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

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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, Trichosanthes cucumerina, Cucurbitaceae, ITS, nrDNA

Introduction
Genus Trichosanthes L. of tribe Trichosantheae, subtribe Trichosantheae, 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]. Trichosanthes anguina (snake gourd or serpent gourd) is an usual cucurbit 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 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’-GTCACGTGAACCTTATCATT-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°C for 5 min, and followed by 40 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C 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 1 hour at 37°C 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 the 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 both the sense and antisense direction. The sequences were analyzed with ABI Sequence Analysis and ABI Sequence Navigator software (PerkinElmer/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 GenBank (www.ncbi.nlm.nih.gov). Gaps were filled 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 in phylogenetic analyses. 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 Bayesian 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 kT = 2.313 (purines) and kV = 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 \( (d_{S} = d_{k}) \) (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 \( d_{S} \) - nonsynonymous substitutions \( d_{k} \)) 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) \( T. \) dioica-\( T. \) 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. \( T. \) anguina clade shows proximity (98 % bootstrap support) with \( T. \) cucumerina (Fig.1). The close relationship of \( T. \) cucumerina var. \( T. \) anguina was also found consistent with BioNJ tree (Figs. 4-5), Parsimony tree (Fig. 6) analysed using SeaView and in Bayesian phylogeny (Fig. 7), but never with 100 % bs. In BioNJ and Parsimony tree, \( T. \) cucumerina var. \( T. \) anguina clade with \( T. \) cucumerina by 89% and 74 % bootstrap support respectively. \( T. \) cucumerina shows close relationship with \( T. \) dioica (74 % bootstrap support). Haines, 1921-1924 [9] recognized \( T. \) cucumerina L. as a wild variant with short fruits and \( T. \) cucumerina var. \( T. \) 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. \( T. \) 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, pubescent or scabrid beneath; Tendrils: 2-3 fid; Plant monocious; 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. \( T. \) 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. \( T. \) 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. \( T. \) anguina clade with \( T. \) cucumerina (98% bootstrap support). Under this clade (branch length 0.009) \( T. \) cucumerina var. \( T. \) 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 Bayesian analysis. Thus, our findings strongly support the recognition of \( T. \) cucumerina var. \( T. \) anguina (L.) Haines as a variety of \( T. \) cucumerina.

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Trichosanthes anguina L. is variety of Trichosanthes cucumerina L.

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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                             | ITS1 | 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                             |       |       |       |       |       |       |
|-----------------------------------|-------|-------|-------|-------|-------|-------|
|                                  |       | Luffa cylindrica |       |       |       |       |
| 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 |
**Trichosanthes anguina** L is variety of *Trichosanthes cucumerina* L.

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

| Taxon                                      | 0.000 | 0.005 | 0.005 | 0.028 | 0.000 | 0.000 | 0.000 | 0.000 |
|--------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| *Trichosanthes lepiniana*                  |       |       |       |       |       |       |       |       |
| *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 |

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 | 0.000 |
| *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 |

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                        | Value 1  | Value 2  | Value 3  | Value 4  | Value 5  | Value 6  | Value 7  | Value 8  | Value 9  | Value 10 |
|------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| 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                        | Value 1  | Value 2  | Value 3  | Value 4  | Value 5  | Value 6  | Value 7  | Value 8  | Value 9  | Value 10 |
|------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| Trichosanthes lepiniana      |          |          |          |          |          |          | 0.504    |          |          |          |
| Luffa cylindrica             |          |          |          |          |          |          | 0.592    | 1.000    |          |          |
| Trichosanthes tricuspidata 2075 |          |          |          |          |          |          | 0.592    | 1.000    | 1.000    |          |
| Trichosanthes tricuspidata 1060 |          |          |          |          |          |          | 0.550    | 0.727    | 1.000    | 1.000    |
| Luffa acutangula             |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |
| Trichosanthes dioica         |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |
| Trichosanthes kirilowii      |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |
| Trichosanthes cucumerina var. anguina |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |
| Trichosanthes cucumerina     |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |
| Trichosanthes cucumerina AM981174 |          |          |          |          |          |          | 1.000    | 1.000    | 1.000    | 1.000    |

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Trichosanthes anguina L. is variety of Trichosanthes cucumerina L.

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. Numbers above the line indicates branch length.
Trichosanthes anguina L. is variety of Trichosanthes cucumerina L.

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.
Trichosanthes anguina L. is variety of Trichosanthes cucumerina L.

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.
Trichosanthes anguina L. is variety of Trichosanthes cucumerina L.

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