposed by Wu and Li (1985). Briefly, all possible quartets of sequences that included an ingroup *Bidens* sequence, *B. cordycarpa*, and two outgroup sequences (*B. prestinaria* and *B. pachyloma*) were extracted and ML estimates of branch lengths were calculated using PUZZLE 4.0.1 (Strimmer and von Haeseler 1996) and the HKY85 model of sequence evolution. The transition-transversion ratio was estimated simultaneously for each quartet in PUZZLE. This technique was employed because it provides estimates for the standard error of the estimated branch lengths. To conduct the relative rates test, the difference in estimated branch lengths was calculated, and the variance of the difference was computed using the sum of the variances for each branch length (see Wu and Li 1985). Since the variances of individual branch lengths were used, it was unnecessary to estimate the covariances of distance estimates.

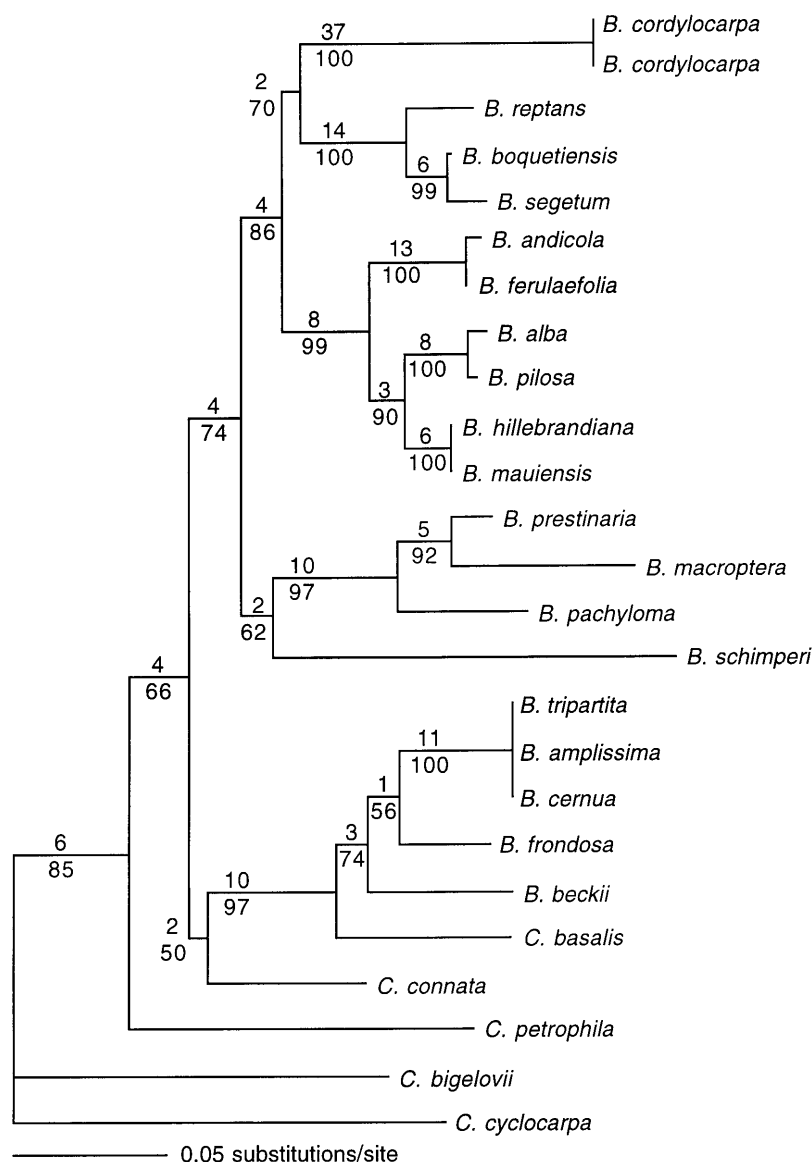
## Results

The two accessions, collected from two different populations, had identical sequences (Genbank accession numbers AF331961 and AF331962 for *Melchert, Sorensen & Crawford 6371 & 6347*, respectively; an alignment can be obtained from the corresponding author). The alignment of ITS 1 and 2 contained 495 sites, of which 281 were variable and 136 were parsimony informative. Base composition was slightly skewed (20.9% A, 26.3% C, 25.9% G, and 26.9% T). A slight transition bias was observed, with an estimated transition-transversion ratio = 1.54. After deleting gaps and sites where gap homology was uncertain, there were 391 sites in the alignment, of which 212 were variable and 136 were parsimony informative. All results from the gaps-deleted data set were topologically identical, and showed similar levels of bootstrap support, to the full data set. As gap treatment appeared to have

little affect on the conclusions, all results presented are from the full data set.

A single MP tree was obtained, with 604 steps, and a consistency index, excluding uninformative sites, of 0.5784. This tree was topologically identical to the ML tree (ln likelihood = -3611.98) presented in Fig. 1. This tree placed *B. cordylocarpa* basally divergent to a clade of Mexican-South American

*Bidens* (sect. *Greenmania* Sherff & Kim et al. 1999), and in a larger clade containing additional Mexican-South American, as well as Hawaiian, *Bidens*. Bootstrap support of 70% has been demonstrated to insure a high degree of confidence in phylogenetic placement (Hillis and Bull 1993), though it has been suggested that higher levels of bootstrap support should be required when taxonomic placement was



**Fig. 1.** Phylogram of the ML tree with parsimony support indices indicated at nodes. Numbers above nodes represent decay indices; numbers below nodes represent percentage of 1000 bootstraps for equally-weighted parsimony

previously uncertain (see Rodrigo et al. 1994 for rationale). Therefore, the placement of *B. cordylocarpa* basally divergent to *B. reptans*, *B. boquetiensis*, and *B. segetum* should be treated with caution, though the placement within the larger clade of Mexican-South American and Hawaiian *Bidens* was well-supported under a more stringent criterion.

A relative rates test comparing the branch lengths of *B. cordylocarpa* with other Mexican-South American and Hawaiian *Bidens* indicated that *B. cordylocarpa* has evolved rapidly at the molecular level (Table 1). In seven of nine comparisons, the branch lengths of *B. cordylocarpa* were significantly longer (greater than two standard deviations from the mean) than those of the other *Bidens*, while in the two non-significant results, *B. cordylocarpa* was longer by more than one standard deviation. Thus, the rapid morphological evolution seen in *B. cordylocarpa*, as evidenced by the large number of apomorphic traits, suggests that morphological and molecular evolution are correlated in this species (see Omland 1997 for other examples).

## Discussion

*Bidens* and *Coreopsis*, the two genera to which the species in this study has been assigned, have been and continue to be recognized as

**Table 1.** Results of the relative rate test between *B. cordylocarpa* and related *Bidens* species. Since the two accessions of *B. cordylocarpa* were identical, only one was used in the relative rates test

Species	Difference	Std. Dev.
<i>B. reptans</i>	0.0428	0.0202*
<i>B. boquetiensis</i>	0.0589	0.0203*
<i>B. segetum</i>	0.0498	0.0212*
<i>B. andicola</i>	0.0449	0.0205*
<i>B. ferulaefolia</i>	0.0491	0.0204*
<i>B. alba</i>	0.0349	0.0208
<i>B. pilosa</i>	0.0383	0.0205
<i>B. hillebrandiana</i>	0.0547	0.0204*
<i>B. mauiensis</i>	0.0561	0.0204*

\* Significantly different at  $p \leq 0.05$

closely related but distinct taxa. Yet, it has not been possible to identify apomorphic characters for each genus or synapomorphies for the two genera (Mesfin 1984, 1986, 1993; Mesfin et al. 1995). The problem surrounding the placement of *B. cordylocarpa* is further exacerbated because cypselae characters have been used in generic delimitation and this species has distinctive cypselae, which “fit” neither genus. While one can never know for certain why Sherff retained this species in *Coreopsis* despite having misgivings, the unusual cypselae probably were an important consideration. In this regard, several studies indicate that cypselae characters such as those distinguishing *B. cordylocarpa* from species of *Bidens* and *Coreopsis* are under simple genetic control. Smith and Parker (1971) showed that the presence or absence of cypselae wings in *Coreopsis tinctoria* is controlled by one or two loci while Smith (1973) demonstrated a simple genetic basis for cypselae characters (such as smooth versus fibrilate wings) in *C. grandiflora*. In Hawaiian *Bidens*, several conspicuous differences distinguishing the cypselae of species, such as smooth versus barbed awns, straight versus coiled bodies, distinct versus decurrent awns, winged versus wingless margins, and setose versus glabrous bodies, are controlled by two loci (Ganders et al. 2000). In *Coreocarpus*, another member of subtribe Coreopsidinae, Smith (1989) showed that the presence of aristae on cypselae is controlled by one locus. Given the results for these genera, it is reasonable to hypothesize that the cypselae features of *B. cordylocarpa*, while very distinctive and obvious, may have a simple genetic basis.

Although *Bidens* and *Coreopsis* may not be monophyletic, and thus it is difficult to assign *B. cordylocarpa* as to one or the other genus, the ITS sequence data show that *B. cordylocarpa* belongs to a strongly supported clade that includes other Mexican and South America species of *Bidens* (Fig. 1). The decision on the most appropriate taxonomic treatment for species now assigned to *Bidens* and *Coreopsis* awaits additional studies, but results of the

present study will be of utility in the ultimate taxonomic disposition of *B. cordylocarpa*. Although, the large number of species of *Bidens* occurring in Latin America precludes adequate taxon sampling for identifying the probable sister species of *B. cordylocarpa* with any confidence, the present results are reasonable from a biogeographic perspective because *B. cordylocarpa* occurs with other *Bidens* species from Mexico and South America. The results presented here conflict with inclusion of the species in *Coreopsis*; also, inclusion of sequences from *Cosmos* and most other genera of the Coreopsidinae in a preliminary phylogenetic analysis does not change the position of *B. cordylocarpa*. Based on the series of distinctive morphological features exhibited by this species and the fact that it does not really “fit” into either *Bidens* or *Coreopsis*, some consideration might be given to recognizing it as a separate genus. However, its nested position within *Bidens* together with the possibility that the distinctive cypselae characters are under simple genetic control suggest that inclusion within *Bidens* is the most appropriate treatment at present.

*Bidens cordylocarpa* occurs in relatively small populations and is rather localized in distribution in the state of Jalisco, Mexico. The distinctive cypselae and other morphological characters may have become fixed in these small populations by a combination of inbreeding and drift. These factors could also account for the apparent high rate of molecular evolution detected in this species, although studies including additional species of *Bidens* in the same clade with *B. cordylocarpa* would be desirable to ascertain whether evolution has been rapid in the entire clade or just in *B. cordylocarpa*. Crawford (1971) suggested that *C. cordylocarpa* may be the remaining element of an ancient polyploid complex, and while the present study does not provide additional evidence in favor of this hypothesis, it does not argue against it. Despite being a high polyploid, there was no indication of heterogeneity in the ITS sequences. Thus, if *B. cordylocarpa* is an allotetraploid, concerted evolution has homogenized the dif-

ferent repeat types. Alternatively, the species could represent an autoployploid. The answers to these questions and others about the origin and relationships of this species await more intensive studies, but for the present it clearly is best recognized as a species, albeit a very distinctive one, of *Bidens*.

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## References

- Baldwin B. R. (1992) Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. *Molec. Phylogenet. Evol.* 1: 3–16.
- Baldwin B. R., Sanderson M. J., Porter J. M., Wojciechowski M. F., Campbell C. S., Donoghue M. J. (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Gard.* 82: 247–277.
- Crawford D. J. (1971) Morphology, chromosome number, and flavonoid chemistry of *Bidens cordylocarpa* (Compositae). *Madroño* 21: 41–47.
- Doyle J. J., Doyle J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochem. Bull.* 19: 11–15.
- Eriksson T. (1998) AutoDecay, version 4.0. (program distributed by the author; Department of Botany, Stockholm University, Stockholm).
- Ganders F. R., Berbee M., Pirseyedi M. (2000) ITS base sequence phylogeny in *Bidens* (Asteraceae): evidence for the continental relatives of Hawaiian and Marquesan *Bidens*. *Syst. Bot.* 25: 122–133.
- Hasegawa M., Kishino H., Yano T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Molec. Evol.* 22: 160–174.
- Hillis D. M., Bull J. J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* 42: 182–192.
- Karis P. O., Ryding O. (1994) Tribe Heliantheae. In: Bremer K. (ed.) *Asteraceae-cladistics and classification*. Timber Press, Portland, Oregon, pp. 559–624.

- Kim H.-G., Keeley S. C., Vroom P. S., Jansen R. K. (1998) Molecular evidence for the African origin of the Hawaiian endemic *Hesperomannia* (Asteraceae). *Proc. Natl. Acad. Sci. USA* 95: 15440–15445.
- Kim S. C., Crawford D. J., Tadesse M., Berbee M., Ganders F. R., Pirseyedi M., Esselman E. J. (1999) ITS sequences and phylogenetic relationships in *Bidens* and *Coreopsis* (Asteraceae). *Syst. Bot.* 24: 480–493.
- Mesfin Tadesse (1984) The genus *Bidens* (Compositae) in N. E. Tropical Africa. *Acta Univ. Ups. Symb. Bot. Upsa.* 24(1): VIII + 138 pp. Uppsala.
- Mesfin Tadesse (1986) The morphological basis for inclusion of African species of *Coreopsis* L. in *Bidens* L. (Compositae-Heliantheae). *Symb. Bot. Upsal.* 26(2): 189–202.
- Mesfin Tadesse (1993) An account of *Bidens* (Compositae: Heliantheae) for Africa. *Kew Bull.* 48: 437–516.
- Mesfin Tadesse, Crawford D. J., Smith E. B. (1995) Comparative capitular morphology and anatomy of *Coreopsis* L. and *Bidens* L. (Compositae), including a review of generic boundaries. *Brittonia* 47: 61–91.
- Omland K. E. (1997) Correlated rates of molecular and morphological evolution. *Evolution* 51: 1381–1393.
- Robinson H. R., Powell A. M., King R. M., Weedon J. F. (1981) Chromosome numbers in Compositae, XII: Heliantheae. *Smithsonian Contr. Bot.* 52: 1–28.
- Rodrigo A. G., Bergquist P. R., Bergquist P. L. (1994) Inadequate support for an evolutionary link between the metazoa and fungi. *Syst. Biol.* 43: 578–584.
- Ryding O., Bremer K. (1992) Phylogeny, distribution, and classification of the Coreopsidinae (Asteraceae). *Syst. Bot.* 17: 649–659.
- Sherff E. E. (1936) Revision of the genus *Coreopsis*. *Field Mus. Nat. Hist. Bot.* 11(6): 279–475.
- Sherff E. E., Alexander E. J. (1955) Compositae-Heliantheae-Coreopsidinae. *N. Amer. Fl.*, ser. 2, 2: 1–149.
- Smith E. B. (1973) A biosystematic study of *Coreopsis saxicola* (Compositae). *Brittonia* 25: 200–208.
- Smith E. B. (1975) The chromosome numbers of North American *Coreopsis* with phyletic interpretations. *Bot. Gaz.* 136: 78–86.
- Smith E. B. (1989) A biosystematic study and revision of the genus *Coreocarpus* (Compositae). *Syst. Bot.* 14: 448–472.
- Smith E. B., Parker H. M. (1971) A biosystematic study of *Coreopsis tinctoria* and *C. cardamine-folia* (Compositae). *Brittonia* 23: 161–170.
- Soltis D. E., Mort M. E., Soltis P. S., Hibsich-Jetter C., Zimmer E. A., Morgan D. R. (1999) Phylogenetic relationships of the enigmatic angiosperm family Podocestemaceae inferred from 18S and 18S + *rbcL* sequence data. *Molec. Phylogenet. Evol.* 11: 261–272.
- Soltis P. S., Soltis D. E., Chase M. W. (1999) Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* 402: 402–404.
- Strimmer K., von Haeseler A. (1996) Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Molec. Biol. Evol.* 13: 964–969.
- Swofford D. L. (1999) PAUP\*: Phylogenetic analysis using parsimony (\* and other methods), version 4.0. Sinauer, Sunderland, MA.
- Thompson J. D., Higgins D. G., Gibson T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.
- Watson S. (1887) Contributions to American botany. XXI. *Proc. Amer. Acad. Arts and Sci.* 22: 396–481.
- White T. J., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Gelfand D., Sminsky J., White T. (eds.) *PCR protocols: a guide to methods and applications*. Academic Press, San Diego, pp. 315–322.
- Wu C.-I., Li W.-H. (1985) Evidence for higher rates of nucleotide substitution in rodents than in man. *Proc. Natl. Acad. Sci. USA* 82: 1741–1745.

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