The generic position of two species of tribe Physaleae (Solanaceae) inferred from three DNA sequences: A case study on *Physaliastrum* and *Archiphysalis*

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ABSTRACT

For the first time, samples of *Physaliastrum* and *Archiphysalis* are included in a molecular phylogenetic analysis of the tribe Physaleae based on the plastid loci ndhF and trnL-F and the nuclear ribosomal internal transcribed spacer (ITS). We selected 38 species from 23 genera of tribe Physaleae (Solanaceae) as ingroup and two genera (*Lycianthes*, *Capsicum*) from Capsiceae as outgroup. Phylogenetic analyses were conducted using maximum parsimony and Bayesian methods. The results indicate that *Physaliastrum* sensu Zhang et al. is not a monophyletic group. Neither *Physaliastrum* sensu Kuang and Lu nor *Archiphysalis* Kuang are members of *Leucophysalis* or *Chamaesaracha* or other genera as had been suggested by previous workers. They are independent genera which belong to subtribes Physalinae and Withaninae respectively.

1. Introduction

Tribe Physaleae (Solanaceae), with approximately 30 genera and 200 species, contains greater generic-level diversity than any other tribe in the family (Olmstead and Bohs, 2007; Olmstead et al., 2008; Whitson, 2012). Three subtribes (Iochrominae, Physalinae and Withaninae) are included within the Physaleae. The tribe is most closely related to the Capsiceae and Solaneae (Hunziker, 1979; D'Arcy, 1991; Olmstead et al., 1999; Bohs and Olmstead, 1997, 2001; Hunziker, 2001; Smith and Baum, 2006; Weese and Bohs, 2007). The morphological characters of the members in tribe Physaleae are relatively similar and provide little insight into the phylogenetic relationships among the several genera of the tribe. For example, some studies suggest that *Margaranthus* should be transferred into genus *Physalis* (Axelius, 1996; Martinez, 1999; Whitson and Manos, 2005), whereas other researchers still keep *Margaranthus* as an accepted genus (Olmstead et al., 1999, Olmstead and Bohs, 2007; Stevens, 2008 onwards). Averett (1979) recognized *Quincula* as distinct from *Physalis*, but Martinez (1999) suggested it should be integrated into genus *Physalis* as a subgenus. A cladistic analysis using 41 morphological characters indicated that *Quincula* is more closely related to *Chamaesaracha* than to *Physalis* (Axelius, 1996), which also is consistent with the results of Whitson and Manos (2005) based on molecular phylogenetic data. Regarding *Withaina*, a widely distributed genus in subtribe Withaninae, it has been debated whether to include *Archiphysalis, Physaliastrum* and *Mellissia* (D'Arcy and Zhang, 1992; Hunziker, 1995, 2001; Stevens, 2008 onwards; Olmstead et al., 2008). This and close relationships with *Athenaea, Aureliana, Larnax* and *Deprea* (Axelius, 1996; Olmstead et al., 2008) have resulted in unstable generic concepts for *Withaina*.

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Physaliastrum Makino was originally segregated from *Chamaesaracha* and contained two species (*P. echinatum* (Yatabe) Makino and *P. savatieri* (Makino) Makino) distributed in Japan (Makino, 1914). Then, Kuang and Lu (1965) expanded it to include seven mainly East Asian species. Averett (1970, 1973) transferred *Chamaesaracha heterophylla* Hemsl. (i.e., *Physaliastrum heterophyllum* (Hemsl.) Migo) into *Leucophysalis*, a previously North American genus which was accepted by Stevens (2001 onwards). Later, Averett (2009) recognized that *Leucophysalis* should only comprise two North American species (*L. grandflora* (Hook.) Rydb. and *L. nana.* (A. Gray) Averett), and excluded *L. viscosa* (Schrad.) Hunz. and the Asian species. This opinion is strongly supported by the molecular data of Whitson and Manos (2005), Olmstead et al. (2008) and the analyses presented herein.

Kuang (1966) established a new eastern Asian genus *Archiphysalis* and published a new species, *Archiphysalis kwangsiensis* Kuang, together with two combinations (*Archiphysalis chamaesarachoides* (Makino) Kuang and *Archiphysalis sinensis* (Hemsl.) Kuang). Kuang and Lu (1978) showed that *Archiphysalis* was morphologically similar to *Physaliastrum*, distinguishing the two genera by the degree to which the berry is enclosed in the persistent calyx and by whether the persistent calyx has greatly thickened ribs. It also should be noted that the persistent calyx is inflated at maturity in *Archiphysalis* and the berry is orange, features that are absent in *Physaliastrum*. Later, D'Arcy and Zhang (1992) suggested that the characters mentioned above are not appropriate for genetic separation of *Archiphysalis* from *Physaliastrum*. Zhang et al. (1994) transferred all the species of *Archiphysalis* into *Physaliastrum* and combined *A. chamaesarachoides* (Makino) Kuang and *A. kwangsiensis* Kuang as a single species (*Physaliastrum chamaesarachoides* (Makino) Makino). However, Stevens (2001 onwards) still recognized the genus *Archiphysalis* and listed *Physaliastrum* as a synonym of *Leucophysalis*.

Clearly, the generic status of *Archiphysalis* and *Physaliastrum* is not well-resolved. Furthermore, the relationship of the two taxa with such genera as *Physalis*, *Leucophysalis* and *Chamaesaracha* of the tribe Physaleae also needs further research before a final disposition can be made. Morphological data alone has not been able to resolve these issues. Here we provide a molecular perspective on the status of *Physaliastrum* and *Archiphysalis* (see Fig. 1) in order to provide additional insight into some of these systematic questions.

2. Materials and methods

2.1. Taxon sampling

Voucher specimens for *Physaliastrum* and *Archiphysalis* are as follows: *Physaliastrum heterophyllum* (Hemsl.) Migo: *Lihq0435*, collected by Hongqing Li et al., at West Tianmu Mountain (Zhejiang, China), November 9, 2010; *Archiphysalis chamaesarachoides* (Makino) Kuang: *Lihq0393*, collected by Hongqing Li et al., at Gutian Mountain (Zhejiang, China), October 27, 2010. Fresh leaves obtained in the field were directly dried by silca-gel. Voucher specimens were deposited in the herbarium of East China Normal University (HSNU). Other samples of tribe Physaleae and those of outgroup (38 species, 90 sequences in total) were retrieved from the GenBank. Details of all the samples are listed in Table S1.

2.2. Molecular methods

2.2.1. DNA extraction, PCR amplification

DNA was extracted either using a modified CTAB method from 10 mg silica-dried leaf material (Doyle and Doyle, 1987; Chase and Hills, 1991) or the Plant Genomic DNA Kit (TIANGEN BIOTECH CO., LTD, China) from 30 mg dried leaves. PCR amplification was performed on TAKARA TP600 thermocycler (TAKARA BIO INC, Japan). The primers and amplification protocols for each DNA region are listed in Table 1. PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN BIOTECH CO., LTD, China).

2.2.2. Sequencing and alignment

The purified products were sequenced bidirectionally for each DNA region at Invitrogen Biotechnology Corporation (Shanghai, China). All sequences were edited and assembled using SeqMan (DNA STAR package, Madison, WI, USA; Burland, 2000). Sequences were initially corrected and aligned using the Clustal W option in Mega 5 (Tamura et al., 2011). Further alignment was optimized manually.

2.2.3. Phylogenetic analyses

Three datasets were selected for our current molecular study: ITS dataset, chloroplast dataset (ndhF, trnL-F) and combined dataset (ITS, ndhF, trnL-F). Each data set was further analyzed using parsimony and Bayesian methods. Gaps were treated as missing characters.

2.2.3.1. Parsimony method. Parsimony analysis was conducted on each data set separately using PAUP* v. 4.0b10 (Swofford, 2003). All characters were assessed as unordered and weighted equally. Heuristic search algorithm was performed with 1000 replicates of random addition, tree bisection-reconnection (TBR) branch swapping, and multiple trees (MULTREES) options. Ten trees were held at each step. All other parameters were conducted with default settings. Bootstrap (BS; Felsenstein, 1985) analyses were conducted using 1000 replicates with TBR branch swapping, each consisting of 100 random
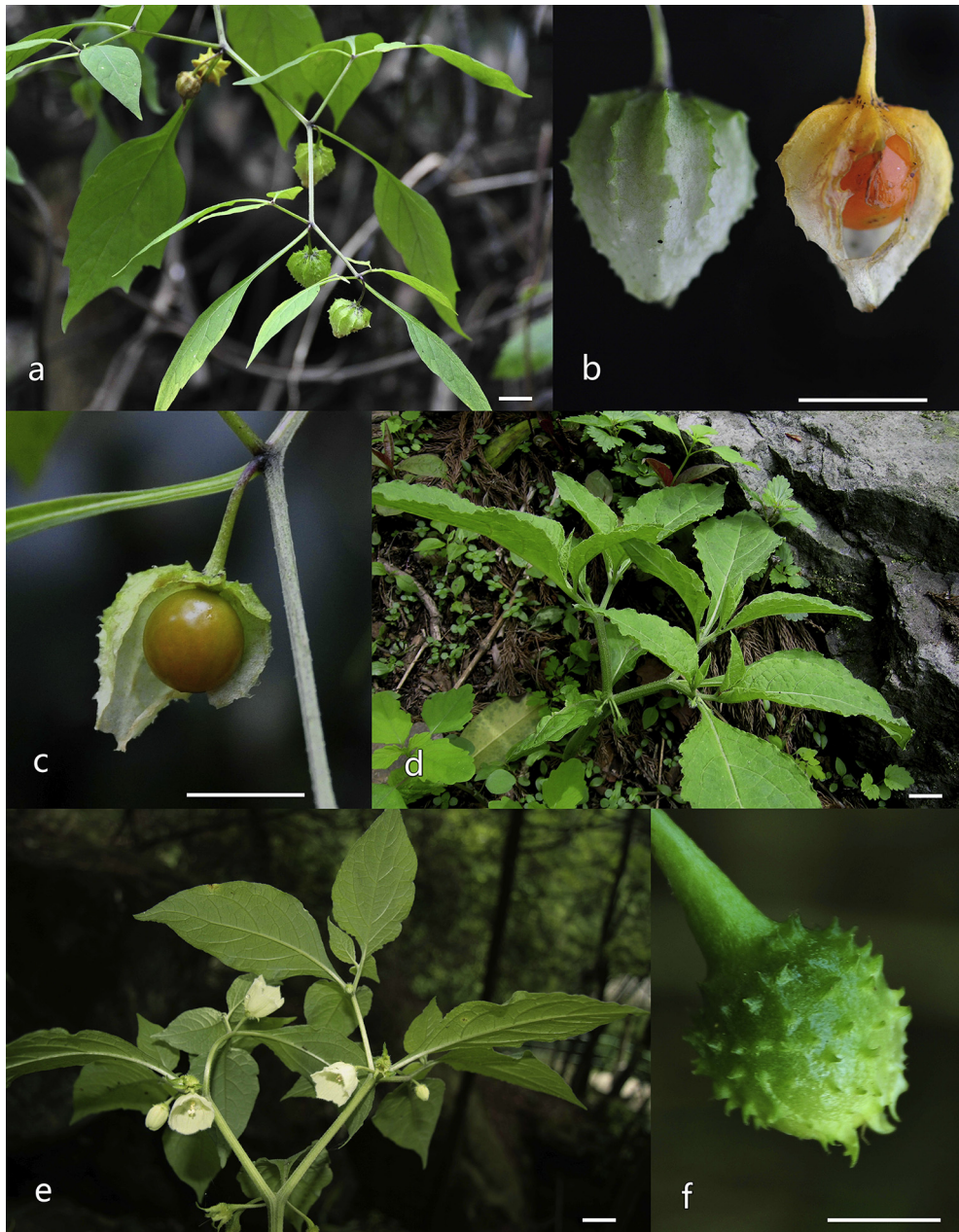
Fig. 1. Morphological features of *Archiphysalis chamaesarachoides* and *Physaliastrum heterophyllum*. White bars shown in the lower right corners indicate 1 cm. a–c, *A. chamaesarachoides*; d–f, *P. heterophyllum*.

addition replicates. Each data set was subjected to a limit of 1000 trees per random addition cycle. Homoplasy levels were assessed by consistency index (CI), retention index (RI) and rescaled consistency index (RC).

2.2.3.2. Bayesian method. MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for the Bayesian analyses of all the data sets. Prior to conducting Bayesian analysis, a general model of nucleotide evolution was selected for each data set. The Akaike information criterion (AIC) (Posada and Buckley, 2004) determined that the GTR + I + G model was the best-fit model as implemented in MrMTgui 1.0 (Nuin, 2005) with MrModelTest 2.3 (Nylander, 2004) and PAUP*v. 4.0b10. The analysis was conducted using four parallel runs of four Markov chains for 3,000,000 generations and sampling every 1000th generation until the average standard deviation of split frequencies was approaching a value of 0.01 (Ronquist et al., 2005). Burn-in values were set to 2000 generations. A 50% majority-rule consensus tree of all sampled trees with posterior probability (PP) values for individual clades was ultimately computed after discarding the trees within the burn-in phase.

Table 1
PCR primers and amplication protocols.

DNA region		Primer name	Sequence (5′–3′)	Reference	Amplication protocol
ITS		ITS 1	CGTAACAAGGTTTCCGTAGG	Venora et al., 2000	97 °C 2 min; 97 °C 1 min, 50 °C 1 min,
		ITS 4	TCCTCCGCTTATTGATATGC	White et al., 1990	72 °C 45 s, 30 cycles; 72 °C 7 min
trnL-F		trnL-Fc	CGAAATCGGTAGACGCTACG	Taberlet et al., 1991	94 °C 5 min; 94 °C 45 s, 55 °C 1 min,
		trnL-Ff	ATTTGAACTGGTGACACGAG	Taberlet et al., 1991	72 °C 90 s, 30 cycles; 72 °C 7 min
ndhF	ndhF1	ndhF-(-47)	AGGTAAGATCCGGTGAATCGGAAAC	Kornhall et al., 2001	94 °C 5 min; 94 °C 1 min, 50 °C 45 s,
		ndhF-925R	CCTCTCTTAATGTCTTTTTGAGCAAGAGCT	Kornhall et al., 2001	72 °C 45 s, 30 cycles; 72 °C 7 min
	ndhF2	ndhF-4F(590)	TTGGATAACGGGGAGTTTCGAATTT	Bremer et al., 2002	92 °C 7 min; 92 °C 1 min, 45 °C 1 min,
		ndhF-1350R	GTAAATAGATCCGAAACATATAAAATG(CGGTT)	Kornhall et al., 2001	72 °C 7 min, 35 cycles; 72 °C 7 min
	ndhF3	ndhF-1200	AGGTACACTTTCTCTTTGCGGTATTCC	Kornhall et al., 2001	94 °C 5 min; 94 °C 1 min, 50 °C 45 s,
		ndhF-2065R	CCAACYCCATTYGTAATTCCATCAAT	Kornhall et al., 2001	72 °C 45 s, 30 cycles; 72 °C 7 min
	ndhF4	ndhF-1811	CAGTCAGTATAGCTTATTTAGGAAT	Kornhall et al., 2001	94 °C 5 min; 94 °C 1 min, 50 °C 45 s,
		ndhF-(+606R)	ACCAAGTTCAATGTTAGCGAGATTAGTC	Kornhall et al., 2001	72 °C 45 s, 30 cycles; 72 °C 7 min

TreeGraph 2.0.47-206 beta (Stöver and Müller, 2010) was used to read trees produced from different phylogenetic analyses with different formats. Both bootstrap proportion (BS) and posterior probability (PP) were computed to provide measures of clade support. Clades with bootstrap proportion of 70% or more (BS $\geqq$ 70%) and posterior probabilities of 0.90 or more (PP $\geqq$ 0.90) were considered as having strong support.

2.2.3.3. Incongruence test. Before combining data sets for analysis, the incongruence length difference (ILD) test (also known as partition homogeneity test; Farris et al., 1994) was conducted to determine whether there were conflicts between the separate data sets. This was implemented in PAUP* using heuristic parsimony searches with 1000 replicates, 100 random-addition replicates, and TBR branch swapping.

3. Results

3.1. ITS dataset

Twenty two genera and 32 species were included in the ITS matrix. The aligned ITS dataset had a total of 697 characters, including 399 constant characters, 97 variable but not parsimony informative characters and 201 parsimony-informative characters. The parsimony analysis resulted in two equally most parsimony trees of 908 steps (CI = 0.5209, RI = 0.6106, RC = 0.3181) from one tree island of shortest trees.

Bayesian analysis summarized a total of 4004 samples from four runs, of which 3880 trees were calculated to be credible. Four parallel runs produced similar lnL values (−5179.368, −5179.132, −5174.326, −5169.799). The strict consensus of most parsimonious trees (not shown) was topologically similar to the 50% majority-rule consensus of Bayesian trees. The Bayesian consensus with the bootstrap percentages of MP is shown in Fig. 2. Although eight out of 23 clades appearing in the Bayesian tree collapsed in the strict consensus tree (BS <50%), they both reflect that *Archiphysalis chamaesarachoides* is nested within a monophyletic Withaninae (PP = 1; BS = 68%). It is sister to *Withania somnifera 1* (PP = 1; BS = 97%) with *Tubocapsicum anomalum* 1 at the base of the clade. The representatives of subtribe Physalinae do not form a monophyletic group. The position of *Physaliastrum heterophyllum* is unresolved, falling among other members of the Physalinae at the base of the tree.

3.2. Incongruence

Although the results of the ILD test of the chloroplast and combined datasets suggested significantly different signals compared to their individual data sets (both *P* = 0.001), the tree topologies were highly congruent with their separate gene trees (not shown) except for unstable genera placement in subtribe Withaninae reflected by the separate trnL-F analysis. This did not affect how the datasets placed our research taxa. Furthermore, an increasing number of studies suggest that the ILD test is more conservative or even unlikely to be an effective measure of congruence when data should be combined (Cunningham, 1997; Yoder et al., 2001; Dowton and Austin, 2002; Barker and Lutzoni, 2002; Quicke et al., 2007). Because there were no strongly supported conflicts between the cpDNA phylogeny and the ITS phylogeny (see Figs. 2 and 3), combined analyses were conducted after the separate analyses.

3.3. Chloroplast dataset

Twenty four genera and 32 species were used for chloroplast dataset analysis. The aligned length had 3181 characters (2092 bp ndhF, 1089 bp trnL-F), together with 2797 constant characters, 230 variable but not parsimony informative characters and 154 parsimony-informative characters. Parsimony analysis produced 116419 trees of 576 steps (CI = 0.797, RI = 0.766, RC = 0.611) from two tree islands of shortest trees. Bayesian analysis obtained a total of 4004 samples from four runs, of which 3989 trees were calculated to be credible. Four parallel runs resulted in similar lnL values (−8116.575,
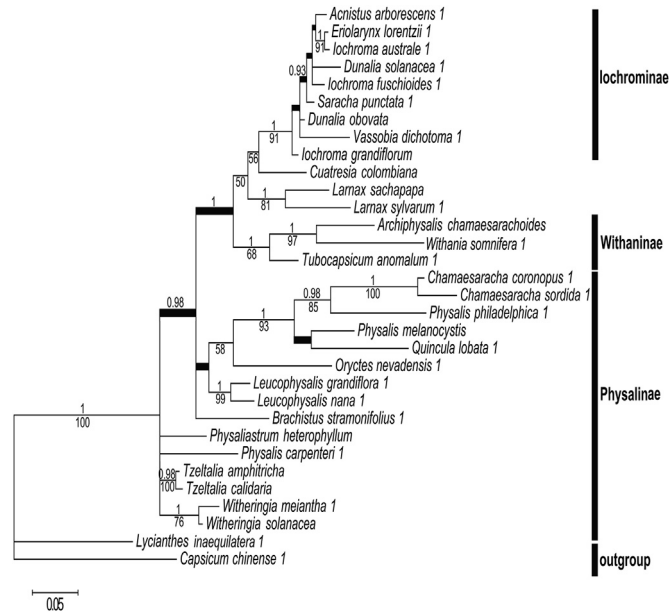
Fig. 2. ITS phylogeny of tribe Physaleae and relatives depicted as a majority-rule consensus of 3880 Bayesian trees. Bold branches collapse in the strict consensus. Posterior probabilities (≅0.90) are presented above branches with MP bootstrap values (≅50%) below them. The subtribe groups are labelled to the right. Unlabeled taxa have not yet been assigned to subtribes.

−8107.683, −8108.660, −8093.341). The strict consensus of most parsimonious trees (not shown) was similar in topology to the 50% majority-rule consensus of Bayesian trees, which is shown with MP bootstrap percentages (see Fig. 3). Two of 20 clades appearing in the Bayesian majority-rule tree collapsed in the strict consensus. Similar to the ITS analysis, *Archiphysalis chamaesarachoides* nests within a monophyletic Withaninae (PP = 0.99; BS = 74%), and together with *Discopodium penni-nervium*, *Tubocapsicum anomalum* and *Nothocestrum longifolium*, forms a well-supported clade (PP = 1; BS = 83%) sister to the other members of subtribe Withaninae. Unlike the ITS analysis, *Archiphysalis* is weakly supported as sister of *Discopodium* (PP = 0.82; BS = 76%) but not *Withania*. The chloroplast dataset analysis strongly indentifies subtribe Physalinae as a monophyletic group with *Physaliastrum heterophyllum* at the base of the clade (PP = 1; BS = 86%).

3.4. Analysis of combined dataset

This combined matrix contained 20 genera, 24 species and the aligned length included 3822 characters (693 bp ITS, 2086 bp ndhF, 1043 bp trnL-F), containing 3218 constant characters, 338 variable but not parsimony informative characters
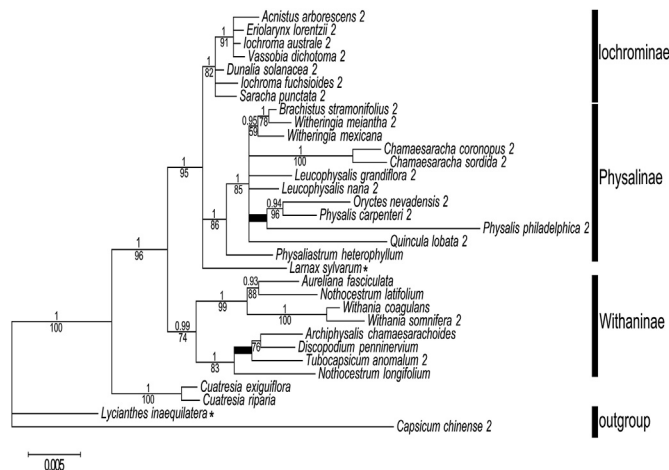

Fig. 3. Combined ndhF and trnL-F phylogeny of tribe Physaleae and relatives depicted as a majority-rule consensus of 3989 Bayesian trees. Bold branches collapse in the strict consensus. Posterior probabilities (≅0.90) are presented above branches with MP bootstrap support (≅50%) below them. The subtribes are labelled to the right. Unlabeled taxa have not yet been assigned to subtribes. Asterisks indicate taxa that combine sequences from different individuals (see Table S1).

and 266 parsimony-informative characters. Parsimony analysis yields two most parsimonious trees of 1124 steps (CI = 0.7109, RI = 0.6377, RC = 0.4533) in one tree island.

Summaries of the Bayesian analysis are based on a total of 4004 samples, of which 423 trees are calculated to be credible. Four parallel runs result in similar lnL values (−11695.130, −11706.391, −11709.860, −11696.634). The strict consensus of most parsimonious trees (not shown) had a similar topology to that of the majority-rule consensus of Bayesian trees, which is shown with the MP bootstrap percentages (see Fig. 4). Four of 19 clades appearing in the majority-rule Bayesian tree collapse in the strict consensus. This analysis shows *Archiphysalis chamaesarachoides* clustered in a monophyletic Withaninae (PP = 1; BS = 76%) and sister to *Tubocapsium anomalum*. As in the chloroplast data analysis, a monophyletic Physalinae has strong support (PP = 1; BS = 93%), with *Physaliastrum heterophyllum* at its base.

4. Discussion

Between them, *Archiphysalis chamaesarachoides* and *Physaliastrum heterophyllum* have been placed in a total of 6 different genera, including *Chamaesaracha*, *Leucophysalis*, *Physalis*, and *Withania* (see Table 2). Though the current *Flora of China* considers *Archiphysalis* a synonym of *Physaliastrum*, our data suggest that these two species merit recognition in their own genera, and that they belong to two different subtribes of the Physaleae: *A. chamaesarachoides* in the Withaninae and *P. heterophyllum* in the Physalineae.

Previous researchers (Makino, 1914; D'Arcy and Zhang, 1992; Averett, 2009) have noted a similarity in morphological characters between *Physaliastrum* (sensu Kuang and Lu, 1965) and other genera of subtribe Physalinae (eg. *Leucophysalis*, *Chamaesaracha* and *Physalis*). Though unresolved in the ITS analysis, our cpDNA and combined analyses (see Figs. 3 and 4) show *Physaliastrum heterophyllum* (Hemsl.) Migo holding a basal position within a monophyletic subtribe Physalinae.

On the other hand, *Archiphysalis chamaesarachoides* (Makino) Kuang is treated as *Physaliastrum chamaesarachoides* (Makino) Makino by Zhang et al. (1994). However, *A. chamaesarachoides* is nested within subtribe Withaninae in all of our analyses (see Figs. 2–4). Placement of the latter species in subtribe Withaninae also has been suggested by Olmstead and Bohs (2007). Though few morphological synapomorphies have been identified for subtribe Withaninae, we find that the axillary inflorescences and lack of peduncles in *A. chamaesarachoides* is similar to that seen in other genera (such as *Withania*, *Tubocapsicum* and *Discopodium*) within subtribe Withaninae. This suggests that *A. chamaesarachoides* is not a member of *Physaliastrum* but is more appropriately placed in subtribe Withaninae. Additionally, Hunziker (1995) transferred *A. chamaesarachoides* (appeared as *P. chamaesarachoides*) to *Withania* and in 2001 treated *Withania* and all of the species of *Physaliastrum*, including those formerly in *Archiphysalis*, within the tribe Solaneae, subtribe Capsicinae. Our combined analysis, however, does not indicate they are sister taxa nor support that disposition (see Figs. 3 and 4).

It is clear that, supported by all the three phylogenetic trees, our molecular results place the two genera (*Physaliastrum* Makino and *Archiphysalis* Kuang, represented by *P. heterophyllum* and *A. chamaesarachoides* respectively) at two different places: *Physaliastrum* at the base of subtribe Physalinae and *Archiphysalis* nested within subtribe Withaninae, each within well-supported clades. Morphological cladistic analysis has demonstrated that *Physaliastrum* is distant from *Archiphysalis* (Axelius, 1996). Here, we also observed in *A. chamaesarachoides* (Makino) Kuang that the diameter and length of fruiting calyx largely exceed the berry, and that the calyx has greatly thickened and bumpy ribs, which exists in the type species (*Archiphysalis sinensis* (Hemsl.) Kuang). Whereas in *P. heterophyllum* (Hemsley) Migo, the fruiting calyx is conspicuously muricate and tightly appressed to the berry wall, the same as in the *P. echinatum* (Yatabe) Makino. So it is obvious that *Physaliastrum* (*P. heterophyllum*) is not closely related to *Archiphysalis* (ie, *A. chamaesarachoides*) and other previously confusing genera
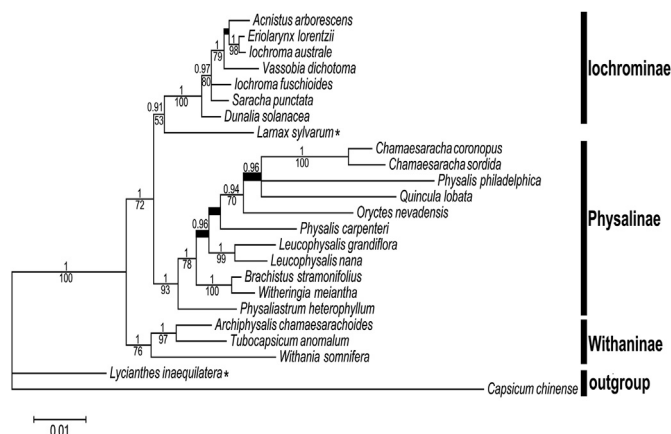

Fig. 4. Combined ndhF, trnL-F and ITS phylogeny of tribe Physaleae and relatives depicted as a majority-rule consensus of 423 Bayesian trees. Bold branches collapse in the strict consensus. Posterior probabilities (≧0.90) are presented above branches with MP bootstrap support (≧50%) below them. The subtribes are labelled to the right. Unlabeled taxa have not yet been assigned to subtribes. Asterisks indicate taxa that combine sequences from three different individuals. Sequences of other species come from two different individuals (see Table S1).

Table 2

Synonyms of *Physa liastrum heterophyllum* (Hemsley) Migo and *Archiphysalis chamaesarachoides* (Makino) Kuang.

Name	Source
Physaliastrum heterophyllum (Hemsley) Migo	J. Shanghai Sci. Inst. Sect. 3, 4: 171.1939.
Chamaesararacha heterophylla Hemsley	J. Linn. Soc., Bot. 26: 174. 1890; Bot. Jahrb. 29: 563. 1900; Bot. Mag. (Tokyo) 28: 21. 1914, in nota. Pl. 7.
Leucophysalis heterophylla (Hemsley) Averett	Ann. Missouri Bot. Gard. 70: 380. 1970.
Withania heterophylla (Hemsley) Hunziker	Lorentzia 8: 7. 1995.
Archiphysalis chamaesarachoides (Makino) Kuang	Acta Phytotax. Sin. 11: 59–63. 1966.
Physalis chamaesarachoides Makino	Bot. Mag. (Tokyo) 22: 34. 1908.
Archiphysalis kwangsiensis Kuang	Acta Phytotax. Sin. 11: 59–63. 1966; Fl. China. 17: 310. 1994.
Physalis linii Y. C. Liu et C. H. Ou	J. Chin. Forest. 7(4): 150. 1974.
Physaliastrum chamaesarachoides (Makino) Makino	J. Jap. Bot. 5(6): 24. 1928; Fl. China. 17: 310. 1994.
Withania chamaesarachoides (Makino) Hunziker	Lorentzia 8: 7. 1995.

(*Leucophysalis*, *Chamaesaracha*, *Withania*), and each should be regarded as an independent genus. As we have only one sample of genus *Archiphysalis* and the representatives of subtribe Withaninae were poorly sampled, the phylogenetic position of *Archiphysalis* in Withaninae is not yet well-resolved.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2013.03.038.

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