# Molecular taxonomy of Chinese truffles belonging to the *Tuber rufum* and *Tuber puberulum* groups

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The taxonomic position of several Chinese *Tuber* species belonging to the *Rufum*- and *Puberulum*-groups were tentatively determined by analyzing the nuclear ITS region and mitochondrial large ribosomal RNA (mt LrRNA) sequences of several dried ascomata harvested all over China in the last 20 years and compared with American and European samples. Within the *Rufum*-group, it was possible to differentiate the specimens harvested on the three continents: Asia (China), Europe, and North America. In China, three species belonging to the *Rufum*-group could be validly recognized: *T. huidongense*, *T. liatongense*, and *T. taiyuanense*. The taxonomy of the *Puberulum* group appeared much more complex. Within the *Puberulum*-group, it was not possible to clearly separate the Chinese specimens from the American and European ones. Nevertheless, at least three new species or subspecies belonging to the *Puberulum*-group are present in China.

**Key words:** China, mitochondrial LrRNA, Nuclear ITS, *Tuber*, *Puberulum*-group, *Rufum*-group

## Introduction

Micheli (1729) was the first to name the genus *Tuber*. Later, numerous taxonomists described different species in Europe. Knapp (1950) recognized 32 species, while Gross (1987) recognized 26 taxa. During the past decades, numerous species have been described from Asia, Europe and North America.

Two new species were discovered in Europe at the end of the twentieth century: *T. malençonii* Donadini, Riousset, G. Riousset & G. Chev. (Donadini *et al*., 1978) and *T. regianum* Montecchi & Lazzari (Montecchi and Lazzari,

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1987). The first North American truffles were discovered in 1878 in California by HW Harkness. Harkness (1899) annotated 13 species, seven of which he described as new: *T. californicum* Harkn., *T. candidum* Harkn., *T. citrinum* Harkn., *T. eisenii* Harnk., *T. gibbosum* Harkn., *T. olivaceum* Harkn. and *T. monticola* Harnk. The first Asian truffle, *T. indicum* Cooke & Massee was described by Cooke and Massee (1892). In the recent years, 31 truffle species have been described from China. However, the validity of several taxa is questionable. In two previous papers (Wang *et al*., 2006a,b), the validity of the Chinese species, *T. himalayense* B.C. Zhang & Minter, *T. indicum*, *T. pseudoexcavatum* Y. Wang, G. Moreno, Riousset, Manjón & G. Riousset, *T. pseudohimalayense* G. Moreno, Manjón, J. Díez & García-Mont. and *T. sinense* K. Tao & Liu (not listed in Index Fungorum) was discussed. It was suggested to consider *T. indicum*, *T. himalayense*, *T. sinense* and *T. pseudohimalayense* as forming one species, *T. indicum*.

Throughout the world, about 140 species and 65 subspecies and varieties of *Tuber* have been reported. Index Fungorum lists 227 species, subspecies and varieties. However, morphological classification within *Tuber* using ascomata and spores characters has led to controversy (Ceruti *et al*., 2003). Approximately, 70-75 species have been validly described (Hawksworth *et al*., 1995; Bougher and Lebel, 2001). In Europe, 28 species are considered to be valid (Ceruti *et al*., 2003).

Fischer (1897) proposed the distinction of two subgenera in *Tuber*. Knapp (1950) continued this idea and classified the species into different groups. This classification was modified by Gross (1987) and Riousset *et al*. (2001). According to these different authors, the following groups were retained: *Aestivum*-, *Excavatum*-, *Macrosporum*-, *Magnatum*-, *Melanosporum*-, *Puberulum*-and *Rufum*-groups.

The *Rufum*-group (areolated or slightly warted or tomented ascomata, echinulate ascospores) seems to occur in America, Asia and Europe. According to Riousset *et al*. (2001), the European species belonging to this group are *T. nitidum* Vittad., *T. panniferum* Tul., *T. requienii* Tul. and *T. rufum* Pico (Riousset *et al*., 2001). *Tuber ferrugineum* Vittad. is considered to be a synonym of *T. requienii* or *T. rufum* Pico, var. *ferrugineum* Vittad. (Ceruti *et al*., 2003). Similarly, *T. requieni* is considered to be a synonym of *T. nitidum* (Ceruti *et al*., 2003). Other forms of *T. rufum* have been described (var. *apicleatum*, *brevisporum*, *lucidum*, *nigrum*, *oblongisporum* and *rutilum*).

The American species belonging to the *Rufum*-group are *T. candidum* Harkn., *T. quercicola* J.L. Frank, Southworth & Trappe and *T. texense* Heimsch, the latter a synonym of *T. lyoniae* Butters.

In China, three species belonging to the *Rufum*-group and originally

described in Europe, *T. nitidum* (Zhang, 1990) and *T. rufum* (Ren, 2003) or in America, *T. texense*, (Zhang, 1990), were recorded. Moreover, three new Chinese species, *T. huidongense* Y. Wang, *T. liaotongense* Y. Wang and *T. taiyuanense* B. Liu, could be recognized as belonging to this group (Liu, 1985, 1994 ; Wang, 1988, 1990; Wang and Li,1991; Wang and He, 2002).

*Tuber taiyuanense* B. Liu was described by Liu (1985). It is characterized by smooth ascomata (0.7-1.5 cm broad), subglobose to tuber-shaped, light brown at the early stage and becoming brown when mature. The ascospores are spinoreticulate with hooked spines (28.4-32.1 × 18.9-24.6 μm). The holotype was deposited in the Herbarium of the Department of Biology, Taiyuan City, Shanxi Province, China. Unfortunately it was destroyed. A neotype was described by Zheng Wang (2001) and deposited in the herbarium of HMAS, Chinese Academy of Sciences (HMAS 75888). Later, it was described by Dejun Ren in his MSc dissertation (Ren, 2003).

*Tuber huidongense* Y. Wang was described by Wang and He (2002). This species was collected two times in November 1989 in Huidong county, Sichuan, under *Pinus yunnanensis* Franch. and *P. armandii* Franch., at an altitude of 2070 m. It is characterized by smooth, whitish to ochreous, ascomata (0.5-2.5 cm broad). The ascospores are spinoreticulate (23-25~17-20 μm). The holotype was deposited in the Herbarium of the Institute of Applied Ecology, Academica Sinica, Shenyang, China, holotype 89923.

*Tuber liaotongense* Y. Wang was described by Wang (1988) from a specimen discovered in Fushun, Liaoning Province (IFS: 87062 holotype). It was also described by Ren (2003). The ascomata are irregular, globose to subglobose (0.6-2.0 cm broad), whitish to brownish-yellow when fresh, yellowish-brown when dried and slightly warted. The ascospores are ellipsoid to subglobose (29-40 × 26-35 μm), reticulate or alveolo-reticulate.

The *Puberulum*-group (smooth ascomata, reticulate spores) also comprises American, Asian and European species. According to Knapp (1950) and Halàsz *et al*. (2005), the European species belonging to this group are *T. borchii* Vittad., *T. foetidum* Vittad., *T. maculatum* Vittad., *T. puberulum* Berk. & Broome and *T. rapaeodorum* Tul. However, other authors consider that *T. asa* (Lespiault) Tul. & C. Tul. (listed in Index Fungorum as *T. asa-foetida* Lesp.) and *T. magnatum* Pico form the *Magnatum*-group, which also includes the species belonging to the *Puberulum*-group (Riousset *et al*., 2001). According to Ceruti *et al*. (2003), another taxon, *T. scruposum* R. Hesse, is closely related to the *Puberulum*-group. *T. oligospermum* (Tul. & C. Tul.) Trappe and *T. dryophilum* Tul. could be also considered as belonging to the *Puberulum*-group. *Tuber puberulum* is sometimes considered as synonym of *T. rapaeodorum* (Riousset *et al*., 2001), while other authors consider that they are

separated species (Pegler *et al*., 1993). According to Ceruti *et al*. (2003), *T. rapaeodorum* is a synonym of *T. puberulum* var. *michaliowskyjanum* Bucholtz and *T. scruposum* is a synonym of *T. puberulum* var. *longisporum* Bucholz. *Tuber puberulum* and *T. borchii* have also been considered to be closely related species. Mello *et al*. (2000) consider that *T. borchii* is closely related to *T. maculatum*. Nevertheless, Halàsz *et al*. (2005), by conducting microscopic and ITS sequence investigations on 31 herbarium specimens including holotypes, isotypes and neotypes, clearly identified ITS clades corresponding to five well defined morphological species: *T. borchii*, *T. foetidum*, *T. maculatum*, *T. puberulum* and *T. rapaeodorum*.

At least, six North American species seem to belong to the *Puberulum*-group: *T. californicum* Harkn., *T. gibbosum* Harkn., (a synonym of *T. giganteum* Gilkey), *T. levessimum* Gilkey, *T. murinum* R. Hesse, *T. shearii* Harkn. and *T. whetstonense* Frank, Southworth & Trappe. The appertaining to defined groups with other American species, such as *T. anniae* W. Colgan & Trappe, *T. besseyi* Gilkey, *T. candidum* Harkn., *T. monticola* Harnk, *T. olivaceum* Harkn, *T. pacificum* Trappe, Castellano & Buschnell, *T. phlebodermum* (Gilkey) Trappe, *T. spinoreticulatum* Uecker & Burds and some other species, is uncertain.

In China, one new species, *T. liui* A.S. Xu, could belong to the *Puberulum*-group. *Tuber liui* was collected from specimens harvested in 1996 under *Quercus sp*. in Miling, Xizang (Tibet) (Xu, 1999). The ascomata are globular, red-brown and pubescent. The ascospores are ellipsoid, reticulate and up to 80 μm long. This is similar to the length of the ascospores of the European species *T. macrosporum* Vittad.. The holotype of *T. liui* was deposited in the herbarium of Xizang Institute of Plateau Ecology, Linzhi, Xizang (HXZE 984) and isotypes in the Herbarium of the Department of Biology, Taiyuan City, Shanxi Province, China.

Several other species first described in Europe or America and belonging to the *Puberulum*-group were recorded in China: *Tuber asa* (Wang, 1988), *T. borchii* (Wang, 1988), *T. californicum* (Tao, 1988), *T. dryophilum* (Wang, 1988), *T. oligospermum* (Zhuang, 1998, 2001), *T. rapaeodorum* (Wang, 1988; Liu *et al*., 1994; Chen and Gong, 2000) and *T. shearii* (Zhang, 1990).

*Tuber oligospermum* was mentioned by Zhuang (1998) and described by Xu (1999) from samples harvested in Tibet. Specimens of *T. oligospermum*, harvested by Xu in Tibet, were examined by Ren, who rejected the existence of this species in China (Ren, 2003).

*Tuber rapaeodorum* has been discussed by several Chinese authors (Wang, 1988; Liu *et al*., 1994; Chen and Gong, 2000). However, their description does not seem congruent with the holotype described by Pegler *et*

*al*. (1993) in Europe (Ren, 2003). It is not certain whether this species exists in China.

*Tuber shearii*, an American species, was mentioned for the first time by Zhang (1990). Later, several Chinese authors mentioned this species (Wang, 1995; Liu, 1994; Chen and Gong, 2000), but without giving any information on the holotype.

Besides species belonging to the *Melanosporum*-, *Rufum*- and *Puberulum*-groups, several other European *Tuber* species have also been recorded in China. *Tuber verrucosum* (not listed in Index Fungorum) was mentioned by Ren (2003) as sp. nov., but without precise description. According to Ceruti *et al*. (2003), *T. verrucosum* Pers. is a synonym of *T. melanosporum*.

*Tuber aestivum* Vittad. was described by Mao (1998), without any indication of location. Moreover, there are no holotypes deposited in Chinese herbarium. This taxon was not mentioned in a more recent publication of the same author (Mao, 2000).

*Tuber excavatum* Vittad. was recorded in China by Wang (1995) and Chen and Gong (2000). But the locations were not recorded and no specimen was deposited in herbarium.

*Tuber brumale* Vittad. was mentioned by Pu (1989) from samples harvested in Yunnan. However, *T. brumale* has not since been recorded in China. The specimens described in 1989 were probably renamed as *T. indicum* by Zang in 1992 (Ren, 2003).

Outside of these probable misidentifications, several new Chinese *Tuber* species were probably invalidly published.

*Tuber gigantosporum* Y. Wang & Z.P. Li was described by Wang and Li (1991).

*Tuber xizangense* A.S. Xu was described by Xu (1999) from specimens harvested in Tibet. The holotypes were studied later by Ren who did not recognize it as a new species (Ren, 2003).

*Tuber formosanum* (not listed in Index Fungorum) was described by Hu (1992). However, no holotype was deposited in a public herbarium.

*Tuber huangshawanense* (not listed in Index Fungorum) and *T. mindense* (not listed in Index Fungorum) were mentioned by Wang (1990). However, there were no detailed descriptions and no holotype was deposited in a public herbarium.

*Tuber tianshanense* (not listed in Index Fungorum) was described by Tao (1988) in his MSc dissertation. This species, was mentioned later by several authors (Liu and Liu, 1994; Chen and Gong, 2000). However, afterwards, this species was never formally published.

*Tuber turbinatosporum* (not listed in Index Fungorum) was described by Zhang (1990) in his PhD dissertation. The holotype seems lost and this species was never published later.

*Tuber leptoperidium* (not listed in Index Fungorum) was described by Ren (2003), but never formally published.

These seven Chinese species, invalidly published, were excluded from this study. The new Chinese species *Tuber umbilicatum* Juan Chen & P.G. Liu, described by Chen *et al*. (2005), was not included in this work.

The objectives of this paper were (i) to study the taxonomic position of several Chinese *Tuber* species belonging the *Rufum*- and *Puberulum*-groups by analyzing sequences of the nuclear ITS region and sequences of the mitochondrial large ribosomal RNA gene (mt LrRNA) of several dried ascomata harvested all over China over the last 20 years and (ii) to improve our understanding of the possible past dispersal patterns within these two truffle groups.

## Materials and methods

### *Source of fungal materials and sequences*

Samples of Chinese truffles were collected by different authors from 1985 to 1999 in several locations of China: Inner Mongolia and Xizang (Tibet) autonomous regions, Gansu, Liaoning, Sichuan, Hebei, Hubei, Jilin and Shanxi Provinces and Beijing City. They were kept dried in Herbaria. Two samples of *T. huidongense* were collected during this study from Panzhihua, Sichuan Province. All samples were identified morphologically and named as new species or named according to their original descriptions and listed in Table 1 (*Rufum*-group) and in Table 2 (*Puberulum*-group). Several DNA sequences deposited in GenBank were also included in this study. Accession numbers are listed in Table 1 and 2.

### *DNA extraction and PCR amplification*

Genomic DNA was isolated from 5 mg of dried sporocarps by using the DNeasy Plant Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions with the following instructions: samples from herbarium were kept in CTAB buffer (100 mM Tris-HCl pH 9, 20 mM EDTA pH 8, 1.4 M NaCl, 2% CTAB, 0.2% B-mercaptoethanol) for 10 days before DNA extraction. Two mg of PVPP and 2 mg of diatomite were added during grinding in liquid nitrogen. An extension of 30 minutes instead of 10 minutes in waterbath at 65°C was performed after grinding. Due to inadequate methods of tissue

**Table 1.** Locations where specimens belonging to the *Rufum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA ).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber aestivum* Vittad. | — | T.aes-eu07 | François Le Tacon 04 06 03 | Aest1 | — | Vaucluse, France | — | — | DQ480473 |
| *Tuber borchii* Vittad. | *Tuber liaotongense* | T.bor-gs01 | Yun Wang Xingyuan He 04 09 1989 | 89196 | — | Zhouqu, Gansu | DQ478665 | — | — |
| *Tuber californicum* Harkn. | *Tuber liaotongense* | T.cal-hr06 | Yun Wang 25 09 1986 | 86 726 | — | Hengren, Liaoning province | DQ478666 | — | DQ480445 |
| *Tuber candidum* Harkn. | — | T.can-AY830856 | J.L. Frank | — | — | Southern Oregon | AY830856 | AY830856 | — |
| *Tuber dryophilum* Tul. | *Tuber liaotongense* | T.dry-hr01 | Yun Wang 26 08 1986 | 86 221 | — | Hengren, Liaoning province | DQ478667 | DQ478630 | DQ480458 |
| *Tuber dryophilum* Tul. | *Tuber liaotongense* | T.dry-hr02 | Yun Wang 26 08 1987 | 87 0127 | — | Hengren, Liaoning province | — | DQ478631 | DQ480459 |
| *Tuber excavatum* Vittad. | — | T.exc-AJ557545 | Halasz *et al*. 2005 | B-2597 | — | Hungary, Miskolctapolca | AJ557545 | AJ557545 | — |
| *Tuber ferrugineum* Vittad. | — | T.fer-AF132506 | Roux et al. 1999 | — | — | unknown | AF132506 | AF132506 | — |
| *Tuber huidongense* Y. Wang | — | T.hui-hd01 | Ziping Li 1988 | 89 924 | | Huidong, Sichuan province | DQ478668 | DQ478632 | DQ480460 |
| *Tuber huidongense* Y. Wang | — | T.hui-pzh08 | Yongjin Wang Zhuming Tan October 2004 | | | Panzhihua, Sichuan province | DQ486032 | DQ486032 | — |
| *Tuber huidongense* Y. Wang | — | T.hui-pzh09 | Yongjin Wang Zhuming Tan October 2004 | | | Panzhihua, Sichuan province | DQ486031 | DQ486031 | — |
| *Tuber nitidum* Vittad. | *Tuber taiyuanense* | T.nit-pw01 | Bincheng Zhang 23 09 1989 | — | HMAS 60 239 | Pingwu, Sichuan | — | DQ478637 | — |

**Table 1 continued.** Locations where specimens belonging to the *Rufum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA ).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber liaotongense* Y. Wang | — | T.lia-fs01 | Yun Wang Xingyuan He 04 09 1989 | 89 300 | — | Fushun, Liaoning province | DQ478670 | — | DQ480462 |
| *Tuber liaotongense* Y. Wang | — | T.lia-mg01 | Yun Wang 08 1988 | 88 059 | — | Inner Mongolia | DQ478672 | DQ478634 | DQ480464 |
| *Tuber liaotongense* Y. Wang | — | T.lia-mg02 | Yun Wang 08 08 1988 | 88 061 | — | Inner Mongolia | DQ478671 | DQ478635 | DQ480465 |
| *Tuber liaotongense* Y. Wang | — | T.lia-fs04 | Yun Wang 20 07 1989 | 89 025 | — | Fushun, Liaoning province | DQ478669 | DQ478633 | DQ480463 |
| *Tuber quercicola* (not listed in Index Fungorum) | — | T.que-AY918957 | J.L. Frank | SOC T33 | — | Oregon, USA | AY918957 | AY918957 | — |
| *Tuber rufum* Pico | — | T.ruf-AY940646 | Iotti *et al*. | — | — | Italy | AY940646 | AY940646 | — |
| *Tuber rufum* Pico | — | T.ruf-AF106892 | Rubini *et al*. | — | — | Italy | AF106892 | AF106892 | — |
| *Tuber rufum* Pico | — | T.ruf-AY112894 | Iotti *et al*. | — | — | Italy | AY112894 | AY112894 | — |
| *Tuber rufum* Pico | — | T.ruf-eu01 | François Le Tacon 04 06 03 | Rufum1 | — | Vaucluse, France, Europe | DQ329375 | DQ329375 | — |
| *Tuber rufum* Pico | *Tuber* sp.1 (new Chinese species or *T. rufum* subspecies) | T.ruf-zq02 | Yun Wang Xingyuan He 21 08 1989 | 89 245 | — | Zhouqu, Gansu province | — | DQ478643 | DQ480456 |
| *Tuber rufum* Pico | *Tuber* sp.1(new Chinese species or *T. rufum* subspecies) | T.ruf-zq03 | Yun Wang Xingyuan He 21 08 1989 | 89 257 | — | Zhouqu, Gansu province | — | DQ478642 | DQ480457 |

**Table 1 continued.** Locations where specimens belonging to the *Rufum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA ).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber* sp. | *Tuber liaotongense* | T.sp-mg01 | — | 88 064 | — | Inner Mongolia | DQ478674 | DQ478646 | DQ480468 |
| *Tuber* sp. | *Tuber liaotongense* | T.sp-mg02 | 08 08 1988 | 88 065 | — | Inner Mongolia | DQ478675 | DQ478647 | DQ480469 |
| *Tuber* sp. | *Tuber huidongense* | T.sp-hd01 | Yun Wang Dacheng Zhang 26 11 1989 | 89 923 | — | Huidong, Sichuan province | — | DQ478644 | DQ480466 |
| *Tuber* sp. | *Tuber liaotongense* | T.sp-hr02 | 25 05 1987 | — | — | Hengren, Liaoning province | — | DQ478645 | DQ480467 |
| *Tuber taiyuanense* B. Liu | *Tuber liaotongense* | T.tai-bj01 | Wang Zheng 01 08 1999 | 2280 | HMAS 76 038 | Dongling Mountain, Beijing | DQ478676 | DQ478648 | DQ480470 |
| *Tuber taiyuanense* B. Liu | — | T.tai-bj02 | Wang Zheng 20 08 1998 | 294 | HMAS 76 888 | Dongling Mountain, Beijing | DQ478662 | — | — |
| *Tuber texense* Heimsch | *Tuber taiyuanense* | T.tex-wl01 | Bincheng Zhang 22 09 1989 | 622 | HMAS 60 239 | Wolong, Sichuan | — | DQ478649 | DQ480471 |
| *Tuber texense* Heimsch | *Tuber taiyuanense* | T.tex-xh01 | Bincheng Zhang 11 10 1988 | 521 | HMAS 60 235 | Xuanhua, Hubei province | DQ478663 | — | DQ480472 |
| *Tuber texense* Heimsch | *Tuber taiyuanense* | T.tex-xh02 | Bincheng Zhang 11 10 1988 | 517 | HMAS 60 234 | Xuanhua, Hubei province | DQ478664 | DQ478650 | DQ480461 |

**Table 2.** Locations where specimens belonging to the *Puberulum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector, name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber asa* Tul. & C. Tul. Listed in Index Fungorum as *Tuber asa-foetida* Lesp. | *Tuber* sp. 2 (new Chinese species) | T.asa kd04 | Yun Wang 10 09 1989 | 89 552 | — | Kuandian, Liaoning province | — | DQ478622 | — |
| *Tuber asa* Tul. & C. Tul. | *Tuber* sp. 2 (new Chinese species) | T.asa-gs01 | Yun Wang 12 08 1989 | 89 175 | — | Gansu province | DQ478655 | — | — |
| *Tuber asa* Tul. & C. Tul. | *Tuber* sp. 2 (new Chinese species) | T.asa-gs02 | Yun Wang Xingyuan He 15 08 1989 | 89 199 | — | Gansu province | — | DQ478621 | DQ480449 |
| *Tuber borchii* Vittad. | — | T.bor -AJ557541 | Halasz *et al*. 2005 | CI-38 | — | Ketvolgy, Hungary | AJ557541 | AJ557541 | — |
| *Tuber borchii* Vittad. | — | T.bor -AJ557540 | Halasz *et al*. 2005 | B 1320 | — | Ruganesti, Romania | AJ557540 | AJ557540 | — |
| *Tuber borchii* Vittad. | — | T.bor -AF003920 | Amicucci *et al*. 1997 | — | — | Near San Angelo, in Vado-Urbino, Italy | AF003920 | AF003920 | — |
| *Tuber borchii* Vittad. | — | T.bor -AF132505 | Roux *et al*. 1999 | — | — | unknown | AF132505 | AF132505 | — |
| *Tuber borchii* Vittad. | — | T.bor-AJ557542 | Halasz *et al*. 2005 | B 1481 | — | Nagyegyhaza, Hungary | AJ557542 | AJ557542 | — |
| *Tuber borchii* Vittad. | *Tuber* sp. 3 (new Chinese species) | T.bor-kd02 | Yun Wang | 89 556 | — | Kuandian, Liaoning province | — | DQ478624 | — |
| *Tuber borchii* Vittad. | *Tuber* sp. 3 (new Chinese species) | T.bor-kd08 | Yun Wang 09 09 1989 | 89 371 | — | Kuandian, Liaoning province | DQ478656 | DQ478625 | DQ480441 |
| *Tuber californicum* Harkn. | — | T.cal-AY558807 | Izzo *et al*. 2005 | | — | Ross Crossing California, USA | AY558807 | — | — |
| *Tuber californicum* Harkn. | *Tuber* sp. 4 (new Chinese species) | T.cal-hr01 | Yun Wang 09 09 1989 | 86 284 | — | Hengren, Liaoning province | — | DQ478626 | DQ480443 |
| *Tuber californicum* Harkn. | *Tuber* sp. 4 (new Chinese species) | T.cal-hr07 | Yun Wang 26 09 1986 | 86 741 | — | Hengren, Liaoning province | — | DQ478627 | DQ480444 |
| *Tuber californicum* Harkn. | *Tuber* sp. 4 (new Chinese species) | T.cal-hr08 | Yun Wang 30 08 1986 | 86 752 | — | Hengren, Liaoning province | DQ478657 | DQ478628 | DQ480447 |

**Table 2 continued.** Locations where specimens belonging to the *Puberulum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector, name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber californicum* Harkn. | *Tuber* sp. 4 (new Chinese species) | T.cal-xb11 | Yun Wang 20 09 1986 | 86 644 | — | Xinbin, Liaoning province | DQ478659 | — | DQ480446 |
| *Tuber californicum* Harkn. | *Tuber* sp. 4 (new Chinese species) | T.cal-hr12 | Yun Wang 22 09 1986 | 86 682 | — | Hengren, Liaoning province | DQ478658 | DQ478629 | DQ480448 |
| *Tuber dryophilum* Tul. | — | T.dry-AF003917 | Amicucci *et al.* 1997 | — | — | Near Urbino, Italy | AF003917 | AF003917 | — |
| *Tuber foetidum* Vittad. | — | T.foe-AJ557543 | Halasz *et al.* 2005 | B-2452 | — | Garé, Hungary | AJ557543 | AJ557543 | — |
| *Tuber foetidum* Vittad. | — | T.foe-AJ557544 | Halasz *et al.* 2005 | B-2489 | — | Szigetujfalu, Hungary | AJ557544 | AJ557544 | — |
| *Tuber liui* A.S. Xu | — | T.liu-ml01 | Asheng Su 01 09 1996 | | HXZE 984 | Miling, Tibet | DQ478660 | DQ478636 | DQ480450 |
| *Tuber maculatum* Vittad. | — | T.mac-AF106889 | Rubini *et al.* 2002 | Mac1 | — | Central Umbria, Italy | AF106889 | AF106889 | — |
| *Tuber maculatum* Vittad. | — | T.mac-AF003919 | Amicucci *et al.* 1997 | — | — | Near Perugia, Italy | AF003919 | AF003919 | — |
| *Tuber maculatum* Vittad. | — | T.mac-AJ557518 | Halasz *et al.* 2005 | B-2756 | — | Aszod, Hungary | AJ557518 | AJ557518 | — |
| *Tuber maculatum* Vittad. | — | T.mac-AJ557520 | Halasz *et al.* 2005 | B-1079 | — | Szeged, Hungary | AJ557520 | AJ557520 | — |
| *Tuber maculatum* Vittad. | — | T.mac-AJ557519 | Halasz *et al.* 2005 | BII-120 | — | Batorliget, Hungary | AJ557519 | AJ557519 | — |
| *Tuber maculatum* Vittad. | *Tuber* sp. | T.mac-AY558809 | Izzo *et al.* 2005 | SNF 54 | — | USA | AY558809 | — | — |
| *Tuber maculatum* Vittad. | — | T.mac-AF003909 | Amicucci *et al.* 1997 | — | — | Near Bologna, Italy | — | AF003909 | — |
| *Tuber oligospermum* Tul. & C. Tul | — | T.oli-AF106891 | Rubini *et al.* 2002 | Oli-5 | — | Italy | AF106891 | AF106891 | — |
| *Tuber puberulum* Berk. & Broome | — | T.pub-AJ557538 | Halasz *et al.* 2005 | B-1076 | — | Ganna, Hungary | AJ557538 | AJ557538 | — |

**Table 2 continued.** Locations where specimens belonging to the *Puberulum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector, name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber puberulum* Berk. & Broome | — | T.pub-AJ557536 | Halasz *et al*. 2005 | CI.-50 | — | Tardosbanya, Hungary | AJ557536 | AJ557536 | — |
| *Tuber puberulum* Berk. & Broome | — | T.pub-AJ557533 | Halasz *et al*. 2005 | B-2658 | — | Tahi, Hungary | AJ557533 | AJ557533 | — |
| *Tuber puberulum* Berk. & Broome | — | T.pub-AJ557537 | Halasz *et al*. 2005 | BI.-32 | — | Abaliget, Hungary | AJ557537 | AJ557537 | — |
| *Tuber puberulum* Berk. & Broome | *Tuber* sp. 3 | T.pub-zq02 | Yun Wang Xingyuan He 08 12 1989 | 89261 | — | Zhouqu, Gansu | — | DQ478639 | — |
| *Tuber puberulum* Berk. & Broome | *Tuber* sp. 3 | T.pub-th01 | Yun Wang Xingyuan He 10 09 1989 | 89 142 | — | Tiaohe, Gansu province | — | DQ478638 | DQ480455 |
| *Tuber puberulum* Berk. & Broome | — | T.pub-it | J. Trappe | — | — | Italy | DQ478661 | — | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557524 | Halasz *et al*. 2005 | B-1284 | — | Jaszszentlaszlo, Hungary | AJ557524 | AJ557524 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557523 | Halasz *et al*. 2005 | B-2113 | — | Felsoszentmarton, Hungary | AJ557523 | AJ557523 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557529 | Halasz *et al*. 2005 | B-1713 | — | Kemence, Hungary | AJ557529 | AJ557529 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557522 | Halasz *et al*. 2005 | B-1360 | — | Szilvagy, Hungary | AJ557522 | AJ557522 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557528 | Halasz *et al*. 2005 | B-1965 | — | Szilvasvarad, Hungary | AJ557528 | AJ557528 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557526 | Halasz *et al*. 2005 | B-2657 | — | Ocsa, Hungary | AJ557526 | AJ557526 | — |
| *Tuber rapaeodorum* Tul. | — | T.rap-AJ557521 | Halasz *et al*. 2005 | B-2139 | — | Simonesti, Romania | AJ557521 | AJ557521 | — |
| *Tuber rapaeodorum* Tul. | *Tuber* sp. 3 | T.rap-zq01 | Yun Wang Xingyuan He 12 08 1989 | 89 173 | — | Zhouqu, Gansu province | — | DQ478641 | DQ480451 |

**Table 2 continued.** Locations where specimens belonging to the *Puberulum*-group were collected. Accession number of sequences from GenBank (ITS1, ITS2, mt LrRNA).

| Fungal name given by the collector | New fungal name given according to this study | Code | Collector, name and collection date | Collection number | Herbarium number | Geographical origin | ITS1 | ITS2 | mt LrRNA |
|---|---|---|---|---|---|---|---|---|---|
| *Tuber rapaeodorum* Tul. | *Tuber* sp. 3 | T.rap-kd01 | Yun Wang 10 09 1989 | 89 719 | — | Kuandian, Liaoning province | DQ478651 | DQ478640 | DQ480454 |
| *Tuber rapaeodorum* Tul. | *Tuber* sp. 3 | T.rap-kd03 | Yun Wang 25 09 1987 | 87 0264 | — | Kuandian, Liaoning province | DQ478652 | — | DQ480452 |
| *Tuber rapaeodorum* Tul. | *Tuber* sp. 3 | T.rap-kd04 | Yun Wang 22 09 1987 | 87 0205 | — | Kuandian, Liaoning province | DQ478653 | — | DQ480453 |
| *Tuber rapaeodorum* Tul. | *Tuber* sp. 3 | T.rap-kd05 | Yun Wang 22 09 1987 | 87 0202 | — | Kuandian, Liaoning province | DQ478654 | — | — |
| *Tuber scruposum* R. Hesse | — | T.scr-DQ011847 | Iotti *et al*. 2005 | — | CMI-UNIBO 2207 | Dilijan, Armenia | — | DQ011847 | — |
| *Tuber scruposum* R. Hesse | — | T.scr-DQ011848 | Iotti *et al*. 2005 | — | CMI-UNIBO 2194 | Dilijan, Armenia | — | DQ011848 | — |
| *Tuber scruposum* R. Hesse | — | T.scr-AJ557539 | Halasz *et al*. 2005 | B-1667 | | Lakitelek, Hungary | — | AJ557539 | — |
| *Tuber whetstonense* Frank, Southworth & Trappe | — | T.wes-AY830855 | J. L.Frank 2004 | — | SOC 756 | Southern Oregon USA | — | AY830855 | — |

conservation (samples kept dry), it was difficult to amplify the complete ITS region. Universal primer pairs of primers ITS1/ITS2 and ITS3/ITS4 were used to amplify separately the ITS1 and ITS2 regions (White *et al*., 1990). Primers MLSU1f (TTGCTTACCTGAGCTGGTATTTAGG) and MLSU1r (ATAAAGGCCAATCTATAGGTTGACC) were designed to amplify a short polymorphic fragment within the region amplified with ML3 and ML4 (White *et al*., 1990) in mitochondrial large subunit ribosomal RNA gene (mt LrRNA).

Amplification reactions were performed in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 µl reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1X PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.7 µg/µl of BSA and 0.5 unit of *Taq* polymerase (Promega). Amplification was conducted under the following conditions: an initial denaturation at 95˚C for 3 minutes, followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 50˚C (ITS) or 58˚C (mt LrRNA) for 45 seconds and extension at 72˚C for 2 minutes for each cycle. The last cycle was followed by a final extension at 72˚C for 10 minutes. PCR products were resolved on a 1.5% agarose gel and visualized by staining with ethidium bromide. For sequencing, the PCR products were purified with MultiScreenTM PCR (Millipore, Molsheim, France) and quantified with Low DNA Mass Ladder (Invitrogen, Cergy Pontoise, France) by electrophoresis on 1.5% agarose gel. Sequencing was performed in CEQ 2000 DNA Analysis System (Beckman, Fullerton, CA) in INRA Centre of Nancy (France) according to manufacturer's protocol.

***Sequence alignment and phylogenetic analysis***

When several identical sequences from GenBank were available, only one was kept. Multiple alignments were performed with CLUSTAL W (Thompson *et al*., 1994) using default settings and manually adjusted with BioEdit version 5.0.9 (Hall, 1999). Genetic distance and phylogenetic analyses were performed with MEGA version 2.1 using Kimura 2-parameter (K2P) model with a transition to transversion ratio (Ti/Tv=2). Pairwise deletion or complete deletion were employed in gap handling. Due to difficulties of amplification, both ITS1 and ITS2 sequences were not available for all samples. ITS1 and ITS2 analysis were performed separately or both together when available. Phylogenetic trees were built using the Neighbour-Joining (NJ) methods (Kumar *et al*., 2001). Bootstrap tests were performed using 500 replicates. In a preliminary work, several general ITS trees were constructed with sequences available in GenBank and sequences obtained in this study with

Chinese samples from herbarium, often misidentified at the species level. The ITS trees (data not shown) displayed a clear distinction among different groups and allowed us to discover taxonomic misidentifications. For further analyses in separated groups, the misidentified specimens were kept in the groups defined by our preliminary work and not in the groups corresponding to their original identifications. Nevertheless we have not modified their original taxonomic identification. All sequence alignments and phylogenetic trees were deposited in TreeBASE, ID: SN2969.

## Results

### *Mt LrRNA analysis, distinction between the Rufum and Puberulum-groups* (Fig. 1)

With 33 mitochondrial sequences of Chinese specimens and one *T. aestivum* sequence, CLUSTAL W generated an alignment of 224 bp. K2P distances ranged between 0.000-0.113. NJ tree clustered the 33 Chinese specimens into two clades: Clade I comprised all the specimens belonging to the *Rufum*-group and described as *T. liaotongense*, *T. huidongense*, *T. rufum*, *T. taiyuanense*, *T. texense*, four specimens not identified at the species level and two specimens identified as *T. dryophilum*. Clade II comprised all the specimens belonging to the *Puberulum*-group and described as *T. asa*, *T. borchii*, *T. californicum*, *T. liu*, *T. puberulum* and *T. rapaeodorum*. Nevertheless, within the two clades, phylogeny was not clearly resolved.

### *Phylogenetic analysis of the Rufum-group*

#### ITS1 analysis (Fig. 2)

Using the 23 ITS1 sequences belonging to the *Rufum* group, and one *T. excavatum* sequence, CLUSTAL W generated an alignment of 243 bp. K2P distances ranged between 0.000 and 0.569 for the *Rufum*-group.

Four clades, supported by high bootstrap values, were distinguished. Clade I comprised only specimens collected from China, which were distributed among two subclades. Subclade I included all the samples described as *T. liaotongense*, two samples not identified at the species level, one sample described a *T. taiyuanense* and three samples described as *T. borchii*, *T. californicum* or *T. dryophilum*. Subclade II comprised the three samples described as *T. huidongense*. Clade IV comprised three specimens collected from China, one described as *T. taiyuanense* and two described as *T. texense*.

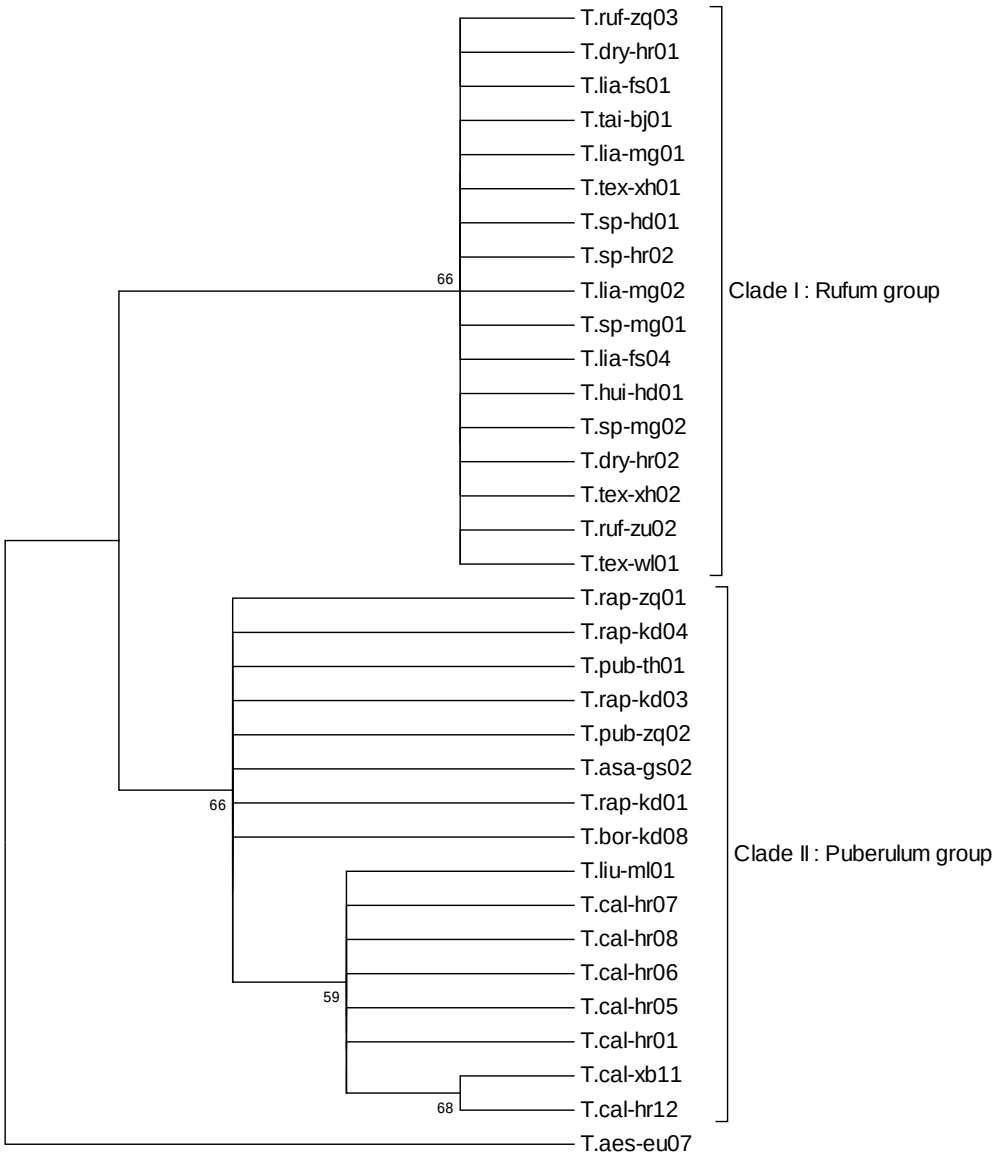


**Fig. 1.** Neighbour-Joining tree generated in Mega from the alignment of mitochondrial large subunit RNA gene (mt LrRNA) sequences of 33 Chinese specimens, using the Kimura two-parameter model with complete deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

All of the Chinese specimens belonging to Clade I and Clade IV shared in common a large and complex indel in the middle of the ITS1 region: one

indel of 53 bp from 71 to 123 for Subclade I, one indel of 53 bp from 65 to 118 for Subclade II and two indels for Clade IV, one of 27 bp from 65 to 91 and a second one of 26 bp from 98 to 123. Moreover, eight of the samples belonging to Subclade I displayed an additional deletion of 18 bp at the beginning of the ITS1 sequence. However, this deletion was not observed in two samples of this subclade described as *T. liaotongense*.

Clade II and Clade III comprised all the European samples, four identified as *T. rufum* and one identified as *T. ferrugineum*, a synonym of *T. rufum*.

The two American specimens, *T. quercicola* and *T. candidum*, belonged to none of these four clades.

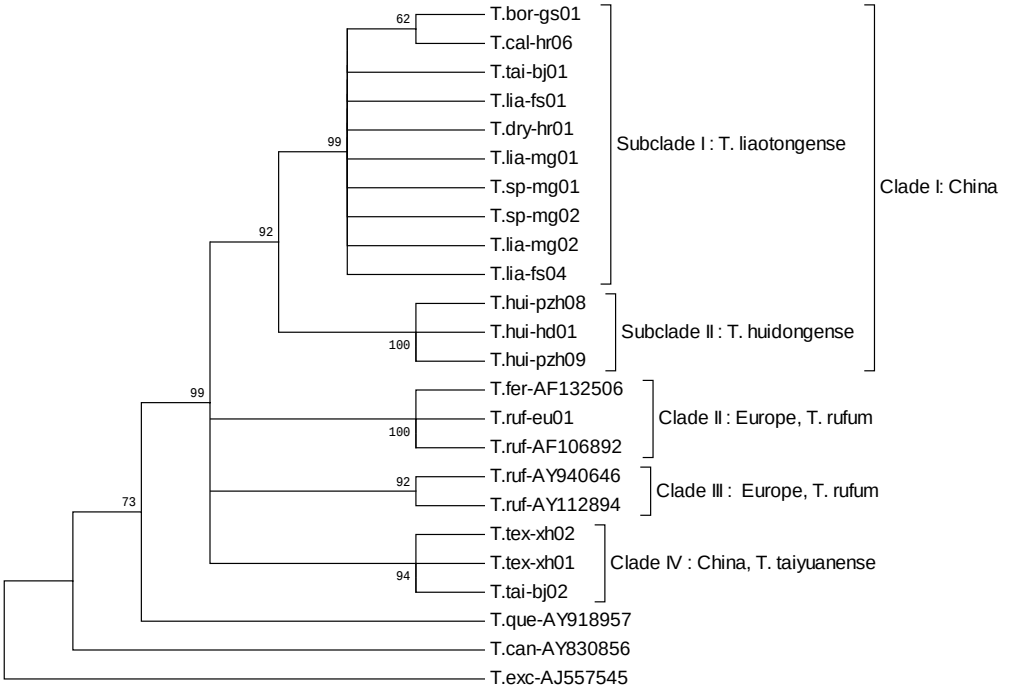


**Fig. 2.** Neighbour-Joining tree generated in Mega from the alignment of ITS1 sequences of specimens belonging to the *Rufum*-group, using the Kimura two-parameter model with complete deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

### ITS2 analysis (Fig. 3)

Using the 25 ITS2 sequences belonging to the *Rufum*-group, and one *T. excavatum* sequence, CLUSTAL W generated an alignment of 258 bp. K2P

distances ranged between 0.000 and 0.437 for the *Rufum*-group.

Five clades, supported by high bootstrap values, were distinguished.

Clade I comprised only specimens from China and was divided into two subclades. Subclade I included the three samples described as *T. liaotongense* (3), three samples not identified at the species level, two specimens described as *T. dryophilum* and one as *T. taiyuanense*. Subclade II comprised the three samples described as *T. huidongense* and one specimen not identified at the species level.

Clade II also comprised specimens from China, one described as *T. nitidum* and two as *T. texense*.

Clade III included only European samples, four identified as *T. rufum* and one identified as *T. ferrugineum*, a synonym of *T. rufum*.

Clade IV comprised two American species, *T. quercicola* and *T. candidum*. Clade V comprised two Chinese samples described as *T. rufum*.

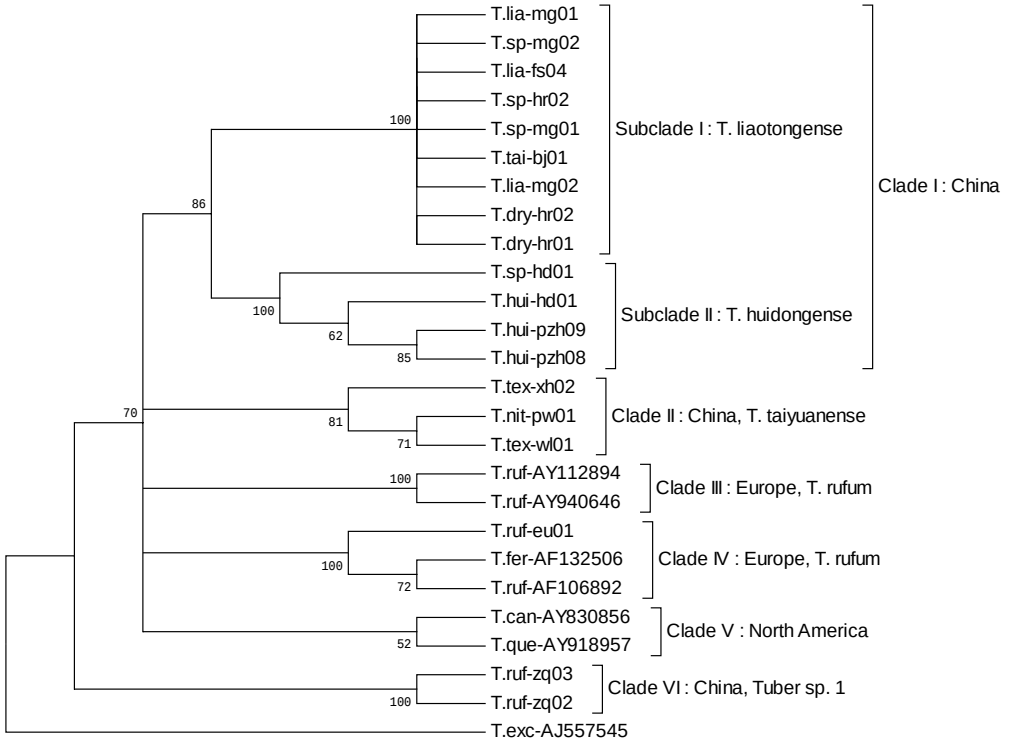


**Fig. 3.** Neighbour-Joining tree generated in Mega from the alignment of ITS2 sequences of specimens belonging to the *Rufum*-group, using the Kimura two-parameter model with pairwise deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

## Complete ITS analysis (Fig. 4)

The ITS1 and ITS2 sequences were manually joined into a complete ITS sequence at the exclusion of the short 5.8S rRNA fragment. With the 18 ITS sequences belonging to the *T. rufum* group, and one *T. excavatum* sequence, CLUSTAL W generated an alignment of 501 bp. K2P distances ranged between 0.000 and 0.398 for the *Rufum*-group.

Two clades were distinguished in this analysis. Clade I comprised all the Chinese specimens except one. It was divided into two subclades. Subclade I corresponded to *T. liaotongense* and Subclade II to *T. huidongense*. Clade II comprised the five European specimens. The two American specimens described as *T. quercicola* and *T. candidum* and a Chinese specimen described as *T. texense* belonged to none of these two clades.

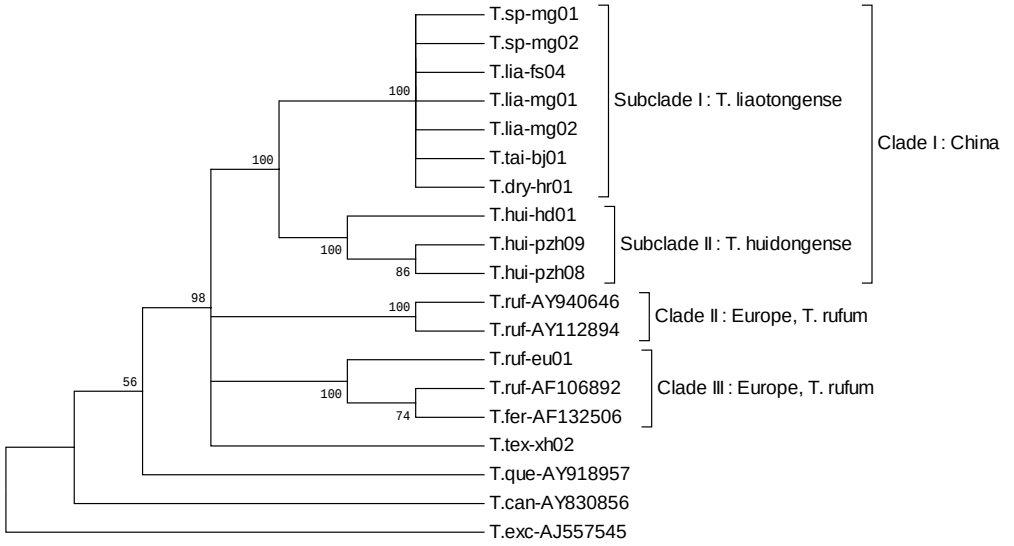


**Fig. 4.** Neighbour-Joining tree generated in Mega from the alignment of ITS sequences of specimens belonging to the *Rufum*-group, using the Kimura two-parameter model with complete deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

### *Phylogenetic analysis of the Puberulum-group*

The preliminary analysis showed that the sequences of *T. magnatum* exhibited a mean genetic distance of 0.570 with the other species of the *Puberulum*-group and were not clustered in that group (data not shown). We have decided to exclude the sequences of *T. magnatum* from the following

analysis and to root the *Puberulum*-group with *T. rufum*.

**ITS1 analysis (Fig. 5)**

Using the 38 ITS1 sequences belonging to the *Puberulum*-group, and one *T. rufum* sequence, CLUSTAL W generated an alignment of 271 bp. K2P distances ranged between 0.000 and 0.337 for the *Puberulum*-group.

Four clades supported by high bootstrap values were distinguished.

Clade I comprised only European samples: seven described as *T. rapaeodorum*, five as *T. maculatum* and two as *T. foetidum*.

Clade II comprised three Chinese samples described as *T. californicum*.

Clade III comprised two Chinese samples described as *T. asa* and *T. liui*, five European samples described as *T. borchii* (3), *T. dryophilum* (1) and *T. oligospermum* (1) and one American species described as *T. maculatum*.

Clade IV comprised five Chinese samples described as *T. borchii* (1) or *T. rapaeodorum* (4). They all displayed a deletion of 23 bp while the two other Chinese samples did not. Subclade II comprised other six European samples described as *T. borchii* (2) or *T. puberulum* (4). Five of these European specimens displayed a large deletion of 83 bp.

The American specimen of *T. californicum* and six European specimens described as *T. puberulum* (4) or *T. borchii* (2) were not clustered within the four clades.

**ITS2 analysis (Fig. 6)**

With the 43 ITS2 sequences belonging to the *Puberulum*-group, and one *T. rufum* sequence, CLUSTAL W generated an alignment of 271 bp. K2P distances ranged between 0.000 and 0.389 for the *Puberulum*-group. Four clades were distinguished.

Clade I was divided into two subclades. Subclade I comprised all the European specimens: seven described as *T. rapaeodorum*, five as *T. maculatum* and two as *T. foetidum*. Subclade II comprised three European specimens described as *T. scruposum* and one American species, *T. whestonense*.

Clade II comprised four Chinese specimens described as *T. californicum*.

Clade III comprised six Chinese specimens, two described as *T. borchii*, two as *T. rapaeodorum* and two as *T. puberulum*.

Clade IV was divided into three subclades. Subclade III comprised two Chinese species described as *T. asa*. Subclade IV comprised five European specimens, three described as *T. borchii*, one as *T. dryophilum* and one as *T. oligospermum*. Subclade V comprised one Chinese specimen described as *T.*

*liui* and seven European specimens described as *T. borchii* (2), *T. maculatum* (1) and *T. puberulum* (4).

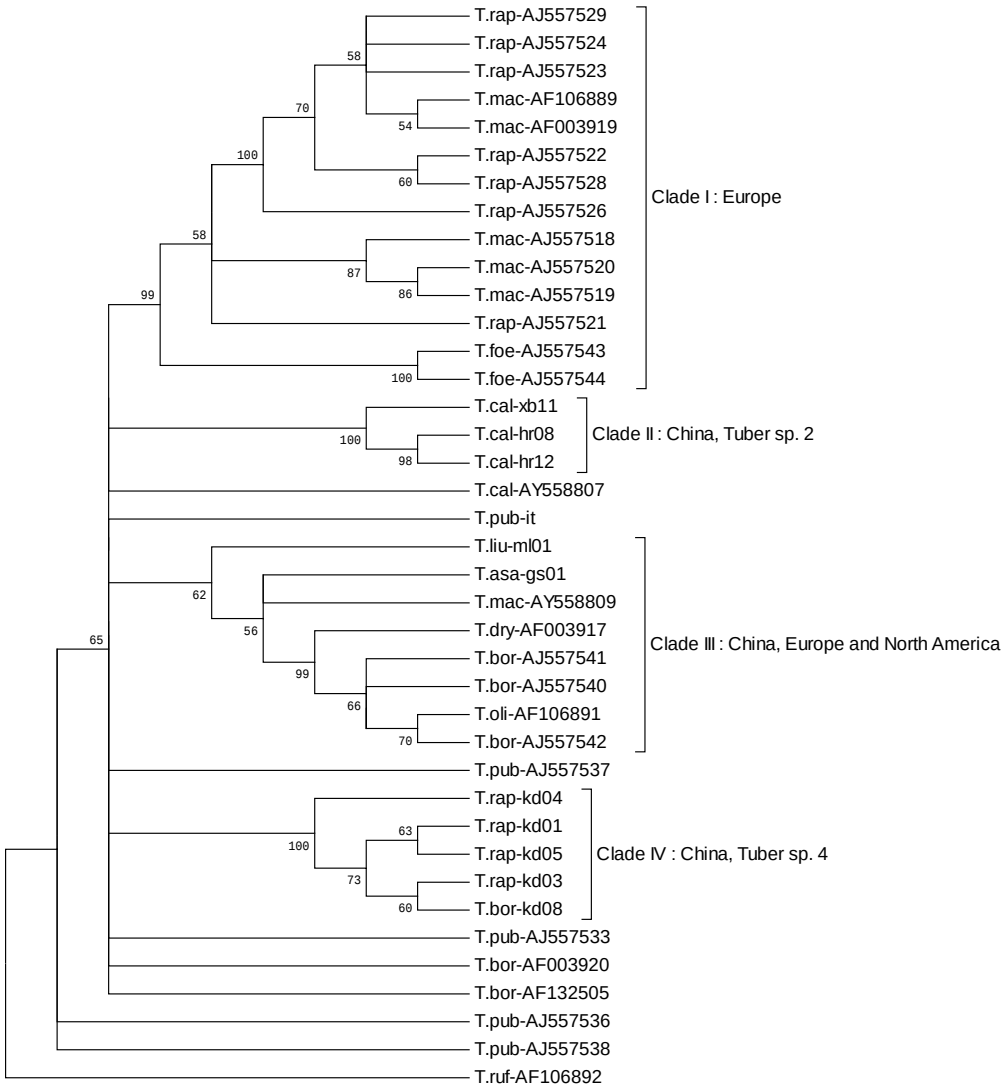


**Fig. 5.** Neighbour-Joining tree generated in Mega from the alignment of ITS1 sequences of specimens belonging to the *Puberulum*-group using the Kimura two-parameter model with pairwise deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

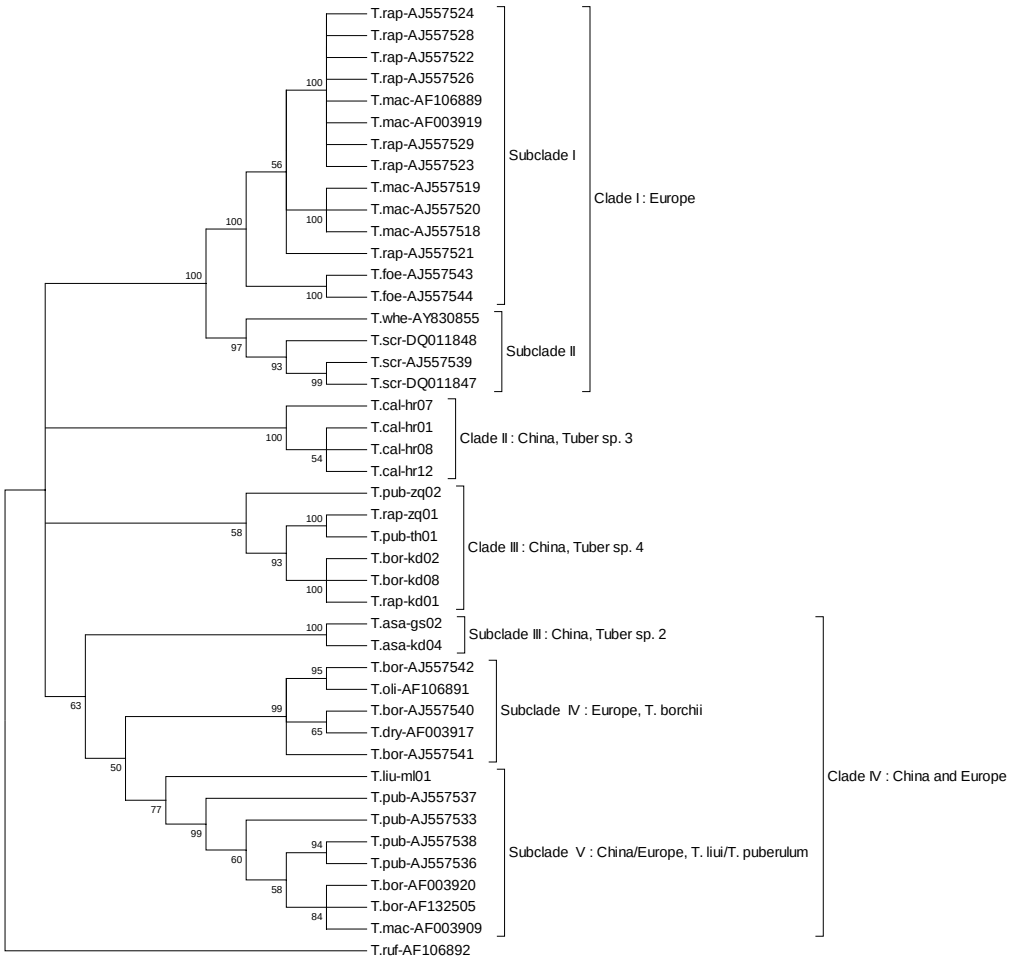


**Fig. 6.** Neighbour-Joining tree generated in Mega from the alignment of ITS2 sequences of specimens belonging to the *Puberulum*-group, using the Kimura two-parameter model with pairwise deletion gap handling and 500-replication bootstrapping. Nodes with bootstrap values inferior to 50% were eliminated. Bootstrap values are indicated next to relevant nodes.

## Complete ITS analysis

The analysis of the complete sequence of the ITS region obtained by manually joining the ITS1 and ITS2 region did not lead to new information (data not shown).

## Discussion

The analysis of the mitochondrial LrRNA sequences allowed to separate

all the Chinese specimens which did not belong to the *Melanosporum*-group into two other groups, the *Rufum*-group (Riousset *et al*., 2001), and the *Puberulum*-group, defined by Knapp (1950) and well defined by Halàsz *et al*. (2005).

The ITS1 and ITS2 phylogenetic analyses of the samples belonging to the *Rufum*- and *Puberulum*-groups led to the existence of different clades, which did not always correspond to the different taxa, suggesting a complex of species with ecological and morphological varieties leading to numerous misidentifications.

Within the *Rufum*-group, according to the ITS2 analysis, which was the most informative, the results were clear and allowed the differentiation of the specimens harvested on the three continents. The ITS1 and the whole ITS analyses led to similar results with some few exceptions. All the Chinese samples of the *Rufum*-group displayed a large deletion of 53 to 58 bp in the ITS1 region, which did not exist in the American and European species, suggesting a differential genetic evolution in the three continents. This also suggests that the European species *T. rufum* is not present in China, although recorded by Ren (2003) and that the Chinese specimens described as *T.rufum* have to be renamed as a new species (*Tuber* sp. 1, table 1). Nevertheless, the two Chinese samples described as *T. rufum* could belong to an Asian subspecies of *T. rufum*. According to ITS1 sequences, the Chinese samples belonging to the *Rufum*-group scattered into three clades or subclades. The 10 samples of Subclade I exhibited exactly the same deletion, whereas named differently by a diversity of collectors. The four *T. liaotongense* specimens were included in this subclade. The ITS2 analysis gave the same results. We suggest that the six other samples described as *T. borchii*, *T. californicum* and *T. dryophilum* were misidentified and to rename them as *T. liaotongense*. Ascospore ornamentation of *T. liaotongense* (reticulate or alveolo-reticulate ascospores) does not exactly fit into the *Rufum*-group. Nevertheless, the three phylogenetic analysis (mitochondrial LrRNA, ITS1 and ITS2) indicate that *T. liatongense* belongs to the *Rufum*-group.

*Tuber huigondense* was clearly differentiated in both ITS1 and ITS2 analysis. According to Wang and He (2002), this species is closely related to *T. borchii* Vittad. and *T. maculatum* Vittad., two species belonging to the Puberulum-group. According to this study, *T. huigondense* belongs to the *Rufum*-group.

According to ITS1 analysis, Clade III comprised one exemplar of *T. taiyuanense* and two examplars of *T. texense*. According to ITS2 analysis, a similar clade comprised two examplars of *T. texense* and one examplar of *T. nitidum*. The specimen described as *T. taiyuanense* in this group is the neotype

collected by Wang (2001) (HMAS 75888). We suppose that all the samples of this subclade belong to the same species: *T. taiyuanense*. Nevertheless, before concluding, it would be necessary to compare the sequences of the Chinese samples described as *T. texense* to sequences of *T. texense* samples harvested in North America. Unfortunately, for the moment these sequences are not available.

The taxonomy of the *Puberulum*-group appeared much more complex. The European species *T. magnatum*, sometimes included in the *Puberulum*-group, was phylogenetically well differentiated from the other species of this group. We suggest that *T. magnatum* does not belong in the *Puberulum*-group. According to ITS1 and ITS2 analyses, the species belonging to the *Puberulum*-group were scattered within different clades or subclades. Moreover, it was not possible to separate well the Chinese specimens from the American and European ones.

However, the ITS2 analysis gave clear results. Clade I and IV were similar to the two clades obtained by Halàsz *et al*. (2005). According to Halàsz *et al*., one of their clades comprised three European species, *T. foetidum*, *T. maculatum* and *T. rapaeodorum*. According to our study, Clade I comprised the same species and *T. scruposum* and one American species, *T. whestonense*. Despite using part of the sequences obtained by Halàsz *et al*. (2005), we did not obtain any distinct separation between *T. maculatum* and *T. rapaeodorum* as obtained by these authors. This is probably due to the fact that some collectors misidentified several samples used in the present work, while Halàsz *et al*. (2005) used well identified specimens. According to ITS1 analysis, it is also obvious that the sample from North America described as *T. maculatum* was misidentified. Nevertheless, despite the clear results obtained by Halàsz *et al*. (2005), it still remains unclear whether *T. foetidum*, *T. maculatum*, *T. rapaeodorum* and *T. scruposum* are four different species, cryptic species, subspecies or varieties. As for the Chinese species belonging to the *Rufum*-group, only a study at the population level could answer this question.

According to ITS2 analysis, Clade IV comprised three Chinese and 12 European samples. Clade IV appeared to correspond well to the second clade obtained by Halàsz *et al*. (2005). If we refer to Halàsz *et al*. (2005) sequences, Subclade IV would correspond to *T. borchii* and Subclade V to *T. puberulum*. This would mean that several sequences from GenBank were obtained from misidentified samples. This would also mean that the two Chinese samples described as *T. asa* have to be renamed as a new Chinese species or subspecies (*Tuber* sp. 2, table 2) and that *T. liui* is very closely related to *T. puberulum*.

According to ITS2 analysis, two clades, Clade II and Clade III comprised only Chinese specimens. Clade II was formed with Chinese samples described

as *T. californicum*. Nevertheless, according to ITS1 sequences, these Chinese samples were different from the American sample described as *T. californicum*. Clade III was formed with Chinese samples described as *T. puberulum* (2), *T. rapaeodorum* (2) and *T. borchii* (2), whereas European samples of *T. rapaeodorum* and *T. borchii* formed the Subclade V (Fig. 6). All the Chinese specimens of Clade II and III have probably to be renamed as two new Chinese species or subspecies (*Tuber* sp. 3 = Clade II and *Tuber* sp. 4 = Clade III, table 2). *Tuber* sp. 3 also corresponded to Clade II of ITS1 analysis and *Tuber* sp. 4 to Clade IV of ITS1 analysis.

It seems obvious that the two European species *T. borchii* and *T. rapaeodorum* do not exist in China.

The confusions existing among the species of the *Puberulum*-group group in Europe and China and the numerous misidentifications, which seem to occur, including in the denomination of the Genbank sequences, indicate the presence of a complex of subspecies or varieties.

Nevertheless, our work supports the recent results of Halàsz *et al*. (2005). The *Puberulum*-group, from which *T. magnatum* has to be excluded, would comprise four subgroups: a subgroup comprising four European species *T. foetidum*, *T. maculatum*, *T. scruposum* and *T. rapaeodorum* and at least one American species *T. whestonense*, two subgroups comprising only Chinese samples which have to be reclassified as two new Chinese species and a fourth subgroup comprising two European species, *T. borchii* and *T. puberulum* and two Chinese samples described as *T. asa*. *Tuber asa*, a European species listed as *T. asa-foetida* Lesp. in Index Fungorum, is probably absent from China, although it was recorded by Wang (1988). This species has also to be renamed as a new Chinese species. *Tuber liui*, which belongs to this fourth subgroup, appears to be closely related to the European species *T. puberulum*.

## Conclusions

All the Chinese *Tuber* specimens harvested by different authors from 1985 in several locations of China and which did not belong to the *Melanosporum*-group scattered into two groups by the analysis of the mitochondrial LrRNA sequences: the *Rufum*-group and the *Puberulum*-group.

We suggest the existence of several Chinese species belonging to the *Rufum*-group: *T. huidongense*, *T. liaotongense*, *T. taiyuanense* and probably two other species corresponding to Clade III and IV of the ITS2 analysis. The samples of these two clades, described as *T. rufum*, *T. nitidum* or *T. texense*, were probably all misidentified. All the Chinese species belonging to the *Rufum*-group are genetically and morphologically close together, leading to

misidentifications. However, *T. liaotongense* Y. Wang has distinct morphological and microscopic characters. One question is remaining: are *T. huidongense*, *T. liaotongense* and *T. taiyuanense* different species or are they three subspecies? Only a study at the population level could answer this.

Despite the complexity of the taxonomic problems within the *Puberulum*-group, we can assume that at least three new species or subspecies belonging to this group are present in China. From this study, it seems also possible to conclude that the existence of the European species, *T. borchii*, *T. dryophilum*, *T. oligospermum* and *T. rapaeodorum*, or the American species *T. californicum* and *T. shearii*, is doubtful in China. Further studies including more sampling for morphological descriptions and molecular analysis are necessary to resolve the taxonomic placement of the Chinese species belonging to the *Puberulum-group*.

In a previous work, we suggested that all black truffles have a common ancestor, located in Europe or Asia (Wang *et al*., 2006a). Similarly, we could propose that the *Puberulum*-group, which displays a complexity of subspecies or varieties, have evolved in very few species during its migration from the original location. This could explain the taxonomical difficulties encountered to differentiate the species in the three continents.

Likewise, the species belonging to the *Rufum*-group seem to have migrated from their original location. Nevertheless, within the *Rufum*-group, according to the ITS1 analysis, it was possible to differentiate the specimens from the three continents, with for the moment the exception of *T. texense*, indicating a clearer allopatric speciation that within the *Puberulum*-group.

*Tuber magnatum*, which has to be excluded from the *Puberulum*-group, exists only in Europe. Rubini *et al*. (2005) found a late genetic and geographic differentiation of *T. magnatum* in Europe following the last postglacial expansion.

Extensive phylogenetic and taxonomic studies of the species belonging to *Rufum* and *Puberulum*-groups are needed in Asia, Europe, and North America to differentiate separate species, to better define appropriate groups or subgroups and phylogeographic structures.

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