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Trichosanthes anguina L. is variety of *Trichosanthese cucumerina* L.- evidence based on molecular phylogenetic analysis of internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA

Ajmal Ali M.* and Fahad M. A. Al-Hemaid
*Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, 11451, Saudi Arabia, ajmalpdrc@gmail.com

Abstract- Phylogenetic relationship among some species of *Trichosanthes* L (*Cucurbitaceae*) was assessed using internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA to infer the taxonomic status of *Trichosanthes anguina*. The parsimony analysis of the entire ITS region resulted in 85 maximally parsimonious trees (MPTs) with a total length of 100 steps, a consistency index (CI) of 0.8800 (0. 8378 excluding uninformative characters), a homoplasy index (HI) of 0.1200 (0. 1622 excluding uninformative characters), rescaled consistency index (RC) of 0.7733 and a retention index (RI) of 0.8788. Our findings support the recognition of *T. cucumerina* var. *anguina* (L.) Haines as a variety of *T. cucumerina*.
Keywords: *Trichosanthes anguina*, *Trichosanthese cucumerina*, *Cucurbitaceae*, ITS, nrDNA

Introduction

Genus *Trichosanthes* L. of tribe Trichosantheae, subtribe Trichosanthinae, family *Cucurbitaceae* include c. 100 species [1-5]. Principally it is an Asiatic genus. The geographic distribution of the genus indicates either an Indo-Malayan or Chinese Centre of origin [6]. *Tichosanthes anguina* (snake gourd or serpent gourd) is an usual cucurbits with long, white spackled fruits that actually in morphology resembles with snake and is widely grown as a vegetable in India and in the Orient. Roots and seeds are used to expel worms and to treat diarrhea and syphilis [7-8]. Haines, 1921-1924 [9] recognized *T. cucumerina* L. var. *cucumerina* (L) Haines as a wild variant with short fruits and *T. cucumerina* var. *anguina* (L.) Haines as cultivated variant with elongated, snake-like fruits. Jeffrey, 1980 [6] also followed Haines, 1921-1924 [9] treatment of *T. anguina* as a variety of *T. cucumerina.* Chakravarty, 1982 [10], however, treated these two varieties as two different species. This treatment has been followed in most of the Indian floras, monographs and research paper published so far. A perusal of literature reveals that taxonomic status of *T. anguina* is controversial [11-17]. Hence, this study was undertaken to compare sequences of the internal transcribed spacer regions of nrDNA in some species of *Trichosanthes* in order to infer taxonomic status of *T. anguina*.

Materials and Methods

Present study sampled 10 accessions of *Trichosanthes* (ingroup) and *Luffa* (outgroup) from the geographical origin of Bihar, West Bengal and Sikkim (India) and South Korea. Voucher specimens are deposited at the BHAG (Herbarium, University Department of Botany, Tilka Manjhi Bhagalpur University, Bhagalpur, Bihar, India) and KRIB (Herbarium, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea). Sources of plant material used in this study, along with voucher information and GenBank accession numbers, are listed in the Table 1. Based on chloroplast DNA sequences [17] and pollen morphology [18] a close relationship between tribe Trichosantheae and Luffeae have been suggested to which the genus *Trichosanthes* and *Luffa* belong. Therefore, the sequences of *Luffa* were used as outgroup. Total DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Amsterdam, Netherlands). ITS sequences of nuclear ribosomal DNA were amplified using primers ITS1 (Forward 5'-GTCCACTGAACCTTATCATTTAG-3') and ITS4 (Reverse 5'-TCCTCCGCTTATTGATATGC-3') [19] via the polymerase chain reaction (PCR) using the AccuPower HF PCR PreMix (Bioneer, Daejeon, South Korea) in 20 µL volumes containing 2 µL of 10X buffer, 300 µM dNTPs, 1 µL of a 10 pM solution of each primer, 1 unit of HF DNA polymerase. The initial denaturation at 94℃ for 5 min, and followed by 40 cycles of 94℃ for 1 min, 49℃ for 1 min, and 72℃ for 1 min, with a final extension step of 72℃ for 5 min. The PCR products were ligated into the pT7Blue cloning vector using Perfectly Blunt Cloning Kit (Novagen, Inc.) according to the manufacturer's instructions. Resulting recombinant plasmids were used to transform competent cells included in the kit. The transformation mix was incubated in 250 µl SOC medium for 1hour at 37℃ on a rotary shaker, then plated on LB agar with 50 µg/mL ampicillin. Colonies were randomly selected and were put into PCR buffer. The PCR products were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea) prior to sequencing. The purified fragments were directly sequenced using dye terminator chemistry following the manufacturer's protocol. Cycle sequencing was conducted using same primers used in amplification and BigDye vers. 3 reagents and an ABI PRISM 3730XL DNA

Analyzer (Perkin-Elmer, Applied Biosystems) by following the manufacturer's instructions. Cycling conditions included an initial denaturing set at 94°C for 5 min., followed by 30 cycles of 96°C for 10 sec., 50°C for 5 sec., and 60°C for 4 minutes. Each sample was sequenced in the sense and antisense direction. The sequences were analyzed with ABI Sequence Analysis and ABI Sequence Navigator software (Perkin-Elmer/Applied Biosystems). Nucleotide sequences of both DNA strands were obtained and compared to ensure accuracy. Initially the sequence alignments were performed using ClustalX version 1.81 [20] with gap opening penalty = 10 and gap extension penalty = 3.0. Sequence alignments were subsequently adjusted manually using BioEdit [21] and SeaView [22]. Insertion-deletions (Indels) were scored as single characters when we had confidence in positional homology (Annexure). The boundaries between the ITS1, 5.8S, and ITS2 were determined by comparisons with earlier published sequences available at National Center for Biotechnology Information (NCBI) GenBank (www.ncbi.nlm.nih.gov). Gaps were treated as missing data in phylogenetic analyses. All sequences generated in the present study were deposited in GenBank and GenBank accession number included in Table 1. Parsimony analyses were performed with PAUP* 4.0b10 [23]. Heuristic searches were conducted using 10,000 random addition sequence replicates, holding 10 trees at each step, and with tree-bisection-reconnection (TBR) branch swapping, characters equally weighted, and gaps treated as missing data. Support for internal nodes was assessed using bootstrap analysis [24] of 1000 replicates with 100 random additions per replicate and holding 10 trees at each step. Phylogenetic and molecular evolutionary analyses (evolutionary divergence between sequences, the number of base substitutions per site from averaging evolutionary divergence over all sequence pairs, homogeneity test of substitution patterns between sequences, base composition bias difference between sequences, maximum composite likelihood estimate of the pattern of nucleotide substitution, codon-based test of neutrality for analysis between sequences, and Fisher's exact test of neutrality for sequence pairs) were conducted using MEGA version 4 [25-28]. The result was verified with BioNJ and Parsimony analysis (using SeaView) and Baseyan analysis (Mr Bayes). For Bayesian analysis, the best-fit model of nucleotide evolution was found using jModelTest v1.0.1 [29]. Bayesian posterior probabilities for the clades were obtained using Metropolis-coupled Markov chain Monte Carlo analysis as implemented in MrBayes. Two simultaneous independent runs with four Markov chains were done for 5 million generations, and trees were sampled every 100th generation, resulting in 50,000 trees. The first 10,000 trees were considered as the burn-in phase and discarded.

Results

Sequence Characteristics- The combined length of the entire ITS region (ITS1, 5.8S and ITS2) from taxa sampled in the present study ranged from 608-616 bp. The length of ITS1 region and %GC ranged from 191-201 bp and 61-62% respectively, the 5.8S gene was 163 bp, the length of ITS2 region and %GC ranged from 235-260 bp and 65-67% respectively (Table 2). Data matrix has a total number of 632 characters of which 554 characters are constant, 25 characters are variable but parsimony-uninformative and 55 characters are parsimony-informative. Insertions and deletions (indels) were necessary to align the sequences. Indels were ranged from 1 to 11 bp.

Phylogenetic analyses- The parsimony analysis (using PAUP) of the entire ITS region resulted in 85 maximally parsimonious trees (MPTs) with a total length of 100 steps, a consistency index (CI) of 0.88 (0. 8378 excluding uninformative characters), a homoplasy index (HI) of 0.12 (0. 1622 excluding uninformative characters), rescaled consistency index (RC) of 0.7733 and a retention index (RI) of 0.8788. The bootstrap values above the line in bootstrap strict consensus tree (Fig. 1) show the relative support of each clade. The number of base substitutions per site from analysis between sequences (evolutionary divergence) is shown in Table 3. The number of base substitutions per site from averaging evolutionary divergence over all sequence pairs was found 0.048. *Homogeneity test of substitution patterns between sequences:* The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. A Monte Carlo test (1000 replicates) was used to estimate the *P*-values, which are shown in diagonal in the Table 4. *P*-values smaller than 0.05 are considered significant. The estimates of the disparity index per site are shown for each sequence pair above the diagonal.

Base composition bias difference between sequences: The difference in base composition bias per site is shown in Table 5. Even when the substitution patterns are homogeneous among lineages, the compositional distance correlates with the number of differences between sequences.

Maximum composite likelihood estimate of the pattern of nucleotide substitution: Each entry shows the probability of substitution from one

base (row) to another base (column) instantaneously. Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. The nucleotide frequencies are 0.186 (A), 0.189 (T/U), 0.353 (C), and 0.272 (G). The transition/transversion rate ratios are k_1 = 2.313 (purines) and k_2 = 7.672 (pyrimidines). The overall transition/transversion bias is R = 3.349 (Table 6).

Codon-based test of neutrality for analysis between sequences: The probability of rejecting the null hypothesis of strict-neutrality ($d_N = d_S$) (Codon-based test of neutrality for analysis between sequences) is shown in Table 7 (below diagonal). Values of *P* less than 0.05 are considered significant at the 5% level. The test statistic (synonymous substitutions d_N - nonsynonymous substitutions d_S) is shown above the diagonal.

Fisher's exact test of neutrality for sequence pairs: The probability (*P*) of rejecting the null hypothesis of strict-neutrality in favor of the alternative hypothesis of positive selection is shown for each sequence pair (Table 8). *P* values smaller than 0.05 are considered significant at the 5% level.

Discussion

All bootstrap strict consensus trees resulting from the analysis of ITS sequences of nrDNA resolves two major clades i.e (1) *T. lepiniana-T. tricuspidata* (100 bootstrap support) and (2) *T. diocia-T. cucumerina* (74% bootstrap support). *T. kirilowii* sampled from South Korea show polytomy at base with both the groups (Fig. 1), however clearly nested with *T. lepiniana-T. tricuspidata* group within NJ tree (Figs. 2-3). *T. cucumerina* var. *anguina* clade shows proximity (98 % bootstrap support) with *T. cucumerina* (Fig.1). The close relationship of *T. cucumerina* var. *anguina* was also found consistent with BioNJ tree (Figs. 4-5), Parsimony tree (Fig. 6) analysed using SeaView and in Baseyan phylogeny (Fig.7), but never with 100 % bs. In BioNJ and Parsimony tree, *T. cucumerina* var. *anguina* clade with *T. cucumerina* by 89% and 74 % bootstrap support respectively. *T. cucumerina* shows close relationship with *T. dioica* (74 % bootrap support). Haines, 1921-1924 [9] recognized *T. cucumerina* L. as a wild variant with short fruits and *T. cucumerina* var. *anguina* (L.) Haines as cultivated variant with elongated, snake-like fruits. Jeffrey, 1980 [6] also followed Haines 1921-1924 [9] varietal concept of *T. anguina* as a variety of *T. cucumerina.* However, these two varieties have been treated as two different species in Indian Flora, monographs and research paper published so far [10, 30-38]. *T. cucumerina* and *T. cucumerina* var. *anguina* share several morphological characters like: Herbs annual; Stem: slender, 5-angled; Petioles: striate, pubescent; Leaves: broadly ovate to orbicular, reniform, cordate at base, deeply 5-7 lobed or angled, distantly glabrous above, pubesent or scabrid beneath; Tendrils: 2-3 fid; Plant monoecious; Flower: white, bracts entire; Male flowers: peduncles, flowers solitary; Male flower Pedicels: 0.5-2.0 cm. long, slender, minutely bracteate, male flower calyx-tube 2.5 - 3.0 cm. long; petals c. 8-9 x 3 mm. white, oblong, 3-nerved, c. 2 mm. long; Female flower: peduncles 0.5 - 5.0 cm. long, flowers solitary; Seeds: thick, ovate-oblong, compressed, corrugated, finely rugulose, undulate, apex round or obscurely truncate, base attenuate. The diagnostic features of *T. cucumerina* var. *anguina* are: anthers ovoid; ovary narrowly fusiform; fruits very long up to 1.5 meter, cylindrical, often twisted or coiled, surface smooth, often 7- 8 white stripes along the length. *T. cucumerina* var. *anguina* vary from *T cucumerina* in having the morphological features i.e., anther and ovary oblong; fruits: 5-6 x 3.5-4.0 cm, ovoid, conical, 8-10-seeded, tapering at both ends with a long sharp beak, red when ripe. In the present study, *T. cucumerina* var. *anguina* clade with *T. cucumerina* (98% bootstrap support). Under this clade (branch length 0.009) *T. cucumerina* var. *anguina* (branch length 0.008) deeply nested with *T. cucumerina* (Fig. 2-3). The relationship was found consistent (Fig. 4-7) when the results were verified with Maximum Parsimony method (using SeaView), and Baseyan analysis. Thus, our findings strongly support the recognition of *T. cucumerina* var. *anguina* (L.) Haines as a variety of *T. cucumerina*.

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References

[1] Huang L., Yang B., and Yue C. (1997) *Acta Phytotax Sin*, 35, 125–135.
[2] Rugayah E. A. and De Wilde W.J.J.O. (1997) *Blumea*, 42, 471–482.
[3] Rugayah E. A. and De Wilde W.J.J.O. (1999) *Reinwardtia*, 11, 227–280.
[4] Jeffrey C. (2005) *Bot Zhurn*, 90, 332–335.
[5] Schaefer H., Kocyan A., and Renner S. S. (2008) *Systematic Botany*, 33(2), 349–355.
[6] Jeffrey C. (1980) *Bot J Linn Soc*, 233-277.
[7] Kirtikar K. and Basu B.D. (1975) *Bishen Singh Mahendra Pal Singh, Dehra Dun.*
[8] Ali M.A. and Pandey A. K. (2007) *Plant Systematics Research Centre (TMBU) Publication, Bhagalpur, India.*
[9] Haines H.H. (1921-1924) *Allard and Son and West Newman Ltd. London*
[10] Chakravarty H.L. (1982) *Botanical Survey of India, Calcutta, India*.
[11] Decker-Whitaker T. W. (1933) *Bot Gaz* 94, 780-790.
[12] Jobst J., King K. and Hemleben V. (1998) *Mol Phylo Evol*, 9, 204-219.
[13] Shanta A. and Radhakrishaniah M. (2000) *Phytomorphology*, 50(1), 103-111.
[14] Chung S.M., Deena S., Walters D. and Staub J.E. (2003) *Can J Bot*, 81, 814-832.
[15] Decker-Walter D.S., Chung S. and Staub J. E. (2004) *J Mol Evol*, 58, 606-614.
[16] Zhang L.B., Mark P.S., Alexander K. and Renner S. S. (2006) *Mol Phylo Evol*, 39, 305–322.
[17] Kocyan A., Zhang L.B., Schaefer H. and Renner S. S. (2007) *Mol. Phylo. Evol.* 44, 553–577.
[18] Pruesapan K. and Raymond V.D.H. (2005) *Grana*, 44, 75–90.
[19] White T. J., Bruns T., Lee S. and Taylor J. (1990) *San Diego, California: Academic Press*, 315-322.
[20] Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997) *Nucl Acid Res*, 24, 4876–4882.
[21] Hall T. A. (1999) *Nucl Acid Symp Ser*, 41, 95-98.
[22] Gouy M., Guindon S. and Gascuel O. (2010) *Mol Bio Evol*, 27(2), 221-224.
[23] Swofford D. L. (2002) *PAUP: Sinauer, Sunderland, Massachusetts*.
[24] Felsenstein J. (1985) *Evol*, 39, 783-791.
[25] Nei M. and Gojobori T. (1986) *Mol Phylo Evol*, 3, 418-426.
[26] Kumar S. and Gadagkar S.R. (2001) *Genetics*, 158, 1321-1327.
[27] Tamura K., Nei M. and Kumar S. (2004) *Proc Nat Acad Sci (USA),* 101, 11030-11035.
[28] Tamura K., Dudley J., Nei M. and Kumar S. (2007) *Mol Bio Evol*, 24, 1596-1599.
[29] Posada D. (2008) *Mol Biol Evol*, 25, 1253–1256.
[30] Mary J.C. and Hopkins C.Y. (1964) *Can J Chem* 42, 560-564.
[31] Rangaswamy N.S. and Shivanna K.R. (1975) *Ann Bot* 39, 193-196.
[32] Sardar A. K. and Mukherjee K.K. (1987 *Theor Appl Genet*, 74, 171-172.
[33] Pasha M.K. and Sen S.P. (1995) *Biochem System and Eco*, 23(4), 399-406.
[34] Shanavas K.R., Elyas K.K. and Vasudevan D. M. (1995) *Bio Plan*, 37 (3), 417-422.
[35] Chow L.P., Chou M.H., Ho C.Y., Chuang C. C., Pan F. M., Wu S. H, and Lin J. Y. (1999) *Biochem J*, 338, 211-219.
[36] Ojiako O.A. and Igwe C. U. (2008) *Pak J Nut* 7(1), 85-89.
[37] Rahman A.H.M.M., Anisuzzaman M., Alam M. Z., Islam A. K. M. R. and Zaman A. T. M. N. (2006) *Res J Agr Bio Sci*, 2(6): 299-302.
[38] Rahman A.H.M.M., Anisuzzaman M., Ahmed F., Rafiul Islam A.K.M. and Naderuzzaman A.T.M. (2008) *J App Sci Res*, 4(5), 555-558.

Table 1- Plant accessions used for the molecular systematic study of Trichosanthes

Taxon	Voucher	Geographic origin	GenBank Accession No.
Luffa acutangula (L.) Roxb.	M. Ajmal Ali and A. K. Pandey 1061 (BHAG)	Bihar, India	GQ183044
L. cylindrica (L.) M. Roem	M. Ajmal Ali and A. K. Pandey 1089 (BHAG)	Bihar, India	GQ183045
Trichosanthes cucumerina L.	M. Ajmal Ali and A. K. Pandey 1113 (BHAG)	Bihar, India	GQ240883
T. cucumerina L.	Dako and Blattner s.n.	Gudja, Ghana	AM981174
T. cucumerina var. *anguina* (L.) Haines	M. Ajmal Ali and A. K. Pandey 1112 (BHAG)	Bihar, India	GQ240882
T. dioica Roxb.	M. Ajmal Ali and A. K. Pandey 1022 (BHAG)	Bihar, India	GQ240881
T. kirilowii Maxim.	M. Ajmal Ali and Joongku Lee 2076 (KRIBB)	Kwang Ju, Korea	GQ240884
T. lepiniana (Naudin) Cogn.	M. Ajmal Ali and A. K. Pandey 20052 (BHAG)	West Bengal, India	GQ183049
T. tricuspidata Lour.	M. Ajmal Ali and A. K. Pandey 2075 (BHAG)	Sikkim, India	GQ240880
T. tricuspidata Lour.	M. Ajmal Ali and A. K. Pandey 1060 (BHAG)	Bihar, India	GQ183050

Table 2- Length and GC Contents of ITS1 and ITS2

Taxon	ITS 1		ITS2	
	Size (bp)	%GC	Size (bp)	%GC
Luffa acutangula	201	61	235	65
L. cylindrica	201	61	258	65
Trichosanthes cucumerina 1113	191	61	258	66
T. cucumerina var. *anguina*	191	61	260	66
T. cucumerina AM981174	191	61	258	66
T. dioica	191	62	257	67
T. kirilowii	193	61	246	67
T. lepiniana	200	61	236	66
T. tricuspidata 2075	199	61	249	65
T. tricuspidata 1060	199	61	235	66

Table 3- Evolutionary divergence between sequences of species of Trichosanthes

Taxon									
Trichosanthes lepiniana									
Luffa cylindrica	0.071								
Trichosanthes tricuspidata 2075	0.010	0.070							
Trichosanthes tricuspidata 1060	0.010	0.070	0.000						
Luffa acutangula	0.077	0.005	0.076	0.076					
Trichosanthes dioica	0.055	0.072	0.056	0.056	0.078				
Trichosanthes kirilowii	0.047	0.054	0.047	0.047	0.056	0.037			
Trichosanthes cucumerina var. *anguina*	0.057	0.070	0.057	0.057	0.076	0.030	0.039		
Trichosanthes cucumerina	0.049	0.060	0.049	0.049	0.066	0.028	0.030	0.009	
Trichosanthes cucumerina AM981174	0.049	0.060	0.049	0.049	0.066	0.028	0.030	0.009	0.000

Table 4- Test of the homogeneity of substitution patterns between sequences of Trichosanthes

Taxon									
Trichosanthes lepiniana		0.000	0.005	0.005	0.028	0.000	0.000	0.000	0.000
Luffa cylindrica	1.000		0.000	0.000	0.007	0.000	0.000	0.000	0.000
Trichosanthes tricuspidata 2075	0.268	1.000		0.000	0.003	0.000	0.000	0.000	0.000
Trichosanthes tricuspidata 1060	0.247	1.000	1.000		0.003	0.000	0.000	0.000	0.000
Luffa acutangula	0.243	0.116	0.398	0.361		0.022	0.031	0.000	0.000
Trichosanthes dioica	1.000	1.000	1.000	1.000	0.278		0.000	0.000	0.000
Trichosanthes kirilowii	1.000	1.000	1.000	1.000	0.214	1.000		0.000	0.000
Trichosanthes cucumerina var. *anguina*	1.000	1.000	1.000	1.000	1.000	1.000	1.000		0.000
Trichosanthes cucumerina	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
Trichosanthes cucumerina AM981174	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 5- Base composition bias difference between sequences of Trichosanthes

Taxon									
Trichosanthes lepiniana									
Luffa cylindrica	0.043								
Trichosanthes tricuspidata 2075	0.016	0.036							
Trichosanthes tricuspidata 1060	0.016	0.036	0.000						
Luffa acutangula	0.098	0.012	0.074	0.074					
Trichosanthes dioica	0.021	0.043	0.033	0.033	0.095				
Trichosanthes kirilowii	0.016	0.040	0.007	0.007	0.084	0.012			
Trichosanthes cucumerina var. *anguina*	0.016	0.022	0.003	0.003	0.057	0.019	0.003		
Trichosanthes cucumerina	0.016	0.022	0.003	0.003	0.057	0.019	0.003	0.000	
Trichosanthes cucumerina AM981174	0.016	0.022	0.003	0.003	0.057	0.019	0.003	0.000	0.000

Table 6- Maximum composite likelihood estimate of the pattern of nucleotide substitution of Trichosanthes

	A	**T**	**C**	**G**
A	-	*2.62*	*4.89*	**8.71**
T	*2.57*	-	**37.53**	*3.77*
C	*2.57*	**20.1**	-	*3.77*
G	**5.95**	*2.62*	*4.89*	-

Table 7- Codon-based test of neutrality for analysis between sequences of Trichosanthes

Taxon										
Trichosanthes lepiniana		0.146	0.384	0.384	0.257	-0.515	-0.061	-0.544	-0.623	-0.623
Luffa cylindrica	0.884		-0.294	-0.294	1.001	-1.055	-0.762	-1.489	-1.727	-1.727
Trichosanthes tricuspidata 2075	0.702	0.769		0.000	-0.187	-0.862	-0.463	-0.863	-0.953	-0.953
Trichosanthes tricuspidata 1060	0.702	0.769	1.000		-0.187	-0.862	-0.463	-0.863	-0.953	-0.953
Luffa acutangula	0.798	0.319	0.852	0.852		-0.938	-0.642	-1.363	-1.591	-1.591
Trichosanthes dioica	0.607	0.293	0.390	0.390	0.350		-0.439	-0.069	-0.252	-0.252
Trichosanthes kirilowii	0.951	0.447	0.644	0.644	0.522	0.661		-0.330	-0.596	-0.596
Trichosanthes cucumerina var. *anguina*	0.587	0.139	0.390	0.390	0.175	0.945	0.742		0.546	0.546
Trichosanthes cucumerina	0.535	0.087	0.342	0.342	0.114	0.802	0.552	0.586		0.000
Trichosanthes cucumerina AM981174	0.535	0.087	0.342	0.342	0.114	0.802	0.552	0.586	1.000	

Table 8- Fisher's exact test of neutrality for sequence pairs of Trichosanthes

Taxon									
Trichosanthes lepiniana									
Luffa cylindrica	0.504								
Trichosanthes tricuspidata 2075	0.592	1.000							
Trichosanthes tricuspidata 1060	0.592	1.000	1.000						
Luffa acutangula	0.550	0.727	1.000	1.000					
Trichosanthes dioica	1.000	1.000	1.000	1.000	1.000				
Trichosanthes kirilowii	1.000	1.000	1.000	1.000	1.000	1.000			
Trichosanthes cucumerina var. *anguina*	1.000	1.000	1.000	1.000	1.000	1.000	1.000		
Trichosanthes cucumerina	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.571	
Trichosanthes cucumerina AM981174	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.571	1.000

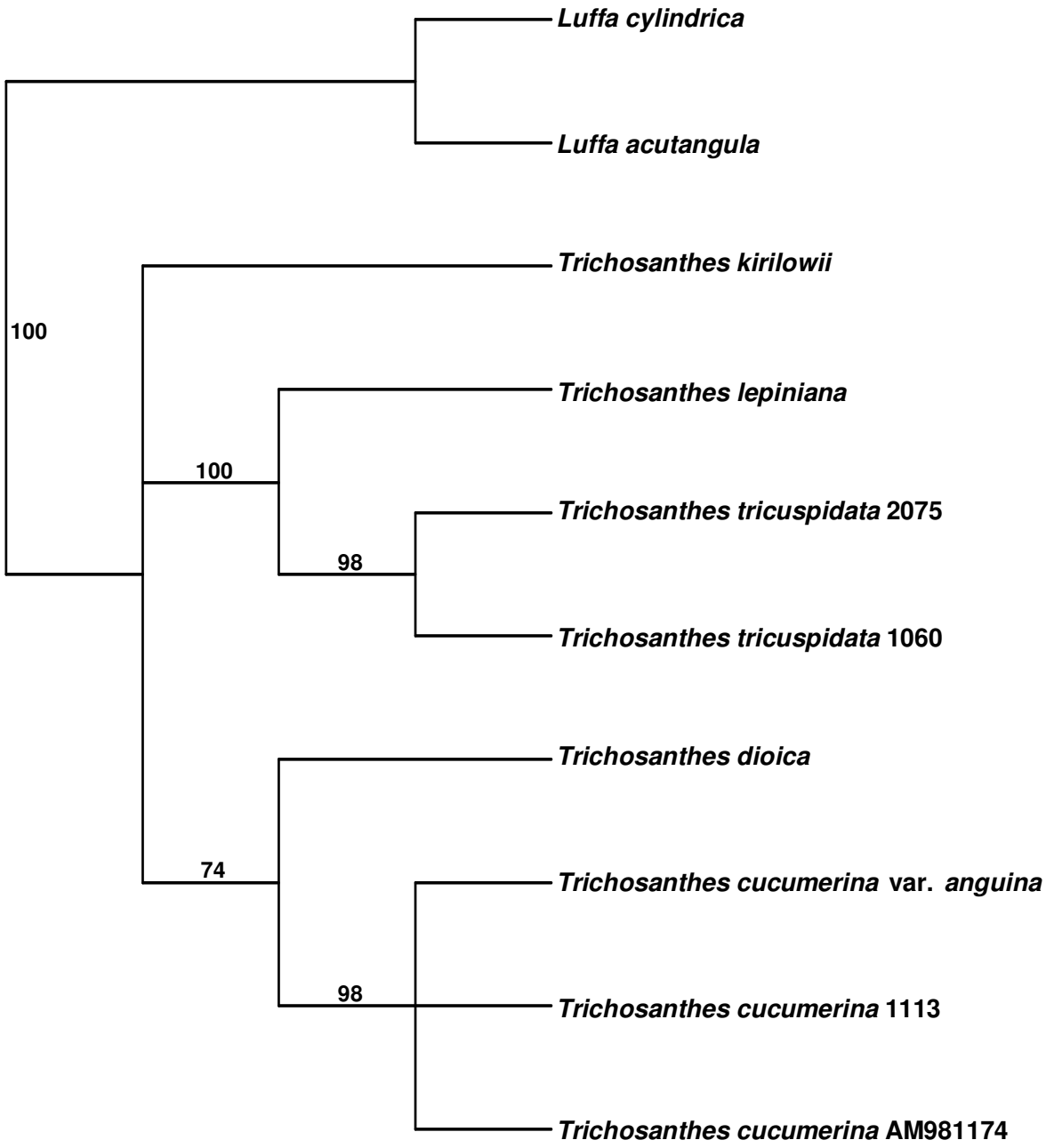

Fig. 1- The strict consensus tree of 85 maximally parsimonious trees of *Trichosanthes* based on the ITS sequences of nuclear ribosomal DNA data set with gaps being treated as missing data (100 steps, a consistency index (CI) of 0.8800 (0. 8378 excluding uninformative characters), a homoplasy index (HI) of 0.1200 (0. 1622 excluding uninformative characters), rescaled consistency index (RC) of 0.7733 and a retention index (RI) of 0.8788. Bootstrap values greater than 50% in 1000 bootstrap replicates are shown above lines

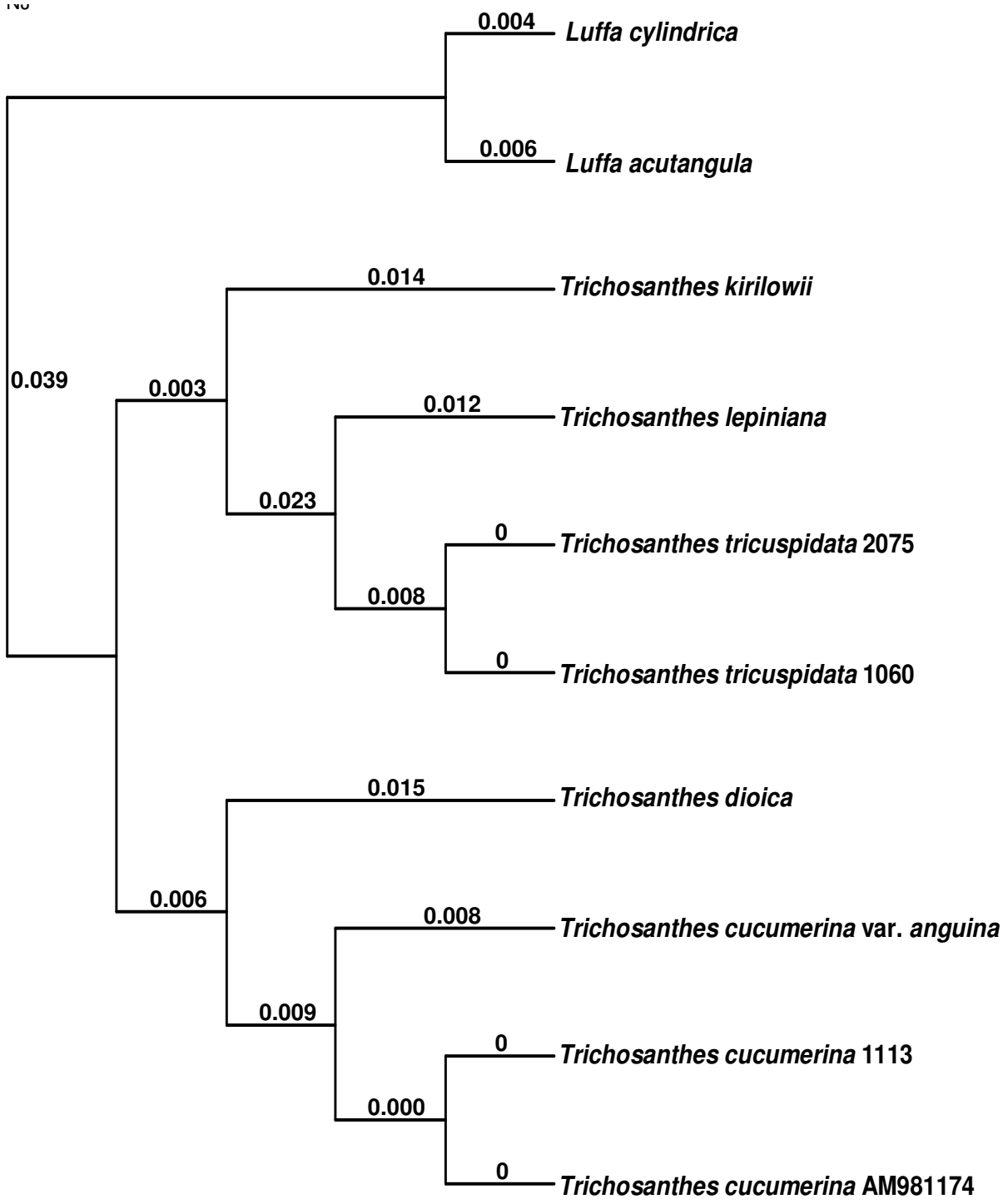

Fig. 2- The neighbor joining tree (rectangular cladogram) of *Trichosanthes* based on the ITS sequences of nuclear ribosomal DNA data set. Nnmbers above the line indicates branch length.

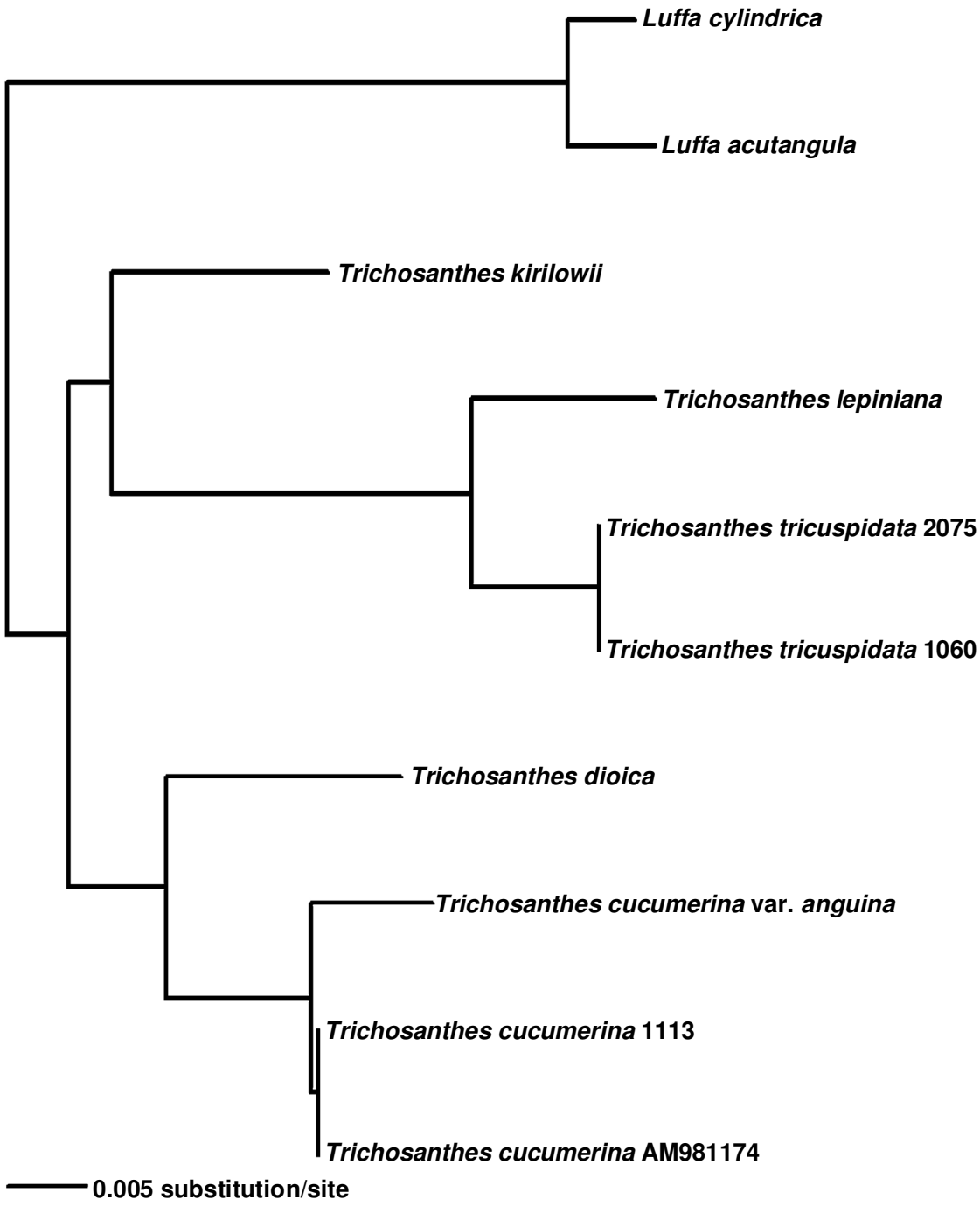

Fig. 3- The NJ phylogram of *Trichosanthes* based on the ITS sequences of nuclear ribosomal DNA data set. The scale bar indicates relative length of the branch.

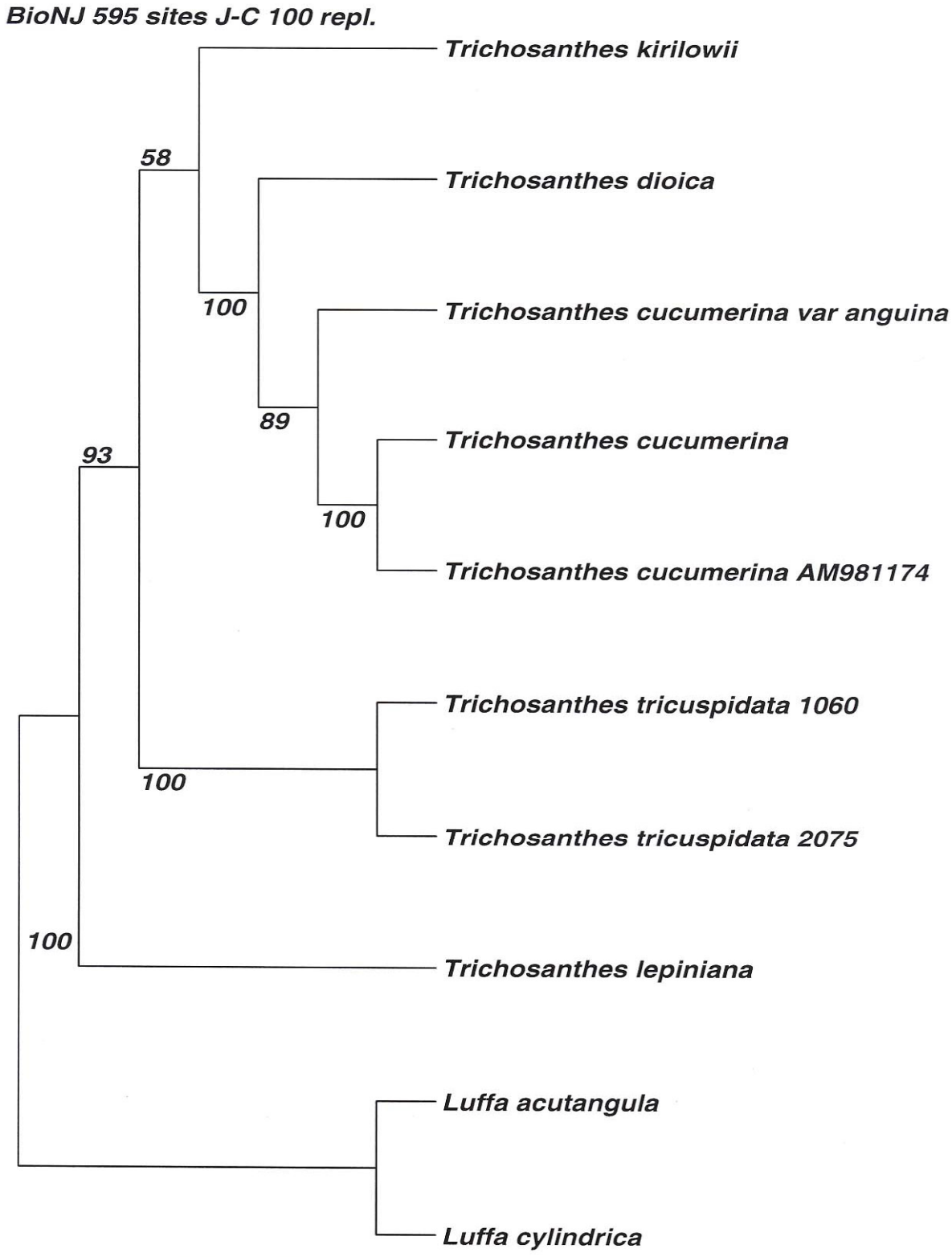

Fig. 4- BioNJ tree inferred from internal transcribed spacer region of nuclear ribosomal DNA. The tree constructed using SeaView after multiple alignment in MUSCLE. The number above the line indicates bootstrap support

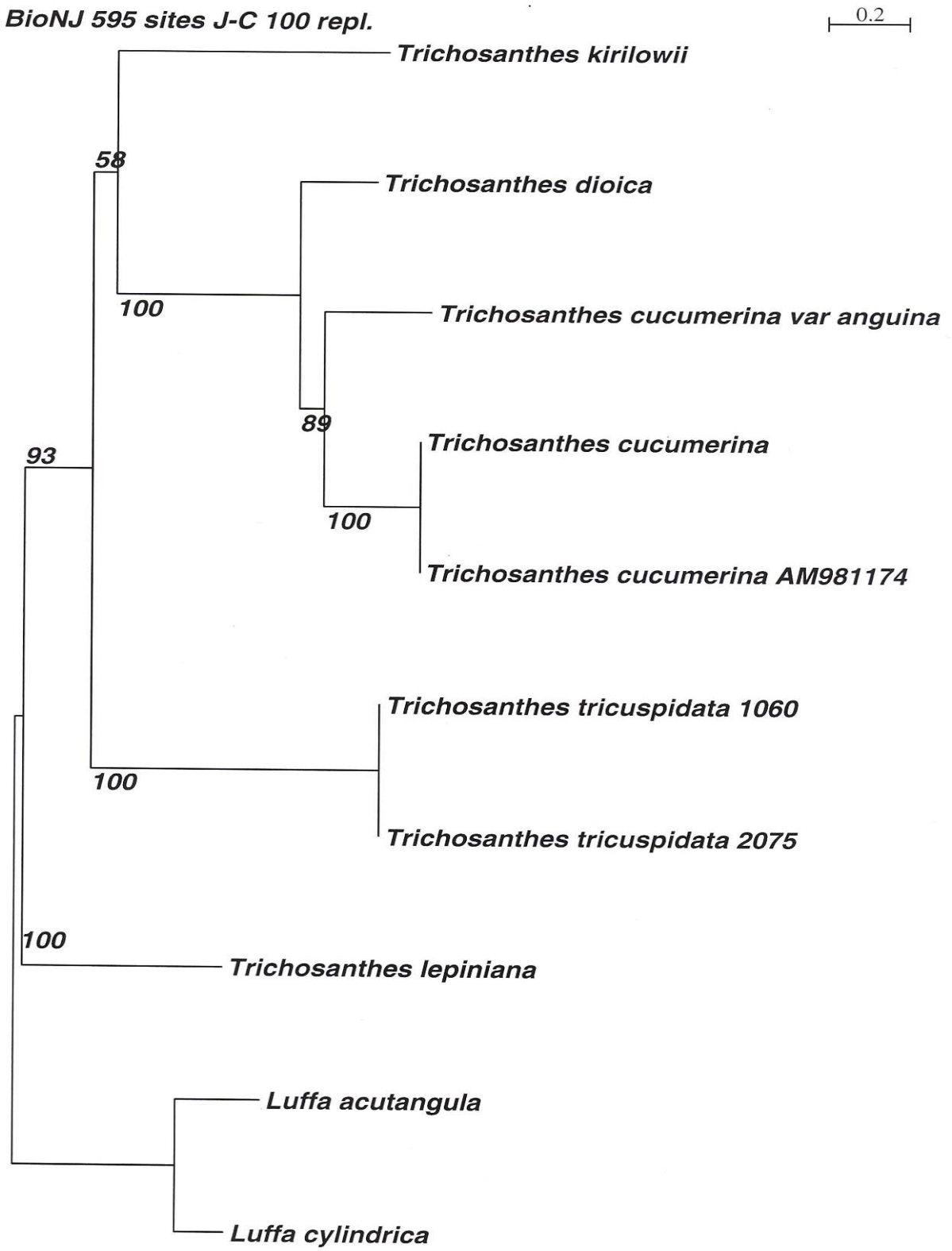

Fig. 5- BioNJ tree (phylogram) inferred from internal transcribed spacer region of nuclear ribosomal DNA. The tree constructed using SeaView after multiple alignment in MUSCLE. The number above the line indicates bootstrap support

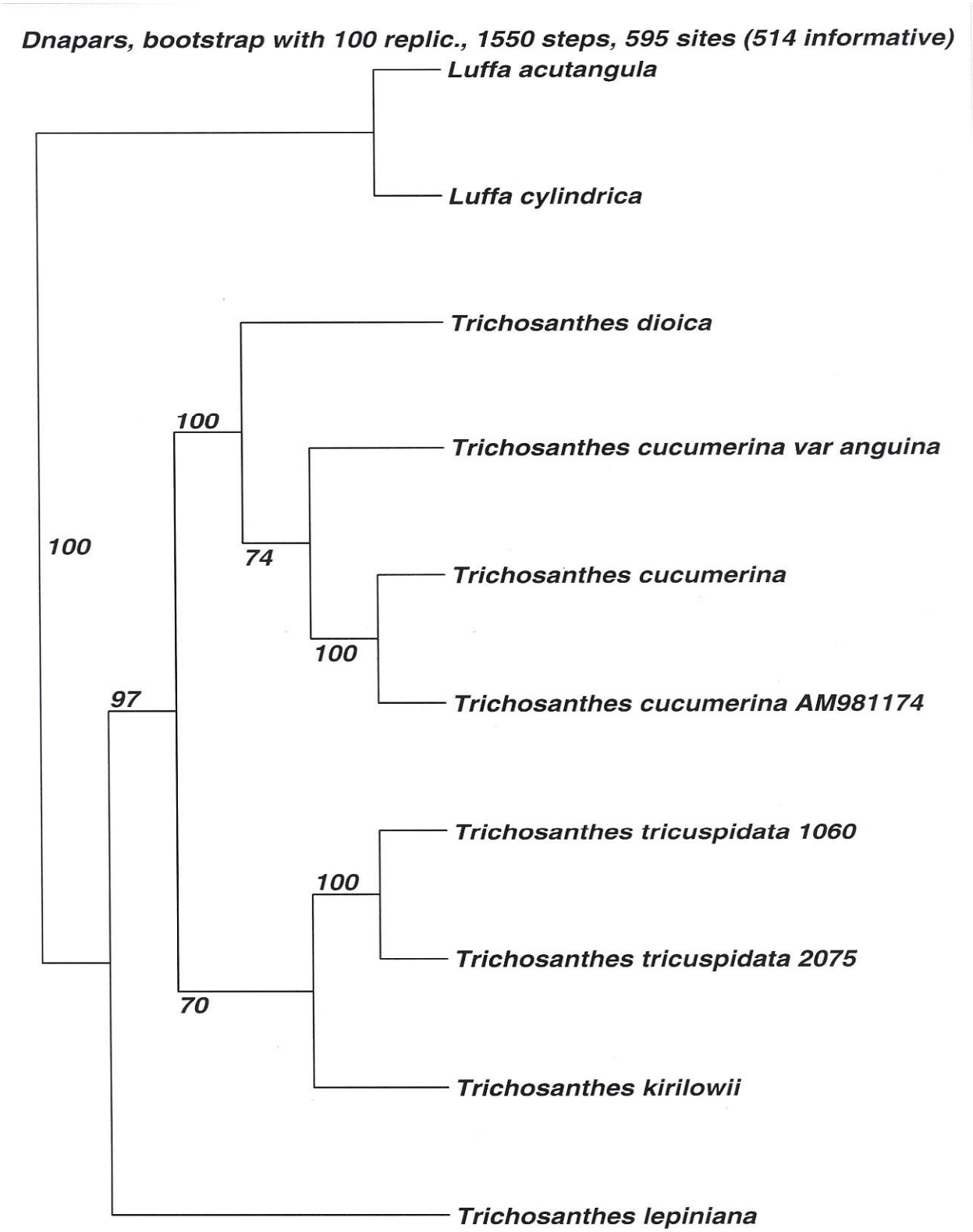

Fig. 6- Bootstrap strict consensus tree based on internal transcribed spacer region of nuclear ribosomal DNA. The tree constructed using Maximum Parsimony method in SeaView after multiple alignment in MUSCLE. The number at nodes indicates bootstrap support in 100 bootstrap replicates

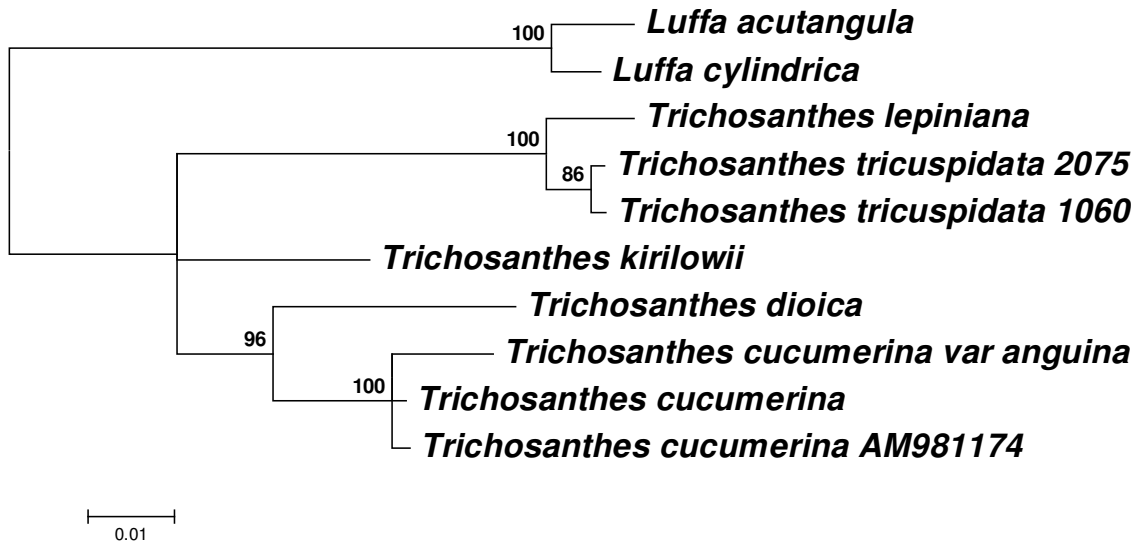

Fig. 7- Bayesian phylogeny with bootstrap support based on analysis of ITS sequences of nrDNA.