Ann. Bot. Fennici 49: 369–376 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 30 November 2012

Dillenia tetrapetala (Dilleniaceae), a new species from HonBa Nature Reserve, Vietnam

Ritesh Kumar Choudhary[1], Tran The Bach[2], Luu Van Nong[3], Do Van Hai[2], Bui Hong Quang[2], You-Mi Lee[4], Sang-Hong Park[1], Changyoung Lee[1] & Joongku Lee[1,*]

[1)] *International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, South Korea (*corresponding author's e-mail: joongku@kribb.re.kr)*
[2)] *Institute of Ecology & Biological Resources, Vietnam Academy of Science & Technology, 18-Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam*
[3)] *HonBa Natural Reserve, Khanh Hoa 57000, Vietnam*
[4)] *Korea National Arboretum, Pocheon-Si, Gyeonggi-Do 487-821, South Korea*

Received 24 May 2012, final version received 26 Sep. 2012, accepted 27 Sep. 2012

Choudhary, R. K., Bach, T. T., Nong, L. V., Hai, D. V., Quang, B. H., Lee, Y. M., Park, S. H., Lee, C. & Lee, J. 2012: *Dillenia tetrapetala* (Dilleniaceae), a new species from HonBa Nature Reserve, Vietnam. — *Ann. Bot. Fennici* 49: 369–376.

A new species *Dillenia tetrapetala* Joongku Lee, T.B. Tran & R.K. Choudhary (Dilleniaceae) is described from HonBa Nature Reserve of the Khanh Hoa province of Vietnam. Detailed illustrations and taxonomic comments are provided along with a table listing the differential characters to the closely similar taxa. Phylogenetic analyses using nrITS region of ribosomal DNA and *psb*A–*trn*H intergenic spacer region of chloroplast DNA sequences were also performed and they supported the status of *D. tetrapetala* as a distinct species. The plant is considered endangered based on the IUCN red list criteria because of its restricted distribution.

Introduction

The genus *Dillenia* (Dilleniaceae) was established by Linnaeus in 1753 for *D. indica*. There are about 60 species distributed from Madagascar and the Seychelles to the Fiji islands, and in the north to the southern slopes of the Himalayas, Yunnan, Kwangsi and Kwantung (Hoogland 1952). In Vietnam, the genus is represented by eight species (Pham 1993).

During an exploration trip to HonBa Nature Reserve in the Khanh Hoa province of south Vietnam, we came across a population of *Dillenia* possessing striking red stamens and four petals and sepals. It first appeared to be *D. blanchardii*, which is known to possess a characteristic narrow and non-amplexicaule winged petiole, a terminal inflorescence and striking red stamens. However, the difference in the number of petals and dehiscence characters of the fruits compelled us to dig deeper. A review of the literature revealed that *D. fischeri* is the only known species in the genus having variable numbers of petals and sepals, from four to six (Hoogland 1952). The possibility of the collected plant to be *D. fischeri* was ruled out because of the

considerable morphological deviations, namely a petiole with completely amplexicaule wings (a feature not recorded in the SE Asian species of *Dillenia*), occurrence of terminal or axillary inflorescences, villous younger parts, number of carpels, etc. (Table 1). Furthermore, we found *D. blanchardii* to be the most morphologically similar species. A close scrutiny of the protologue and the type specimen however revealed a number of morphological differences (Table 1). Intrigued by the morphological differences from *D. blanchardii*, we decided to analyze the possible molecular differences between these two. We surveyed the type locality of *D. blanchardii* at Con Dao Island (earlier known as Poulo Condor and then Con Son) of Vietnam, and collected fresh vouchers and leaf material for the DNA extraction. Some apparently closely allied SE Asian *Dillenia* that shared common ancestral characters were also included in our analysis. For this, we generated sequences for *D. hookeri*, *D. ovata* and *D. indica* from the voucher samples (Table 2) housed in the herbarium of the Korea Research Institute of Bioscience and Biotechnology, South Korea (KRIB).

In this study, we chose a combination of two markers, namely the *psb*A–*trn*H intergenic spacer region of chloroplast DNA and the internal transcribed spacer (ITS) region of the ribosomal cistron (18S-5.8S-26S) because of their expected sequence variation and easy PCR amplification (Table 3). ITS has been frequently used to construct phylogenetic relationships at the inter-specific level in angiosperm taxa (Alvarez & Wendel 2003). The *psb*A–*trn*H spacer region has also been proved to be a successful marker in delimitation of many closely related species (Sang *et al*. 1997, Azuma *et al*. 1999). Kress *et al*. (2005) suggested that the sequences of *psb*A–*trn*H, along with ITS, have the potential to discriminate among the largest number of plant species for barcoding purposes. With the morphological and molecular variations found, we concluded that our collection from HonBa represented a new species, which we describe here.

Material and methods

Morphological study

The morphological studies were based on the field observations and the samples collected from the HonBa Nature Reserve and the type locality of *D. blanchardii* in the Con Dao dis-

Table 1. Morphological comparison of *Dillenia tetrapetala* with its allied species.

Character	*D. tetrapetala*	*D. blanchardii*	*D. fischeri*
Young twigs	Glabrous	Pubescent	Sparsely hirsute
Petiole	With non-amplexicaule wings	With non-amplexicaule wings	With completely amplexicaule wings
Inflorescence	Terminal, glabrous, 1–2 flowered	Terminal, glabrous, 1–3 flowered	Terminal or lateral, 2–4(–6) flowered
Number of petals	4	5	4–6 (usually 5)
Color of petals	Yellow	Yellow	White
Size of petals	Double of the length of sepals, 3–5 cm	Almost equal to the size of sepals. 2.5–3 cm	ca. 3 cm
Number of sepals	4	5	4–6
Leaves	Glabrous below	Pubescent below	Sparsely strigose to glabrescent below
Fruits	Dehiscent	Unknown in the original description; mentioned as 'probably indehiscent' by Hoogland (1952)	Unknown
Number of carpels	5	7	4–6 (usually 5)
Ovules	2–5	12	8–10
Seeds	Light buff colored with brown patches to light brown	Dark brown	Unknown

trict of South Vietnam. Specimens housed in the herbaria of the Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea (KRIB) and Institute of Ecology and Biological Resources, Hanoi, Vietnam (HN) were also studied.

Sampling of material for molecular analysis

Samples of *D. tetrapetala*, *D. blanchardii* and the outgroup *Tetracera scandens* were collected from the field and stored in plastic scintillation tubes with silica gel. Samples of *D. hookeri*, *D. indica* and *D. ovata* were obtained from the voucher specimens housed in KRIB. The voucher specimens for all sequenced plants are deposited in KRIB and HN.

DNA isolation, PCR amplification and sequencing

Dried plant material (10–15 mg) was used for the DNA isolation. The plant tissue was ground to fine powder using the TissueLyser and 3-mm tungsten beads. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's protocol (the final elution step was carried out using 2×50 μl elution buffer). The quality of the DNA extractions was verified by electrophoresis on 1% agarose gel.

The nuclear ribosomal Internal Transcribes Spacer region (including ITS1, 5.8S and ITS2) was amplified using the primers ITS1 and ITS4 (Table 3). The following reaction-mix composition was applied in a total volume of 20 μl. A touchdown cycling profile was applied, including 5 min at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 56 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C.

The *psb*A–*trn*H region (ca. 350 bp) was also amplified with the primers indicated in Table 1. One cycle of a PCR reaction consisted of 4 min at 95 °C, 30 sec at 94 °C, 1 min at 55 °C (35 cycles) for primer annealing, and 1 min at 72 °C, and a final extension step of 10 mins at 72 °C.

Prior to sequencing, the PCR products were purified with the SolGent PCR Purification Kit (SolGent, Daejeon, Korea) or QIAquick (Qiagen). The sequencing reaction

Table 2. Information on the *Dillenia* samples used in the study.

Taxa	ITS	*psb*A–*trn*H	Voucher information
D. blanchardii-DBC1	JX852680	JX852690	*J. Lee & Bach*, *0040507* (KRIB)
D. blanchardii-DBC2	JX852681	JX852691	*J. Lee & Bach*, *0040508* (KRIB)
D. blanchardii-DBC4	JX852682	JX852692	*J. Lee & Bach*, *0040509* (KRIB)
D. tetrapetala-DBH1	JX852684	JX852694	*Nong et al.*, *VK-044* (HN)
D. tetrapetala-DBH2	JX852685	JX852695	*J. Lee et al.*, *0040505* (KRIB)
D. tetrapetala-1G	JX852683	JX852693	*J. Lee et al.*, *0040506* (KRIB)
D. ovata-DO7	JX852686	JX852697	*Kongmany*, *0033787* (KRIB)
Dillenia hookeri-DV7	JX852679	JX852689	*Bach et al.*, *0028190* (KRIB)
Dillenia indica-DO2	JX852687	JX852696	*Bach et al.*, *0018007* (KRIB)
Tetracera scandens	JX852688	JX852698	*J. Lee et al.*, *HIKK-132* (HN)

Table 3. Primers and references used in amplification and sequencing of ITS and *psb*A–*trn*H intergenic spacer regions.

Primers	Seq. (5´→3´)		References
ITS	ITS1	GTCCACTGAACCTTATCATTTAG	White *et al.* 1990
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* 1990
*psb*A–*trn*F	TRHF	CGCATGGTGGATTCACAATC	Sang *et al.* 1997
	PSAR	AGACCTAGCTGCTATCGAAG	Sang *et al.* 1997

was performed using a BigDye Terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems) according to the manufacturer's instructions, in an ABI PRISM 3730XL DNA Analyzer (Perkin-Elmer, Applied Biosystems).

Analyses

The sequences were checked and edited with Sequence Navigator (Parker 1997) and aligned with ClustalX (Thompson *et al*. 1997) followed by manual adjustments. The parsimony analyses were conducted using PAUP* ver. 4.b10 (Swofford 2002), and the character states were specified as unordered and unweighted. The indels were coded as missing data. All most-parsimonious trees (MPTs) were saved. The bootstrap analyses (Felsenstein 1985) were performed with 10 000 replications and a heuristic search as a default option.

The Bayesian inference (BI) estimation was calculated with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) using the GTR + τ model (Yang 1996). Two simultaneous and independent analyses were performed; for each analysis, four Markov Monte Carlo chains were run simultaneously starting from random trees. Each analysis was run for 200 000 generations sampling one out of every 200 generations, which resulted in 10 000 sample trees. A critical aspect of the Bayesian analysis is to ensure that the Markov chain has reached its stationary distribution. With this aim, the first 1000 trees (burn-in) were excluded to avoid trees that might have been sampled prior to convergence of the Markov chains, before computing the majority-rule consensus tree. Posterior probability support (PP) was estimated to be significant for nodes with PP > 0.95.

Results and discussion

Dillenia tetrapetala Joongku Lee, T.B. Tran & R.K. Choudhary, *sp. nova* (Figs. 1 and 2)

TYPE: Vietnam. Khanh Hoa Province, HonBa Nature Reserve, 5 April 2009 *Nong et al*., *VK-044* (holotype HN!; isotype KRIB!). — PARATYPES: Vietnam. Khanh Hoa Province, HonBa Nature Reserve, 12°07'N, 109°00´E, ca. 221 m, 14 April 2011 *J. Lee et al*., *HIKK-524* (KRIB!); Khanh Hoa prov., Cam Lam district, 31 March 2010 *Bach et al*., VK 3616 (KRIB!).

ETYMOLOGY: *Dillenia tetrapetala* takes its specific epithet from its four petals, which is a character encountered only in two species of *Dillenia* (i.e. *D. fischeri* and *D. tetrapetala*).

Evergreen trees, 16–20 m high. Bark grey-brown to brown. Branches sympodial. Leaf scars clasping about 3/5 of the branch, subfalcate with ca. 7–9 leaf-traces. Leaves elliptic-oblong to obovate, (4–)6–14(–18) × (2–)3–7 cm, with (7–)9–12(–14) nerves on either side; slightly retuse or rounded to obtuse at apex, rounded at base, decurrent; margin entire to slightly undulate, nerves curving upwards, not reaching margin, glossy, glabrous on both sides. Petiole (0.6–)1–2(–3) cm, glabrous, with 0.5 to 1.5 mm broad, not amplexicaule wings. Inflorescences terminal, usually 2-, rarely 1- or 3-flowered racemes, 3.5–5 cm long; axis ca. 1.5 mm thick, glabrous; bracts caducous, elliptic, somewhat concave, ca. 6 × 3 mm. Flowers 5.5–6.5 cm across. Pedicel 1.5–4 cm, ca. 1 mm thick, thickened to 4 mm at apex, glabrous, without bracteoles. Sepals 4, oval, 16–20 × 8–12 mm, glabrous inside, sparsely hirsute outside. Petals 4, yellow, membranaceous, 30–32 × 24–27 mm, obovate, narrowed towards base, rounded and shriveled at apex. Stamens 100–170, pink-purple, outermost ones slightly curved in bud, 10–12 mm long, innermost ones with apical part reflexed outward in bud, 7–12 mm long, with many of intermediate lengths; filament 2.5–4 mm long; anther 0.6–1 mm broad, truncate at apex; thecae linear, opening with apical pore. Carpels 5–7, pink-purple, arranged around narrow conical receptacle, oblong, 5–6 × 1.2–1.5 mm, glabrous, each with ca. 7–15 ovules; styles recurved, 8–10 mm long, 0.6 mm thick at base, 0.1–0.2 mm near apex. Pseudocarps dehiscent, oval, 20–28 × 15–22 mm; carpels 12–14 × 6–7 mm, each with 1–6 seeds. Seeds reniform, 5–5.5 × 3–4 mm, light buff with brown patches to light brown, enclosed by a fleshy aril. Seed surface glabrous, colliculate. Flowering and fruiting in April–November.

DISTRIBUTION AND HABITAT: Vietnam, Khanh Hoa Province, HonBa Nature Reserve, south Vietnam, mostly near streams. Subtropical forests in association with plants such as *Polygala*

Fig. 1. *Dillenia tetrapetala.* — **A**: Habit. — **B**: Bark. — **C** and **F**: Petiole, abaxial and adaxial view. — **D**: Flowering twig. — **E** and **G**: Sepals. — **H** and **I**: Stamens. — **J**: Twig showing dehiscent fruit. — **K**: Carpel. — **L**: Seed.

karensium, *Smilax corbularia*, *Croton caudatus*, *Psychotria rubra*, *Dischidia major*, *Ficus vasculosa*, *Micromelum minutum*, *Litsea monopetala*, *Maesa acuminatissima,* etc. Local inhabitants know this plant for its good timber value.

Molecular data

Alignment of the combined nrITS and *psb*A–*trn*F dataset yielded 1025 nucleotide sites. Of these, 689 sites were constant, 241 were variable

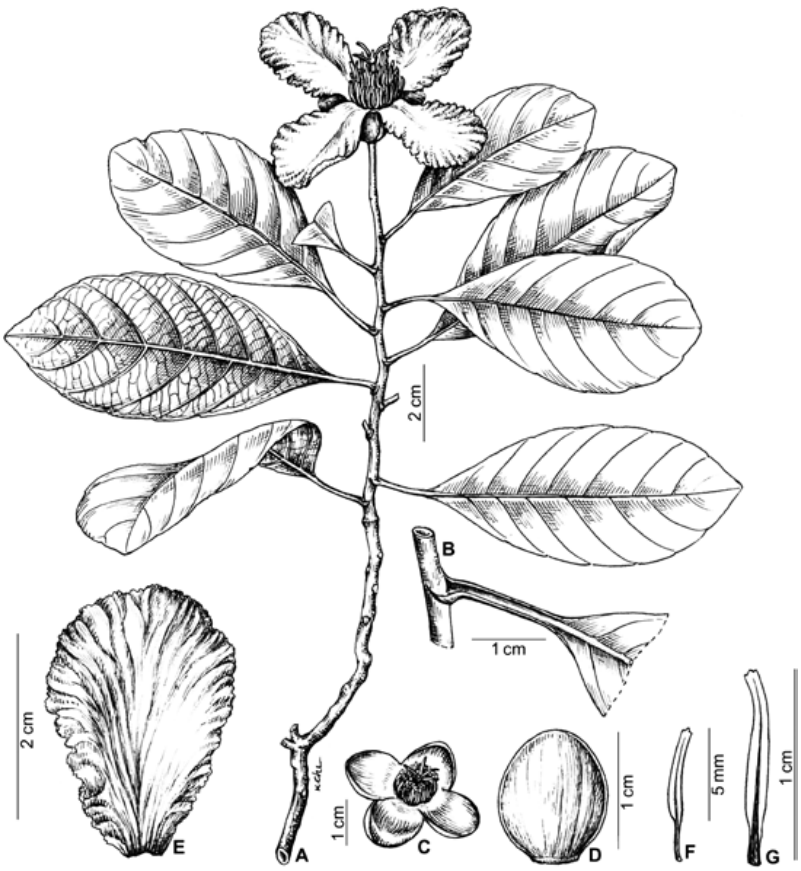

Fig. 2. *Dillenia tetrapetala* (drawn from the holotype by Mrs. Kim Chi). — **A**: Flowering twig. — **B**: Petiole. — **C** and **D**: Sepals. — **E**: Petal. — **F** and **G**: Stamens.

but parsimony uninformative, and 95 were parsimony informative. The total length of the majority rule consensus tree was 394, with CI = 0.934, RI = 0.862 and HI = 0.069. The entire ITS region, including both spacers and the 5.8S gene obtained from the studied samples, varied from 594–602 bp and the length of the *trn*H–*psb*A spacer region varied from 333 to 336 bp. There were eight indels ranging from 1 to 20 bp and four site substitutions on ITS, and six indels and twenty site substitutions on *trn*H–*psb*A. The phylogenetic analysis of the sequences using *Tetracera scandens* as outgroup showed that *D. tetrapetala* is a distinct species and closely related to *D. blanchardii* (Fig. 3). The remaining three species, *viz*. *D. hookeri*, *D. indica* and *D. ovata*, formed a sister clade. The sequences produced during the study were submitted to GenBank (Table 2).

Key to the *Dillenia* species of Vietnam (modified from Hoogland 1952)

1. Inflorescence or solitary flowers typically terminal on leaf-bearing branches, often later lateral 2
1. Flowers either axillary on leaf bearing branches or solitary or in fascicles on leafless branches 7
2. Stamens about equally long, only slightly curved in bud ..... *D. hookeri*
2. Innermost stamens distinctly longer than outer ones, with apical part reflexed outward over the latter 3
3. Stamens of outer group gradually increasing in size towards center of flower, outer group not sharply distinct from inner one 4
3. Stamens of outer group approximately equally long, outer group sharply distinct from inner one 5
4. Leaves glabrous below; petals and sepals 4; seeds light buff-colored with dark brown patches to light brown .... *D. tetrapetala*
4. Leaves pubescent below; petals and sepals 5; seeds dark brown ..... *D. blanchardii*
5. Number of carpels 5–12 6
5. Number of carpels 14–20 ..... *D. indica*

6. Flowers in 2–4-flowered racemes, ca. 10–13 cm across. Leaves obovate ..... *D. turbinata*
6. Flowers solitary, ca. 16 cm across; leaves ovate to oval . ..... *D. ovata*
7. Thecae opening with longitudinal slit; flowers up to ca. 5 cm across 8
7. Thecae opening with apical pore; flowers ca. 10–12 cm across ..... *D. obovata*
8. Pedicels without bracteoles ..... *D. pentagyna*
8. At least part of pedicels with caducous bracteoles .... *D. scabrella*

Conservation

Dillenia tetrapetala has a very narrow distribution range in Vietnam, which fortunately falls under the reaches of the protected forest areas of the HonBa Nature Reserve. However, the plants are very sparsely distributed throughout the region. During our frequent field visits to the HonBa Nature Reserve, almost 80 individuals were found. The habitat of this species is well protected from anthropogenic activities, but the site within the reserve needs to be marked for long-term monitoring in close coordination with the management authorities. Furthermore, the species is sought after for its strong wood. If care is not taken, the species will possibly be lost. The limited distribution of the species and an estimated population size of less than 250 mature individuals justify an assessment of a preliminary (not yet submitted to IUCN/the Vietnam Red List authority) conservation status as Endangered category under criterion D, according to IUCN Red List Criteria (http://intranet.iucn.org/webfiles/doc/SSC/RedList/RedListGuidelines.pdf).

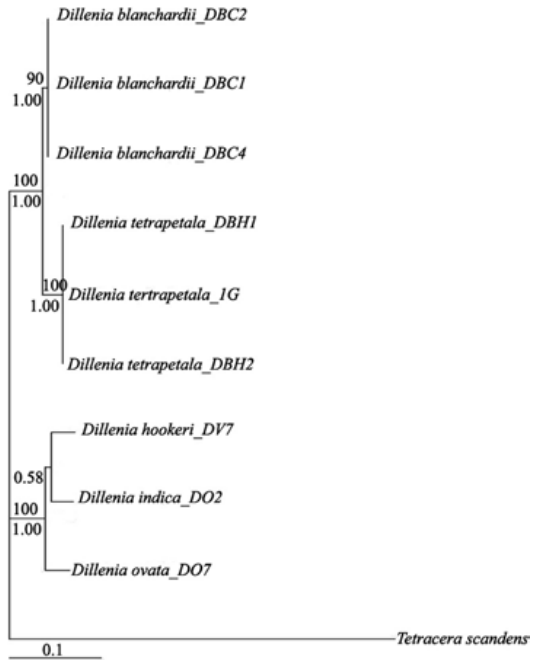

Fig. 3. Consensus tree obtained from the Bayesian analysis of the combined ITS and *trn*H–*psb*A dataset. Bayesian posterior probabilities are shown below the branches, > 65% bootstrap values from the parsimony analyses are shown above the branches.

Acknowledgements

Funding support received from Korea National Arboretum and International Biological Material Research Center, KRIBB, Korea for this study is greatly acknowledged. The authors also express their thanks to Mrs. Kim Chi, IEBR, Vietnam for making illustration of *D. tetrapetala* for the present communication. Thanks are also due to the forest administration of HonBa Nature Reserve for their support.

References

Alvarez, I. & Wendel, J. F. 2003: Ribosomal ITS sequences and plant phylogenetic inference. — *Molecular Phylogenetics and Evolution* 29: 417–434.

Azuma, H., Leonard, B. T. & Shoichi, K. 1999: Molecular phylogeny of *Magnolia* (Magnoliaceae) inferred from CpDNA sequences and evolutionary divergence of the floral scents. — *Journal of Plant Research* 112: 291–206.

Felsenstein, J. 1985: Confidence limits on phylogenies: an approach using the bootstrap. — *Evolution* 39: 783–791.

Hoogland, R. D. 1952: A revision of the genus *Dillenia*. — *Blumea* 7: 1–145.

Huelsenbeck, P. & Ronquist, F. 2001: MRBAYES: Bayesian inference of phylogenic tree. — *Bioinformatics* 17: 754–755.

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A. & Janzen, D. H. 2005: Use of DNA barcodes to identify flowering plants. — *Proceedings of the National Academy of Sciences* 102: 8369–8374.

Parker, S. R. 1997: Sequence navigator. Multiple sequence alignment software. — *Methods in Molecular Biology* 70: 145–154.

Pham, H. H. 1993: Dilleniaceae. — In: Pham, H. H. (ed.), *Cay co Viet Nam: an illustrated flora of Vietnam*, vol. 2(2): 910–949. Mekong Printing, Montreal.

Ronquist, F. & Huelsenbeck, J. P. 2003: MrBayes 3: Bayesian phylogenic inference under mixed models. — *Bioinformatics* 19: 1572–1574.

Sang, T., Crawford, D. J. & Stuessy, T. F. 1997: Chloroplast

DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). — *American Journal of Botany* 84: 1120–1136.

Swofford, D. L. 2002: *PAUP*: phylogenetic analysis using parsimony (and other methods)*, 4.0 b10. — Sinauer Associates, Sunderland, MA.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997: The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. — *Nucleic Acids Research* 24: 4876–4882.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phyologenetics. — In: Innis, M. A., Gelfand, D. H., Sninksky, J. J. & White, T. J. (eds.), *PCR protocols: a guide to methods and amplifications*: 315–322. Academic Press, San Diego, California.

Yang, Z. 1996: Among-site rate variation and its impact on phylogenetic analyses. — *Trends in Ecology and Evolution* 11: 367–372.