A phylogenetic study of commercial Chinese truffles and their allies: Taxonomic implications

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Abstract

Phylogenetic relationships of commercial Chinese truffles and their allies were investigated mainly by morphological studies and analyses of the sequences of ITS regions of nuclear ribosomal DNA. Two species, *Tuber indicum* and *T. himalayense*, closely related to the European *T. melanosporum* (the Perigord Truffle), are recognized among commercial Chinese black truffles. Both *T. pseudohimalayense* and *T. sinense* should be regarded as synonyms of *T. indicum. Tuber* species producing excavated ascomata are not monophyletic, suggesting that excavation of ascomata may have evolved more than once, or evolved once during the evolution of truffle species and then was lost once during the evolution of *Tuber* species.

Keywords: *Tuber*; Internal transcribed spacer; Systematics; Synonyms

1. Introduction

Truffles are very renowned and are of high economic importance because of their cuisinary value. Although the Chinese truffles, resembling *Tuber melanosporum* Vitt. (the European prized Perigord Truffle), are inferior in taste and odor, truffles exported from China to Europe have increased dramatically since about 1993 [1]. For a long time, commercial Chinese truffles were lumped as *T. indicum* Cooke and Massee [2], but in recent years, several new species have been recognized: *T. himalayense* Zhang and Minter [3], *T. pseudohimalayense* Moreno et al. [4], *T. sinense* Tao and Liu [5]. The taxa mentioned above are similar to each other and it seems to be challenging to identify them based on subtle morphological characters alone. Previous molecular studies [6–10] have repeatedly found two main monophyletic groups or haplotypes (A and B) among the Chinese truffles identified as *T. indicum*. It was unclear whether such a simple, two-part partitioning was related to differences in populations of diverse collecting sites as proposed by Paolocci et al. [7] or the partitioning reflected repeated misidentifications of several taxa as *T. indicum* [8]. Another opinion is that most of the truffles exported from southwestern China to Europe similar to *T. melanosporum* and regarded as *T. indicum* or *T. himalayense* were *T. sinense*, while *T. pseudohimalayense* was considered to be very similar to *T. sinense* [1]. These controversial views made it worthwhile to clarify the taxonomy of commercial Chinese truffles similar to *T. melanosporum*.

T. pseudoexcavatum Wang et al., another newly published commercial species from China, is macroscopically

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similar to *T. excavatum* Vitt. and *T. mesentericum* Vitt. from Europe [1,11]. These three species are characterized by their deeply excavate ascomata (with an evident basal cavity), but their phylogenetic relationships have never been considered.

In this study a phylogenetic investigation of commercial Chinese truffles (including *T. indicum*, *T. himalayense*, *T. pseudohimalayense*, *T. sinense* and *T. pseudoexcavatum*) and their relative taxa was conducted based both on morphological and molecular analyses. Parsimony, likelihood and distance inferences were applied on a large number of internal transcribed spacer (ITS) sequences of Chinese truffles with different geographical origins in order to evaluate their relationships, the interspecific and/or intraspecific divergence in the *T. indicum* "complex," and to propose phylogenetic relationships between *T. pseudoexcavatum* and phenetically similar species.

2. Materials and methods

2.1. Morphological studies and sample source

Standard techniques were employed [11,12] for macro-morphological and anatomical studies. Types of

Table 1
Taxa included in DNA analysis

Taxon	Voucher	Geographic origin	GenBank
Tuber indicum in Group A	HKAS 39501	Kunming, Yunnan, China under *Pinus armandii*	[b]AY514305
	HKAS 39506	Chuxiong, Yunnan, China under *Pinus yunnanensis*	[b]AY514306
	HKAS 39515	Kunming, Yunnan, China	[b]AY514307
	HKAS 39516	Kunming, Yunnan, China	[b]AY514308
	HKAS 39507	Gongshan, Yunnan, China	[b]AY514309
	HKAS 38933	Kunming, Yunnan, China under *Pinus yunnanensis*	[b]AY773357
	–	Imported from China	Y09791
	–	Imported from China	Y09792
	–	Imported from China	AF106881
	–	Imported from China	AF300822
	–	Imported from China	AF300824
	–	Imported from China	U89362
T. indicum in Group B [*T. himalayense*][a]	–	Huili, Sichuan, China	AF132502
	–	Imported from China	U89360
	–	Imported from China	AF106882
	–	Imported from China	AF106883
	–	Imported from China	AF300823
	–	Imported from China	AF106884
T. himalayense [*T. indicum*[a]]	–	Huidong, Sichuan, China	AF132503
T. himalayense	HKAS 25689	Huize, Yunnan, China under *Pinus yunnanensis*	[b]AY773356
T. pseudohimalayense? [*T. indicum*[a]]	–	Imported from China	U89361
T. melanosporum	–	Italy	AF106873
	–	Italy	AF106874
	–	Italy	AF106875
	–	Italy	AF106876
	–	Spain	AF106877
	–	France	AF106878
	–	France	AF106879
	–	France	AF132501
	–	Unknown	AF167096
	–	Unknown	AF167097
	–	France or Italy	AF300825
	–	France or Italy	AF300826
	–	France or Italy	AF300827
	–	Unknown	U89359
T. pseudoexcavatum	HKAS 39504	Chuxiong, Yunnan, China	[b]AY514310
T. excavatum	–	Hungary	AJ557545
T. mesentericum	–	Italy	AF106887
T. brumale	–	Unknown	AF106880
	–	Unknown	AF132504
T. brumale f. *moschatum*	–	Unknown	AF001010
T. borchii	–	Unknown	AF132505
	–	Unknown	AJ557538
	–	Unknown	AJ557536

[a] Recent epithets denoted within brackets are according to our conclusions.
[b] Sequences obtained in this study. The others were from GenBank.

T. indicum [K (M) 39493], *T. himalayense* [K (M) 32236], and the isotype of *T. sinense* (HMAS 60222) were examined and morphologically compared with the collections sequenced in this study. Over 20 additional specimens made for this study were examined and were deposited in the Cryptogamic Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS).

The samples used for molecular analysis, their geographic origin, depository and the GenBank accession numbers are listed in Table 1. All sequences labeled *T. indicum* and its allies in GenBank were included in our study. Sampling localities of *T. indicum* and *T. himalayense* are shown in Fig. 1. The outgroup was *T. borchii* Vitt., a white truffle in the genus *Tuber*. The type of *T. indicum* and *T. himalayense*, and the isotype of *T. sinense* were not sequenced in consideration of their poor condition or in order to minimize sampling from them.

2.2. DNA extraction, PCR amplification and sequencing

Total DNA was obtained directly from fresh or dried specimens using a modified CTAB procedure of Doyle and Doyle [13]. The primers ITS 4 and ITS 5 [14] were used for amplification of the ITS region. Reaction volumes were 20 μl and contained 1.5 U of AmpliTaq DNA polymerase (Perkin–Elmer), Replitherm TM buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} dNTP, 0.1 μmol L^{-1} primer, 25–60 ng sample DNA. PCR was performed in a GeneAmp 9600 thermal cycler (Perkin–Elmer, Applied Biosystems). Cycling conditions were set as follows: initial denaturation at 97 °C for 4 min, 35 cycles of 30 s at 94 °C, 1 min at 52 °C, 1 min at 72 °C, and a final extension of 5 min at 72 °C.

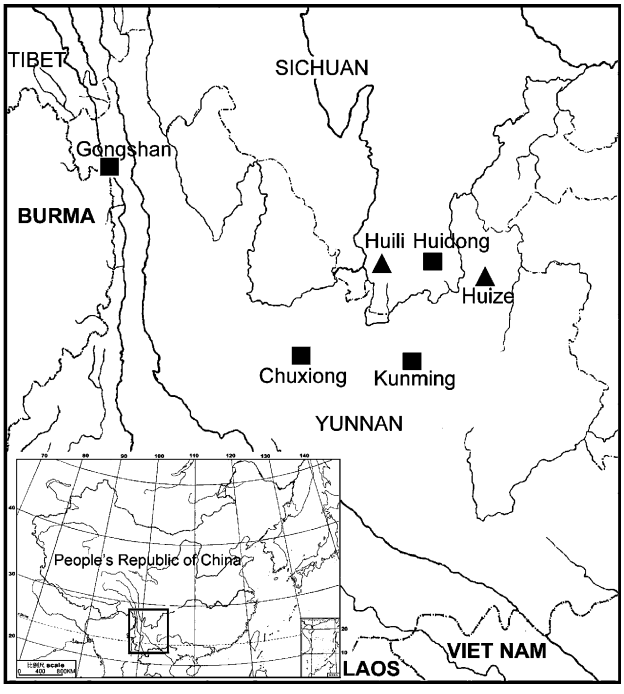

Fig. 1. Sampling localities of *Tuber indicum* (black squares) and *T. himalayense* (black triangles).

Amplified PCR products were purified using the Watson's purification kit (Watson, China), followed by ethanol precipitations. Both DNA strands of amplicons were sequenced using Dideoxy Chain Termination method with an ABI PRISM™ Bigdye Terminator cycle sequencing kit on an ABI 310 automatic sequencer. The same primers as described above for PCR were used for the sequencing reactions.

2.3. Alignment and phylogenetic analyses

DNA sequences were edited and aligned with SeqMan and Megalign (DNASTAR Package), and manually modified where necessary. Ambiguous regions (characters 24, 41, 51–54, 86–87, 102, 121–123, 146–147, 149, 354, 370–371, 419, 433–434, 474, 491, 493–494) were excluded from the analyses. Gaps were treated as missing data. All unambiguous characters and character-transformations were weighted equally. Analyses of maximum-parsimony (MP), maximum-likelihood (ML) and neighbor-joining (NJ) were performed with PAUP version 4.0b10 [15]. ML model parameters (Base = equal, Nst = 2, TRatio = 1.8972, Rates = gamma, Shape = 3.3712, Pinvar = 0.3349) were selected by hLRT in Modeltest Version 3.06 [16]. To construct the distance tree with neighbor-joining method we used Kimura two-parameter model [17]. Heuristic searches were performed with these settings: MAXTREES set to 1000, TBR, zero length branch collapsed, gaps treated as missing. A heuristic search option of 1000 random addition sequence replicates was used for MP, of asis addition sequence for ML. To assess the relative support for each clade, bootstrap values were calculated from 1000 (100 for ML tree) replicates analyses.

3. Results

ITS sequence data of 44 samples were analyzed. The final alignment contained 522 characters of which 25 ambiguous characters were excluded.

3.1. MP analysis

Of the remaining 497 included characters, 194 characters were constant, 67 were variable, and 236 were parsimony-informative. A single MP tree of 670 steps, CI = 0.7657, RI = 0.8910, was found (Fig. 2).

The ITS phylogenetic tree indicated that sampled specimens labeled *T. indicum* were not monophyletic. Two main groups, A and B, can be recognized. Bootstrap support for each group was strong. The ITS region of group B differed in length from group A by insertion of a few base pairs in the ITS 2 (not shown), whereas

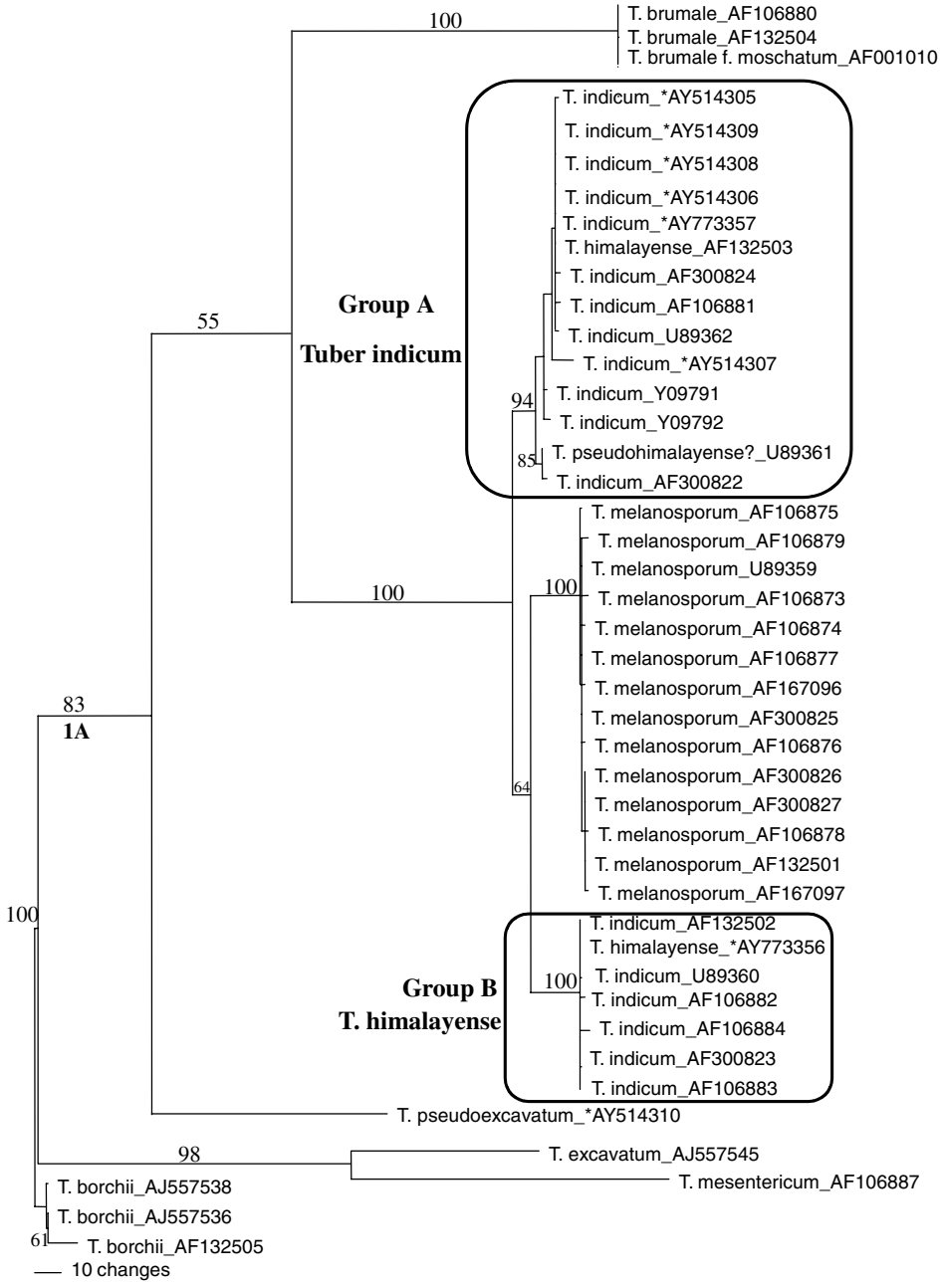

Fig. 2. The single most parsimonious tree generated for the commercial Chinese truffles and their allies based on ITS sequence data. Numbers above each internode are the percentage of 1000 bootstrap replicates supporting that binary partition (value >50%). GenBank accession number preceded by an asterisk was obtained in this study.

within a single group sequences were quite homogeneous (more than 98% nucleotide identity). In group A, *T. pseudohimalayense* together with AF300822 showed a slight difference from the other samples. *Tuber brumale* Vitt. formed a sister group to the clade comprising groups A, B and *T. melanosporum* with weak support (55%). Relationships among the three highly supported groups, A, B and *T. melanosporum* were not resolved. *T. excavatum* and *T. mesentericum* constituted a highly supported monophyletic group (bootstrap = 98%), while *T. pseudoexcavatum*, also with excavate ascomata, is nested in clade 1A, supported with a bootstrap value of 83%.

3.2. ML analysis

The tree (Fig. 3) produced by ML analysis exhibited a topology that was somewhat congruent with those from the MP analysis: each of groups A and B was well supported as a monophyletic group. Subtle differences between the ML and MP topologies were not supported.

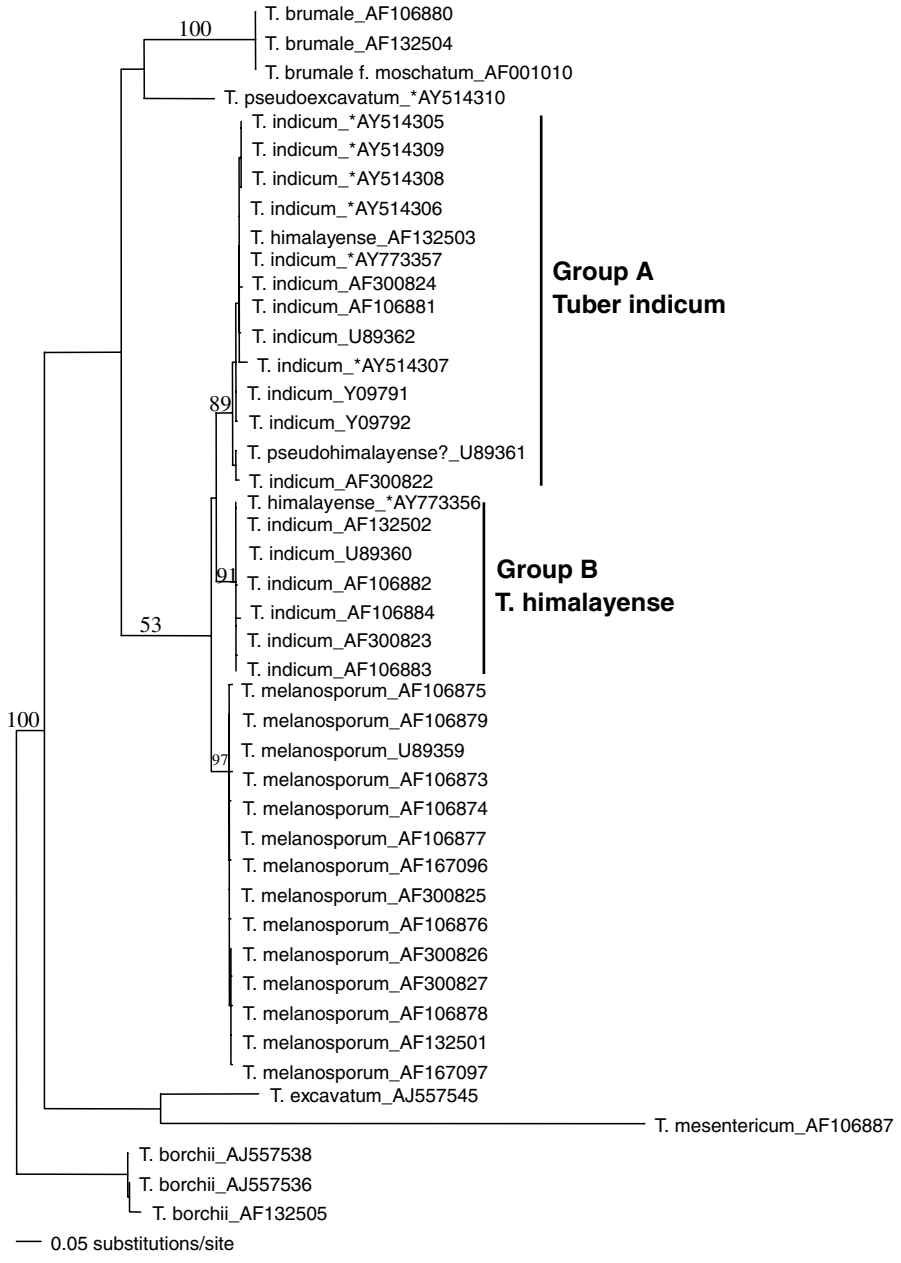

Fig. 3. The most likelihood tree generated for the commercial Chinese truffles and their allies based on ITS sequence data. Numbers above each internode are the percentage of 100 bootstrap replicates supporting that binary partition (value >50%). GenBank accession number preceded by an asterisk was obtained in this study.

3.3. NJ analysis

NJ analysis yielded a topology (not shown) that was similar to those from the ML and MP analyses.

3.4. Taxonomic implications

The original descriptions of *T. indicum*, *T. himalayense*, *T. pseudohimalayense* and *T. sinense* were usually based on a single collection, i.e., the type, or few collections. Thus morphological variation within a species was unclear. In this study, over 20 collections of the *T. indicum* complex including the types of *T. indicum* and *T. himalayense*, and the isotype of *T. sinense* were examined. In the light of the molecular phylogenetic analyses and given the hypothesis that the distinctly separated clades in the trees produced from these analyses represent a single species each, one is driven to the conclusion that morphologies of all species mentioned above are variable. Past attempts at distinguishing the taxa have not been successful because the complexity of the situation was not taken into account. The size and form of

Table 2
Morphological characters of commercial Chinese species of *Tuber* recognized in this study

Taxon	Peridium surface	Ascospore ornamentations
Tuber indicum	With pyramidal warts, sometimes warts less regular, thin, acuate, rarely wide and very coarse	Mostly partially reticulate, sometimes spiny, rarely alveolate but no crater-like structure at sporal poles
T. himalayense	With polygonal shallow splits and flattened warts	Alveolate, often forming a crater-like structure at one or both sporal poles
T. pseudoexcavatum	With small verrucous warts, ascomata deeply excavate	Spinoreticulatae (spines usually with broad basal connections)

the ascomata, the asci and even the ascospores are relatively variable from collection to collection and cannot be used alone as morphological characters with which to separate species. Within a given species, variations in the ornamentation of the surfaces of peridia and ascospores are also present. Examination of the morphology of the taxa of concerned strongly supported the hypothesis based on the molecular data analysis. Morphological character states of the isotype of *T. sinense* are within the ranges of variation of the same states in *T. indicum*, and that the ranges of states of morphological characters in *T. indicum* and *T. pseudohimalayense* overlap.

On the other hand, morphological characters of the surface of the peridium in combination with the forms of the ornamentations of the ascospores appear to be useful tools for determining the taxonomy of the *T. indicum* complex. Unique morphological characters for commercial Chinese truffles recognized in this study are summarized in Table 2.

4. Discussion

4.1. Phylogeny and taxonomy of the complex of T. indicum

Two main groups can be consistently identified within the commercial Chinese truffles that closely resemble *T. melanosporum* in all analyses, which conforms to the observations of other studies [6–10].

Paolocci et al. [7] proposed different geographical origins for groups A and B. However, as showed in Table 1 and Fig. 1, our samples in group A were collected from diverse locations and exhibit minimal variation of their ITS sequences. For example, samples yielding sequences AY514305, AY514308 and AY773357 were from Kunming, while AY514306 and AY514309 were extracted from material from Chuxiong and Gongshan, respectively, but their ITS sequences are identical. On the contrary, some samples collected in closer proximity showed consistent genetic differences. For instance, the samples made from Huili (AY132502) and Huize (AY773356) differ from the sample AY132503 collected from Huidong, a locality between the first two places. Thus, the morphological and molecular differences between the two groups do not appear to relate to geographical distributions.

Is the diversity due to ectomycorrhizal host-specificity? Although the voucher collections for sequences AY514305, AY514306 and AY773357 in group A were collected in forests dominated by different species of *Pinus*, they showed no differences in ITS sequences. Conversely, the vouchers for sequences AY514306, AY773357 and AY773356 were all collected in forests dominated by *P. yunnanensis*; and they did not cluster in the same group. It seems there is no strict host-specificity between the truffles and the trees.

Our data suggest that the two groups, A and B, are closely related but separate species, if *T. melanosporum* is recognized. Then the question is: what is the correct scientific name corresponding to each group? Roux et al. [8] suggested sequences in group A could belong to species like *T. himalayense* and *T. pseudohimalayense* while those in group B should be *T. indicum*. Our morphological comparative studies of the types of *T. indicum* [K (M) 39493] and *T. himalayense* [K (M) 32236], and the specimens sequenced in this study and other collections kept in HKAS lead to a different conclusion, i.e., that the isolates in group A are *T. indicum* and those in group B are *T. himalayense*. This conclusion was based on the following considerations. First, the sample of AY773356 in group B is identical with the type of *T. himalayense* in the key characters separating *T. himalayense* from *T. indicum*: the surface of the peridium only with polygonal shallow splits and flattened warts (that of *T. indicum* usually has distinct pyramidal warts), like a parched riverbed (see Figs. 9–10 in Zhang and Minter [3]), and the ornamentations of the ascospores include a crater-like structure at one or both sporal poles, a unique form of ornamentation in the taxa studied (see Fig. 14 in Zhang and Minter [3], Fig. 3 in Moreno et al. [4]). Second, our samples in group A resemble the type of *T. indicum* in macro- and micromorphology and none of them possess both characters unique to *T. himalayense*.

It is worth emphasizing again that there are variations in the ornamentations of the peridium and ascospores among collections of a single species. Some of our samples of *T. indicum* (such as the vouchers for

sequences AY514305 and AY514308), have less regular, thin, acuate pyramidal warts while some (such as the voucher for sequence AY514307 and HKAS 44318), possess wide, coarsely pyramidal warts, but none of them are as flat as those in *T. himalayense*. The spines of the ascospores of the holotype of *T. indicum* are almost free or sometimes connected by low ridges attached to the very base of the spines, while the ascospores of most of our samples of *T. indicum* show partial reticulation (pseudoreticulum) by anastomosis of the broad bases of a few spines (also see Yamanaka et al. [18]). An extreme case is the ornamentations of the ascospores in the sample of AY773357, which are alveolate; but the crater-like structures of *T. himalayense* spores are absent; and the surface of the voucher's peridium is coarsely pyramidally warted.

Although the ascospore ornamentation of our voucher for sequence AY773357 comprises spines with broad basal connections that form a distinct reticulum, which is very similar to that described in the protologue for *T. pseudohimalayense* [4], the voucher strongly resembles *T. indicum* in ITS sequence and other morphological characters. Because the morphological differences between *T. indicum* and *T. pseudohimalayense* are overlapping, we suppose *T. pseudohimalayense*, whose type is unfortunately unavailable for our analysis, is probably conspecific with *T. indicum*. Thus, sequence U89361 , which has been supposed to be *T. pseudohimalayense*, is very likely to be *T. indicum*.

The ITS sequence AF132503 from GenBank extracted from material determined as *T. himalayense* [8] is nested in the clade of *T. indicum*, which suggests that the voucher for AF132503 has been misidentified and is in fact *T. indicum*.

Wang and Hall [1] suggested that most of the truffles similar to *T. melanosporum* and exported from southwestern China to Europe were *T. sinense*. We have restudied the isotype of *T. sinense* (HMAS 60222), which was mentioned by Zhang [12]. Only half an immature ascoma is preserved. Its peridium possesses pyramidal warts, and spines of its ascospores are somewhat connected by broken lines but without the unique ornamentation of *T. himalayense*. Since the macro-morphological and anatomical characters of the isotype can be covered within the variation range of *T. indicum* (Table 2.), *T. sinense* is probably a synonym of *T. indicum*.

T. indicum and *T. himalayense*, which only occur in Asia, have a close relationship with *T. melanosporum*, which only occurs in Europe. They probably share a common origin, as suggested in a recent study based on southern and dot-blot hybridization [19]. Because the lengths of the branches that link them are so short, it is conceivable that the diversification and differentiation among *T. indicum*, *T. himalayense* and *T. melanosporum* occurred within a relatively short time.

4.2. Phylogenetic relationships among the species with excavate ascomata

The three species, *T. excavatum*, *T. mesentericum* and *T. pseudoexcavatum*, which have excavated ascomata, did not group together in any of the analyses. The ITS lineages within *Tuber* were not well supported by ascoma excavation. Thus, the ''ascoma with orifice or cavity'' may have evolved more than once; or if it evolved only once, then it was lost once during the evolution of *Tuber* species. The homoplasy of ''ascoma with orifice or cavity'' indicates that this character lacks phylogenetic information at higher taxonomic levels, such as at the rank of section. It should not be used alone in separating taxa. However, this does not decrease the value of this character in distinguishing *T. pseudoexcavatum* among commercial Chinese truffles.

4.3. Concluding remarks

A molecular phylogenetic investigation of commercial Chinese truffles and their relatives was conducted in combination with morphological studies. It illustrates that two groups of commercial Chinese truffles macroscopically similar to *T. melanosporum* correspond to two closely related but separate species, i.e., *T. indicum* and *T. himalayense*. It is also shown that *Tuber* species producing excavated ascomata are not monophyletic.

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