Delimitation of *Taxus fuana* Nan Li & R.R. Mill (Taxaceae) based on morphological and molecular data

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The taxonomic status of *Taxus* in Pakistan was confused and uncertain due to opposing views about specimen identities. We used 27 morphological characters of 62 selected herbarium specimens of *T. baccata*, *T. wallichiana* and tentatively identified *T. fuana* to investigate the taxonomic status of *Taxus* in Pakistan by principal component analysis (PCA). Three discrete clusters representing the three species were identified in the PCA scatter plot. Character combinations allowed the reliable identification of specimens of *T. fuana*, *T. baccata* and *T. wallichiana*. Sequence data of nuclear ribosomal DNA ITS and the chloroplast *trnL-F* region were used to further delimitate *T. baccata*, *T. wallichiana* and *T. fuana*. Using maximum parsimony analysis three distinct clades were obtained representing the three species with strong bootstrap support. Based on both morphological and molecular sequence data, the *Taxus* species occurring in the western Himalaya including Pakistan was shown to be *T. fuana* and not *T. wallichiana* or *T. baccata*. The distinct species status of *T. fuana*, *T. baccata* and *T. wallichiana* was also well supported and found to be well correlated with their geographic distributions. The geographic ranges of the three species were updated based on the present study.

KEYWORDS: ITS, maximum parsimony analysis, molecular data, morphometric multivariate analysis, PCA, taxonomy, *Taxus baccata*, *Taxus fuana*, *Taxus wallichiana*, *trnL-F*

INTRODUCTION

Species of *Taxus* are mostly distributed in the temperate zone of the Northern Hemisphere but extending into the tropics and south of the Equator into Malaysia (Farjon, 2001). The nomenclature of *Taxus* is largely based on the geographic distribution of individual taxa. Partly because of the few reliable morphological characters available for diagnosing species and more morphological similarities than differences between species, the taxonomy of the genus is difficult and controversial (Fu & al., 1999; Li & al., 2001; Möller & al., 2007). Since Pilger (1903) treated all previously described *Taxus* species as seven geographical subspecies of a single species, many other authors have proposed alternative schemes. Ten species and two varieties have been generally adopted in the genus *Taxus* in recent work (Farjon, 2001). However, as the vegetative characters used for identification are quite variable and overlapping between species, the species concepts vary and the total number of distinct species of *Taxus* occurring in Asia is still not fully determined (Li & Fu, 1997).

The nomenclature and taxonomic status of *Taxus* in Pakistan is especially confused. Three names, viz. *Taxus wallichiana* Zucc., *T. baccata* L. and *T. fuana* Nan Li & R.R. Mill have been used for *Taxus* species occurring in the western Himalaya. *Taxus wallichiana* was widely and traditionally recognized by most taxonomists (Kitamura, 1960, 1964; Nasir & al., 1969; Nasir & Ali 1972, 1987; Farjon, 2001), while the name *T. baccata* has also been used for *Taxus* specimens from this region (Aitchison, 1979). *Taxus fuana* has recently been established as a new species based on specimens from southwest Xizang, China, with a distribution from southwest Xizang to Nepal and north India, extending westward to Pakistan (Li & Fu, 1997; Fu & al., 1999). This view was not widely accepted and Farjon (2001) restricted *T. fuana* to the southwest of Xizang, China, excluding specimens from the western Himalaya (including Pakistan). According to Farjon (2001) *T. wallichiana* occurs from Afghanistan (Hindu Kush) along the Himalaya to southwest China (thus including or overlapping with *T. fuana*), and *T. baccata* is native to Europe, N Africa (Atlas Mts.), the Caucasus and western Asia, from Turkey to North Iran, never extending into the western Himalaya.

Morphological data of herbarium specimens of *Taxus* from China and the Himalaya were recently analysed to identify morphological taxonomic entities occurring

there, including *T. fuana* (Möller & al., 2007). *Taxus fuana* proved to be a distinguishable species with a distribution from Central Nepal westwards into Kashmir, extending the concept of Farjon (2001). The species possibly reaches into Pakistan, however, no specimens of *Taxus* from Pakistan were included in the analysis by Möller & al. (2007) as were no specimens of *T. baccata*, the species occurring to the west of Pakistan. Thus, the delimitation of *T. fuana* is still uncertain.

Such controversial views about the taxonomic status of *Taxus* in Pakistan and *T. fuana* per se stimulated a proper methodical examination of this species here, combining morphometric and molecular data. This combination of techniques has proven to be very successful in untangling uncertain taxonomic groups (MacMaster & al., 2005). In the present study we wished to investigate the number and identity of *Taxus* species in Pakistan and the western Himalaya. We further wanted to determine the taxonomic and geographic boundaries between the *Taxus* species implicated in the problem, *T. baccata* and *T. wallichiana*, with respect to *T. fuana*. To address these objectives, 27 morphological characters from 62 specimens of the three species from localities covering most of their distribution ranges were gathered and analysed using principal component analyses (PCA). The selection of reliable distinguishing characters to discriminate these three species was also attempted. Additionally, molecular sequence data of nuclear ribosomal DNA (nrDNA) internal transcribed spacers (ITS) and chloroplast DNA (cpDNA) *trnL-F* intron-spacer regions were also used to investigate the distinctiveness of the three species.

MATERIALS AND METHODS

Plant materials. — Sixty-two herbarium specimens referable to the three species, held at the herbaria of KUN, E, ISL and LAH were used for morphological analysis in the present study, including 11 specimens of *Taxus baccata*, 19 specimens of *T. wallichiana*, and 32 of *T. fuana* (Appendix 1). Specimens from the western Himalaya were tentatively identified as *T. fuana*. Within the *T. fuana* specimens, one originated from southwest Xizang, China (the isotype of *T. fuana*), five from Nepal (including one paratype of *T. fuana*), six from northwest India (including one paratype of *T. fuana*) and twenty from Pakistan.

Of the *Taxus fuana* samples from Pakistan two specimens (PK1 and PK2) were selected for analysis in this study from 46 examined specimens of *Taxus* deposited in six herbaria of Pakistan (PMNH, RAW, ISL, PPFI, LAH, PES), which had been identified as *T. baccata* and *T. wallichiana* according to the labels on the specimen sheets (data not shown), because of the morphological similarity among these samples. During 2005 and 2006, 332 samples from 13 individual localities of *T. fuana* from Pakistan were collected, covering most of the distribution of *Taxus* in Pakistan. Nineteen out of the 332 individual specimens from the 13 individual localities of *T. fuana* were randomly selected for morphological data analysis.

To depict a detailed overview of the geographic distribution of the three species, the localities of all individuals were mapped onto a contour map using DIVA GIS v5.2.0.2 (Fig. 1), except for two specimens (one *Taxus fuana* and one *T. baccata*, Appendix 1) for which no detailed locality information was available other than country or province.

Considering their geographic distribution, twenty samples representing the three species were selected for molecular analysis (Appendix 1). Within these molecular samples, eight represented *T. fuana*, six *T. wallichiana* and six *T. baccata*.

Morphometric analysis. — Characters and character states as defined by Möller & al. (2007) were used in the present study on 62 specimens. A total of 27 characters, including 26 leaf characters and 1 bud scale character, were used in the morphometric analyses including those that play a key role in the discrimination of *Taxus* species. Of these characters, seven were continuous (characters 2 to 7 and 25) while the remaining were discrete. Taking into account variation on herbarium sheets between youngest and oldest growth of a particular year and the specimens per se, leaf observations were limited to the mid section of 2 to 3 year-old branches wherever possible to obtain standardized measurements (Möller & al., 2007). Measurements were scored by hand with ruler, protractor and high-power binocular microscope. Morphological variation was assessed using univariate statistics (mean, maximum and minimum values) and multivariate analysis. Coding of discrete character states was not attempted to reflect evolutionary progressions (Möller & al., 2007).

Morphometric data were subjected to a PCA clustering analysis (Sneath & Sokal, 1973; Krzanowski, 1990) using R-pack Le Progiciel R.4.d 10 (Casgrain & al., 2005) following Poulsen & Nordal (2005). PCA requires a normal distribution of the continuous character states which was tested prior to analysis: no character showed a significant skewness and no data transformation was necessary. R-pack was used to generate a correlation matrix (by dividing each value by the variable's standard deviation) of the 27 characters, because of the different nature of the characters (Casgrain & Legendre, 2001: 108). This eliminates the effect of the different measurement scales used and produces variables without physical dimensions (Casgrain & al., 2005).

Molecular analysis. — Genomic DNA was extracted from silica-gel dried leaves of all accessions using a modified CTAB method (Doyle & Doyle, 1987) by adding an ammonium acetate wash as described by Weising & al. (1995) for additional purification. The extracted total

DNA was diluted to a final concentration of 20–30 ng/μL for subsequent use.

The universal primers "c" and "f" (Taberlet & al., 1991) were used to PCR amplify the *trnL-F* intron-spacer region. The PCR reactions were carried out in 25 μL volumes. The reaction mix contained 0.625 U AmpliTaq DNA polymerase, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.3 μmol/L primer and 20–60 ng genomic DNA. PCR reactions were performed in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norfolk, Connecticut). The PCR condition consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C, and finished with an extension step of 10 min at 72°C. The nrDNA ITS region was amplified using the primers ITS4 and ITS-Leu, and the PCR condition followed Li & al. (2001). An additional primer 5.8S-GYM was used for sequencing (Liston & al., 1999). The PCR products were purified using a Sangon Purification kit according to the manufacturer's protocol for sequencing PCR reactions. Sequencing reactions were performed using PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). The sequencing products were run on an ABI 3700 automated sequencer (Perkin Elmer). The sequences were submitted to GenBank (ITS, Accession No. EF680236–EF680255; *trnL-F*, Accession No. EF680256–EF680275) (Appendix 1).

Contiguous DNA sequences were edited using SeqMan (DNASTAR package). Sequences were aligned using Clustal X (Thompson & al., 1997) and adjust manually where necessary. Maximum parsimony (MP) analysis was performed using PAUP*4.0b10 (Swofford, 2002) treating gaps as missing data, using heuristic search options with 1,000 random replications of stepwise taxon addition and TBR swapping and "MulTrees on" with "unlimited MaxTrees". All characters were weighted equally and unordered. Relative clade support for MP analysis was estimated by bootstrap analysis of 10,000 replicates of "heuristic searches" with "random sequence addition", "Multrees" and TBR branch-swapping on.

RESULTS

Morphometric analysis

PCA. — In the PCA, the first two axes of the 62 included specimens of *Taxus wallichiana*, *T. baccata* and *T. fuana* explained 33.33% and 17.94% of the total variance respectively (51.27% cumulative variance), while the third axis contained only 8.67% of the variance. Thus, the major proportion of variance was detected in the first two axes, and a graphical display of the first two axes would satisfy the "scree test" (Cattell, 1966; Nelson, 2005), and thus the data are displayed in 2D plots.

The 2D PCA scatter plot shows a distinct spatial structure among the specimens included in this study. *Taxus wallichiana*, *T. baccata* and the putatively identified *T. fuana* samples formed discrete species specific clusters respectively. In the PCA plot, all the specimens representing the putative *T. fuana* from the western Himalaya, including the type specimens (isotype ITC from southwest Xizang, China, and two paratypes, PTN from Tukucha, Nepal and PTI from Garhwal, India) formed a

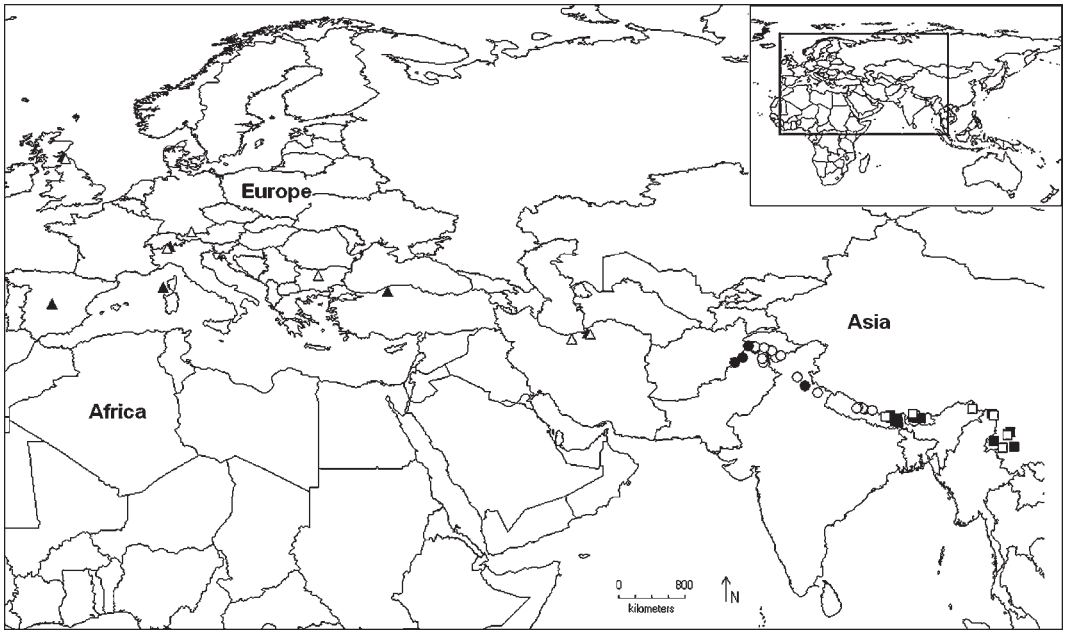


Fig. 1. Localities of 60 out of 62 herbarium specimens of *Taxus* used for morphometric and molecular sequence analysis. Circle = *T. fuana*; triangle = *T. baccata*; square = *T. wallichiana*. Solid symbols indicates samples for sequence analysis.

compact cluster without any overlap with specimens of *T. wallichiana* and *T. baccata* (Fig. 2). All the specimens of *T. wallichiana* from the eastern Himalaya formed another distinct group. Among the *T. baccata* samples, two specimens from Iran (IR1 and IR2) fell somewhat distant from the remaining *T. baccata* samples, which altogether formed a somewhat loose cluster. Although the two isolated Iranian samples were geographically close to the western Himalaya samples, they clustered distantly from the *T. fuana* and *T. wallichiana* clusters.

Descriptors. — Analysing the value of the individual characters (descriptors) in separating the three species, seven had very low descriptor values in the first two axes (characters 12, 15, 17, 18, 20, 22, 26) (Appendix 2, Fig. 3). Eleven characters (characters 2, 5–11, 14, 25, 27) were found to have strong effects on separating the specimens in the first axis separating mainly *Taxus wallichiana* from *T. fuana* (Appendix 2, Fig. 3). Ten characters, including characters 1, 3, 4, 7, 13, 16, 19, 21, 23 and 24, separated *T. wallichiana* and *T. fuana* from *T. baccata* in the second axis (Appendix 2, Fig. 3). These strongly discriminating characters can thus be used to differentiate *T. fuana*, *T. wallichiana* and *T. baccata*.

Most characters, even though they exert a strong influence on the clustering of the samples in the PCA, showed considerable overlaps of character states between the three taxa (Tables 1–2). Several characters showed some consistency of character states that allowed a discrimination of a pair of taxa against the third. Among these were characters 2, 6, 7, 10, 11, 14 and 27 predominantly separating *Taxus wallichiana* and *T. baccata* from

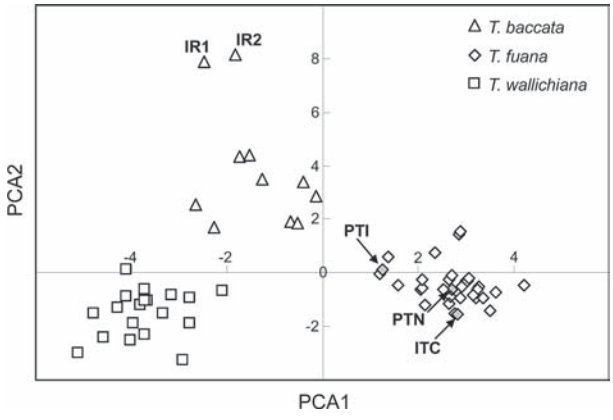


Fig. 2. 2D PCA scatter plot based on 27 vegetative characters of 62 *Taxus* herbarium specimens. ITC, isotype specimen of *T. fuana*; PTI and PTN, paratype specimens of *T. fuana* (filled grey symbols); IR1 and IR2 represent *T. baccata* samples from Iran.

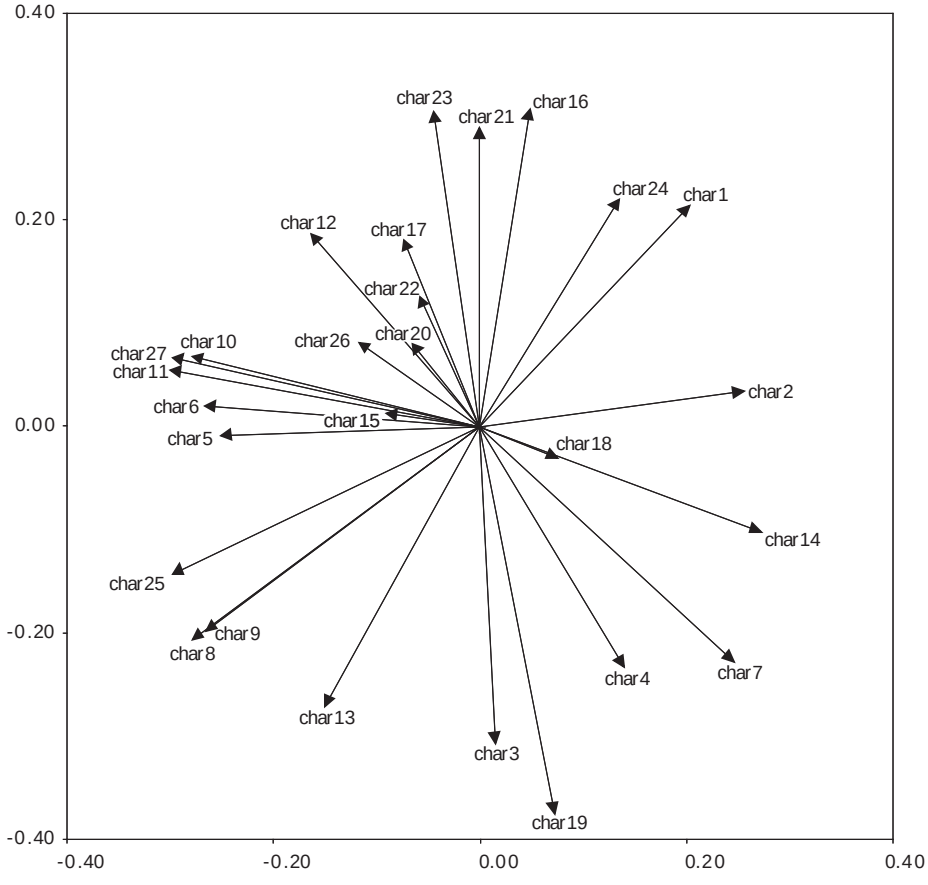


Fig. 3. 2D descriptor loading plot of the PCA based on 27 vegetative characters of 62 *Taxus* herbarium specimens. Character numbers refer to Tables 1 and 2.

T. fuana, characters 8 and 13 separating *T. baccata* and *T. fuana* from *T. wallichiana* and character 19 separating *T. wallichiana* and *fuana* from *T. baccata*. Character 9 (leaf margins) allowed a separation between *T. wallichiana* and *T. fuana*, while character 25 (number of stomatal bands) showed almost no overlap between all three taxa, *T. wallichiana* (11.5 to 15.5), *T. baccata* (8 to 9) and *T. fuana* (5.5 to 8) (Tables 1–2). Character 1 (leaf angle) showed a great degree of variation within, and overlap between the taxa, but was predominantly between 60°–90° for *T. wallichiana*, 40°–70° for *T. baccata* and similarly 40°–80° for *T. fuana*.

Species characteristics. — Focusing on reliable characters, as defined by their consistent discrimination between taxa, the taxa can be described as follows: *T. wallichiana* possesses a low leaf density (character 2: range 8–12, average 9.9), broad leaves (character 6: 2.2–3.2, 2.8), with medium length/width ratio (character 7: 8.2–14.1, 10.1), and high number of stomatal bands (character 25: 11.5–16.5, 14.1). The leaves are mainly sigmoid (character 8), with mostly tapering margins (lanceolate leaf) (character 9), asymmetrical base (character 10), and pectinate insertion (character 11), with indistinct mucro (character 14) and dense papillation on the midrib (character 19), and most bud scales are persistent (character 27).

Taxus baccata is characterized by a medium leaf density (character 2: 9–15, average 11.5), broad leaves (character 6: 2.1–3.2, 2.7), with a low length/width ratio (character 7: 6.6–11.3, 9.0), and medium number of stomatal bands (character 25: 8–9, 8.5). The leaves are straight or falcate (character 8), with mostly parallel margins (character 9), asymmetrical base (character 10), and mostly pectinate insertion (character 11), with indistinct mucro (character 14) and scattered papillation on the midrib (character 19), and most bud scales (character 27) are persistent.

Taxus fuana has a high leaf density (character 2: 12–17, average 13.3), narrow leaves (character 6: 2–2.4, 2.2), with a high length/width ratio (character 7: 11.4–15.9, 13.2), and low number of stomatal bands (character 25: 5.5–8, 6.9). The leaves are predominantly straight (character 8), with mostly parallel margins (character 9), mostly symmetrical base (character 10), and spiral insertion (character 11), with distinct mucro (character 14) and dense papillation on the midrib (character 19), and only some bud scales (character 27) are persistent.

Table 1. Summary of results for continuous characters used in the morphometric analysis of 62 *Taxus wallichiana*, *T. baccata* and *T. fuana* herbarium specimens.

No.	Character/taxon		*T. wallichiana*	*T. baccata*	*T. fuana*
2	Leaf density (per 2 cm branch)	Minimum	8	9	12
		Maximum	12	15	17
		Average	9.9	11.5	13.3
		SD	1.2	1.9	1.3
3	Min. leaf length (mm)	Minimum	15.0	11.0	17.0
		Maximum	26.0	24.0	28.0
		Average	21.7	17.5	21.4
		SD	3.4	3.8	3.0
4	Max. leaf length (mm)	Minimum	23.0	17.0	27.0
		Maximum	39.0	37.0	41.0
		Average	28.7	25.9	32.4
		SD	3.9	7.1	4.1
5	Min. leaf width (mm)	Minimum	1.8	1.6	1.7
		Maximum	3.0	2.8	2.1
		Average	2.3	2.1	1.8
		SD	0.3	0.3	0.1
6	Max. leaf width (mm)	Minimum	2.2	2.1	2.0
		Maximum	3.2	3.2	2.4
		Average	2.8	2.7	2.2
		SD	0.3	0.4	0.1
7	Ratio (average)	Minimum	8.2	6.6	11.4
		Maximum	14.1	11.3	15.9
		Average	10.1	9.0	13.2
		SD	1.6	1.9	1.3
25	Number of stomatal bands	Minimum	11.5	8.0	5.5
		Maximum	15.5	9.0	8.0
		Average	14.1	8.5	6.9
		SD	0.9	0.5	0.8

Sequence data analysis

Among the 20 accessions, the length of the entire ITS region (including ITS1, 5.8S and ITS2) was identical for *Taxus fuana* and *T. baccata* with 1,155 base pairs (bp) while that of *T. wallichiana* was 1,156 bp. The aligned ITS matrix contained 1,156 characters and included 25 variable sites (Appendix 3). Within the 8 samples of *T. fuana*, six ITS sequences were identical and two had autapomorphies, unique mutations (CH8 at position 192 and FU2 at position 541). Seven further nucleotide substitutions (at position 144, 380, 392, 537, 729, 1,082 and 1,137) were restricted to all *T. fuana* samples. While seven nucleotide changes were unique to the *T. baccata* samples (at position 35, 268, 489, 1,004, 1,079, 1,138 and 1,144), six nucleotide mutations were only present in *T. wallichiana* samples (at position 282, 354, 448, 529, 552 and 1,077) (Appendix 3). All ITS sequences of the six sampled *T. baccata* specimens were identical. Among the six *T. wallichiana* accessions,

Table 2. Summary of scores for discrete characters used in the morphometric analysis of 62 *Taxus wallichiana*, *T. baccata* and *T. fuana* herbarium specimens.

No.	Character	*T. wallichiana*	*T. baccata*	*T. fuana*
1	Leaf angle	60°–90°	40°–70°	40°–80°
8	Leaf curvature	Mainly sigmoid **3**/2	Mostly straight, some falcate **0**/1	Mostly straight, some falcate **0**/1
9	Margin taper	Tapering (2)/**3**	Mostly parallel **0**/2	Parallel, some not **0**/1
10	Leaf base	Asymmetrical **1**	Asymmetrical **1**	Symmetrical **0**/1
11	Leaf insertion	Pectinate **1**	Mostly pectinate 0/**1**	Spiral **0**/(1)
12	Apex symmetry	Asymmetrical or symmetrical 0/1	Asymmetrical **1**	Mostly symmetrical **0**/1
13	Apex shape	2* 1/**2**/(3)	0,1* 0/1	1* **1**
14	Mucro	Indistinct **0**/(1)	Indistinct **0**	Distinct 0/**1**
15	Texture	Medium (0)/**1**	Medium **1**	Medium 0/**1**
16	Leaf edges	Mostly revolute 0/**1**	Incurving or revolute 1/2	Revolute **1**
17	Midrib appearance (abaxial)	Elevated **0**/(1/2)	Sunken, some elevated 0/(1)/2	Elevated **0**(1)
18	Midrib appearance (adaxial)	Elevated **0**/(1)/2	Elevated **0**(1)	Elevated or level 0/1
19	Papillation on midrib	Dense **2**	Scattered **1**	Dense **2**
20	Midrib colour	Same, some different **0**/1	Same **0**(1)	Mainly same **0**/1
21	Midrib shininess	Same **0**	Mainly same **0**/1	Same **0**(1)
22	Margin colour	Same **0**(1)	Mainly same **0**/1	Same **0**(1)
23	Margin shininess	Same **0**	Mainly same **0**/1	Same **0**
24	Margin width up to midrib	Equal or narrower 0/1/(2)	Narrower, some equal **1**/2	Equal, some broader **1**/2
26	Stomata density	Mostly dense 0/**1**/(2)	Dense **1**	Dense or less dense 0/1
27	Bud scales persistence	Most persistent **2**	Most persistent (1)/**2**	Some persistent **1**/(2)

*Characters and character state definitions follow Möller & al. (2007). In bold = predominant states; in brackets = rare states.

the ITS sequences of five samples (ND14, BT7, XZ1, GS1, YD4) were identical while sample DL25 differed by four nucleotide substitutions from the other *T. wallichiana* samples; one shared nucleotide with *T. baccata* at position 190, two autapomorphies at position 226 and 654, and one polymorphic site at position 1,082 (Appendix 3). Pair wise distances among *T. fuana*, *T. baccata* and *T. wallichiana* samples ranged from 1.13% to 1.22% between *T. fuana* and *T. wallichiana*, 1.21% to 1.30% between *T. fuana* and *T. baccata* and 0.97% to 1.20% between *T. baccata* and *T. wallichiana*. The sequence divergence within species ranged from 0 to 0.26%.

The sequence length of the *trnL-F* intron-spacer region was 833 bp for all samples of *T. fuana*, while the *trnL-F* length of the *T. wallichiana* samples ranged from 841 to 881 bp and that of *T. baccata* from 842 to 843 bp. The aligned *trnL-F* matrix was 883 characters long, and included 18 variable sites with indels (insertions/deletions) included as individual evolutionary events (Appendix 4). Among the eight samples of *T. fuana*, seven had identical sequences. Only SW12 had two unique mutations (at positions 131 and 795). There were three mutations (at positions 326, 719 and 785) unique to *T. fuana*. Four nucleotide changes were shared among *T. baccata* samples (at positions 374, 535, 582 and 612), five nucleotide mutations were shared by *T. wallichiana* samples (at positions 357, 367, 737, 801 and 806). There were two variable indels (at positions 538 and 785) among the *T. wallichiana* samples. At position 538 a 20 bp duplication motif was absent in sample DL25, inserted once in samples XZ1 and YD4, and inserted twice in the remainder of the *T. wallichiana* samples. At position 785 a mononucleotide microsatellite region was missing in the *T. fuana* samples; it had invariably seven A's in all *T. wallichiana* samples but was variable among the *T. baccata* samples (nine A's in IR3, and eight A's in all remaining samples). Beside these indels, only one nucleotide site (XZ1, at position 477) differed among the *T. wallichiana* samples. Among *T. baccata*, the *trnL-F* sequence of five samples were identical and one (IR3) showed two mutations (at positions 37 and 608), apart from the variable indel (Appendix 4). The sequence divergence ranged from 0.84% to 1.20% between *T. fuana* and *T. wallichiana*, 0.84% to 1.08% between *T. fuana* and *T. baccata* and 1.19% to 1.31% between *T. baccata* and *T. wallichiana*. The sequence divergence within species was 0 to 0.24%.

A single phylogenetic tree was obtained in the MP analysis for each separate ITS and *trnL-F* data set with the indel gaps treated as missing. For simplicity the trees are depicted as unrooted cladograms (Figs. 4–5). The two data sets resulted in very similar tree topologies, and the selected accessions of the three species formed distinct species-specific clades with strong bootstrap support respectively. In the ITS tree, *T. baccata* and *T. fuana* were equidistant with seven nucleotide changes while the branch leading to *T. wallichiana* was shorter with five steps. A similar genetic relatedness was revealed by the *trnL-F* data, with *T. fuana* possessing the shortest branch with two steps, *T. baccata* with four and *T. wallichiana* the longest with five steps (Figs. 4–5).

DISCUSSION

Before *Taxus fuana* was described as a new species by Li & Mill (in Li & Fu, 1997), the taxonomic status of *Taxus* in the western Himalaya was confused, and the names *T. baccata* or *T. wallichiana* were used (e.g., Nasir & Ali 1972, 1987; Aitchison, 1979). *Taxus fuana* has not been widely recognized and adopted for the genus in this region since its description (Farjon, 2001). The limited detailed character analysis and taxonomic treatments of *Taxus* in this region, and especially in Pakistan (Nasir & Ali 1972), may be responsible for this unsatisfactory situation. However, our detailed morphological and molecular data analysis has greatly clarified the situation.

The fact that the included samples of the three species, *Taxus baccata*, *T. wallichiana* and the tentatively identified *T. fuana*, formed distinct groups in the PCA, with no overlap of the specimens among the three species clusters, strongly supported their morphological distinctness. All the tentatively identified *T. fuana* specimens from the western Himalaya clustered, together with the isotype and paratype specimens of *T. fuana*, supporting the morphological integrity of the *T. fuana* samples. Further support for the taxonomic distinctness of the three taxa came from the molecular analysis of the nrDNA ITS and chloroplast *trnL-F* sequence data. As the sequence variation within species was very low to zero (maximal 3 steps in ITS and 2 steps in *trnL-F*), and the divergence between the three species higher (maximal 7 steps in ITS, and 5 steps in *trnL-F*), the majority of divergence was distributed between the species. This indicated that *T. wallichiana*, *T. baccata* and *T. fuana* represent three independent genetic entities. The genetic distance between the three species was similar, about equidistant in both ITS and *trnL-F* sequences, suggesting that they diverged at the same or similar time (although the *T. fuana* branches were slightly shorter, suggesting a slightly younger age). The maximum inter-specific ITS divergence was 1.21% and was similarly low as previously indicated for the genus (Li & al., 2001). Within the species, the sequence divergence was very low or non-existent in both molecular markers. This may also suggest that the species are of very recent origin.

We have included samples covering the majority of the distribution range of the three species in our analysis and based on both morphological and molecular sequence data, the independent species status of all three taxa is strongly indicated. Thus, the species identity of *Taxus* in

Fig. 4. The single most parsimonious tree depicted as unrooted cladogram of three *Taxus* species based on nrDNA ITS sequence data. Small numbers along the branches indicate branch lengths, and numbers in bold italics indicate bootstrap values.

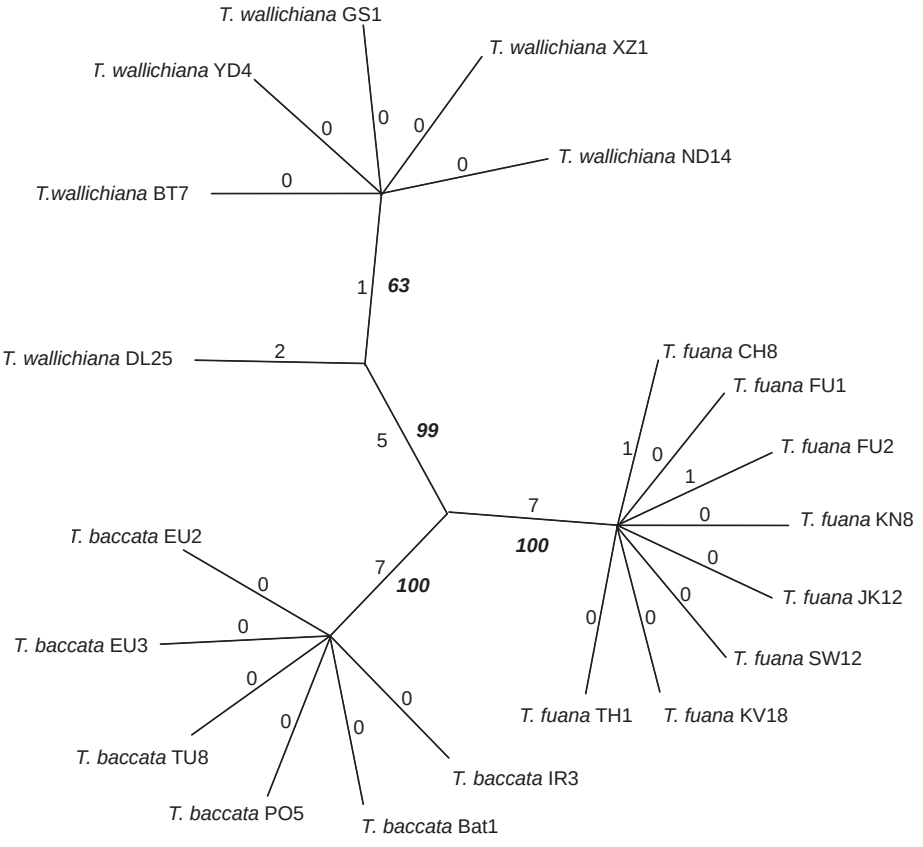
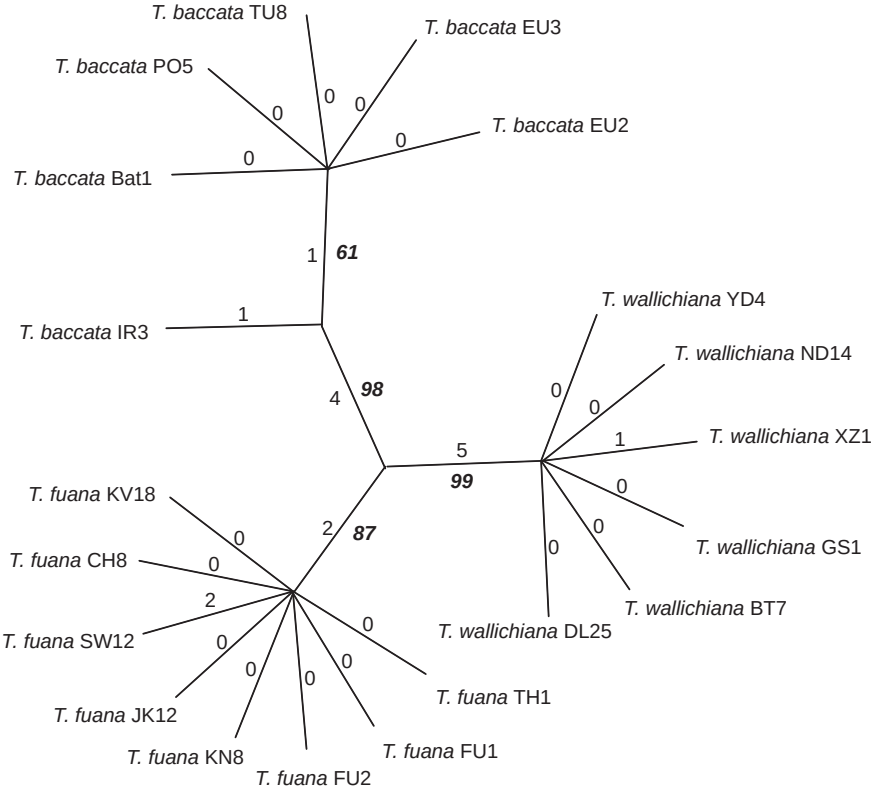


Fig. 5. The single most parsimonious tree depicted as unrooted cladogram of three *Taxus* species based on chloroplast DNA *trnL-F* sequence data. Small numbers along the branches indicate branch lengths, and numbers in bold italics indicate bootstrap values.



the western Himalaya, including Pakistan, can be confirmed as *Taxus fuana*, and neither *T. wallichiana* nor *T. baccata* are present in this area. Therefore *T. fuana* is endemic to the western Himalaya, and its distribution range extends from SW Xizang along the Himalaya westwards into Pakistan to the border with East Afghanistan. In Nepal *T. fuana* is restricted to the western part, while *T. wallichiana* occurs in the eastern parts through to southwestern China. In Central Nepal, *T. fuana* seems to be replaced by *T. wallichiana*. In our results no overlap between the two species was indicated. However, our sampling in this area was limited and whether an area of sympatry exists, as indicated by a previous morphometric study (Möller & al., 2007), requires further sampling there and data analyses. Although the included specimens of *T. baccata* included in our PCA analysis formed a loose cluster, the two specimens from Iran (IR1 and IR2) fell apart from the rest of the *T. baccata* specimens, they were morphologically most distant to *T. wallichiana* and *T. fuana* indicating a morphological discontinuity between Iran and Pakistan and that the eastern limit of *T. baccata* is in North Iran. The morphological characters of *T. baccata* were more variable and diverse which may be due to its wider distribution (Fig. 1) (Farjon, 2001).

No individual morphological character state was shared for all specimens belonging to a taxon, and only the combination of characters allowed a reliable identification of specimens. *Taxus wallichiana* can be distinguished from the other two taxa by a low leaf density, large leaf width, and high number of stomatal bands, sigmoid leaf curvature and tapering leaf margin. *Taxus fuana* is unique by the possession of a high leaf density, narrow leaves, high leaf length/width ratio, lowest number of stomatal bands, symmetrical leaf base, spiral leaf insertion, distinct mucro, and few persistent bud scales. *Taxus baccata* is intermediate between the two other taxa in most of the reliable characters, but may be distinguished from these by its short leaves and low leaf length/width ratio, and the scattered papillation on the midrib of the abaxial leaf surface.

CONCLUSION

The independent species status of *Taxus fuana* as well as the uniqueness of the other two species *T. wallichiana* and *T. baccata* were well supported by both our PCA on morphological data and by molecular sequence data. The taxonomic identity of *Taxus* in Pakistan (western Himalaya) was proven to be *T. fuana*, and neither *T. wallichiana* nor *T. baccata* occur there. *T. wallichiana* is restricted to the eastern Himalaya and southwestern China, while *T. baccata* is distributed in Europe, northern Africa and western Asia with its easternmost occurrence in North Iran, never extending into the western Himalaya. In Nepal *T. fuana* is replaced by *T. wallichiana* in the central region. Although our study utilized only a small proportion of herbarium specimens of the three species, our sampling strategy covered the entire distribution area of the species, and should thus reliably represent the taxon delimitation. Our study has resolved the taxonomic confusion surrounding the distribution and taxonomic status of *Taxus* in Pakistan. This study has demonstrated the power of combining morphometric and molecular sequence data to address complex taxonomic scenarios.

ACKNOWLEDGEMENTS

We are grateful to Robert R. Mill and two anonymous reviewers for their helpful comments on the manuscript. We also thank Rizwana Aleem Qureshi of Quaid-e-Azam University, Pakistan, John H. Wiersema, curator of GRIN, U.S.A., Sahibzada Adnan, Waseem and Kamran Shah from WWF Pakistan and Rashid Khan from Quaid-e-Azam University Islamabad, Pakistan for their help in collecting materials or allowing us to study specimens. This study was supported by the National Basic Research Program of China (973 Program, grant number: 2007CB411600), the Royal Society of London and the Chinese Academy of Sciences, and also supported by the National Natural Science Foundation of China (NSFC, grant number: 30700042) and the Natural Sciences Foundation of Yunnan (NSFY, grant number: 2005C0050M), the Ministry of Science and Technology of China (grant number: 2005DKA21006), and the Higher Education Commission of Pakistan. The Royal Botanic Garden Edinburgh is supported by the Rural and Environment Research and Analysis Directorate (RERAD).

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Appendix 1. Specimens of *T. fuana*, *T. wallichiana* and *T. baccata* used for morphometric and molecular analysis, with locality and voucher information.

Species (code); locality; latitude and longitude; voucher; herbarium; ITS GenBank no.; *trnL-F* GenBank no.

T. fuana (ITC); China: Xizang, Jilong (isotype); N 28°40′ E 85°15′; *Qing-Zang Exped., 7032*; KUN. *T. fuana*; Nepal: Marsyandi Khola, Bardang; N 28°33′ E 84°12′; *Ohba H. & al., 8311046*; E. *T. fuana*; Nepal: Chimgaon, Kali Gandaki valley; N 28°43′ E 83°40′; *Stainton & al., 7832*; E. *T. fuana* (PTN); Nepal: Lete, south of Tukucha (paratype); N 28°38′ E 83°37′; *Stainton & al., 734*; E. *T. fuana** (FU2); Nepal: Gandaki zone, Manang; N 28°32′ E 84°28′; *Suzuki M. & al., 9455291*; E; EF680237; EF680257. *T. fuana*; Nepal: Dhaulagiri Zone, Mustang Dist.; N 28°36′ E 83°35′; *Mikage M. & al., 9550282*; E. *T. fuana** (FU1); India: Great Himalayan National Park; N 31°00′ E 78°00′; *Howick C. & al., 1912*; E; EF680236; EF680256. *T. fuana* (PTI); India: Trijugi Narainet, Garhwal (paratype); N 30°15′ E 79°20′; *Ram K., 8894*; E. *T. fuana*; India: Bushreo Pass, Kulu, Punjab; N 31°58′ E77°06′; *Koelz W., 3119*; E. *T. fuana*; India: Kashmir, Kootihar valley; N 34°10′ E 74°45′; *Johnston H.H., 85*; E. *T. fuana*; India: Kashmir, Sind valley; N 34°18′ E 75°18′; *Polunin O., 56/622*; E. *T. fuana*; India: Bashahr, NW Himalaya (no details available); *Lace J.H., 301*; E. *T. fuana*; Pakistan: Punjab, Galehra Gali; N 33°54′ E 73°22′; *Amin, 25147*; KUN. *T. fuana*; Pakistan: Punjab, Galehra Gali; N 33°54′ E 73°22′; *Amin, 25170*; KUN. *T. fuana** (JK12); Pakistan: Punjab, Jhikka Gali; N 33°55′ E 73°40′; *Amin, 25127*; KUN; EF680239; F680259. *T. fuana*; Pakistan: Punjab, Jhikka Gali; N 33°55′ E 73°40′; *Amin, 25135*; KUN. *T. fuana*; Pakistan: N.W.F.P., Miranjani; N 34°05′ E 73°23′; *Amin, 25183*; KUN. *T. fuana*; Pakistan: N.W.F.P, Nathia Gali; N 34°03′ E 73°24′; *Amin, 25028*; KUN. *T. fuana*; Pakistan: N.W.F.P, Mukshpuri; N 34°04′ E 73°25′; *Amin, 25040*; KUN. *T. fuana*; Pakistan: N.W.F.P., Hazara; N 34°33′ E 73°23′; *Amin, 25216*; KUN. *T. fuana*; Pakistan: N.W.F.P., Swat; N 35°02′ E 72°35′; *Amin, 25229*; KUN. *T. fuana** (SW12); Pakistan: N.W.F.P., Swat; N 35°02′ E 73°35′; *Amin, 25237*; KUN; EF680240; EF680260. *T. fuana*; Pakistan: N.W.F.P., Swat; N 35°02′ E 72°35′; *Amin, 25244*; KUN. *T. fuana*; Pakistan: N.W.F.P., Palas Valley; N 35°16′ E 72°35′; *Amin, 25259*; KUN. *T. fuana** (CH8); Pakistan: N.W.F.P., Chitral; N 35°21′ E 71°48′; *Amin, 25278*; KUN; EF680243; EF680263. *T. fuana** (TH1); Pakistan: F.A.T.A., Tirah; N 34°05′ E 71°10′; *Amin, 25291*; KUN; EF680242; EF680262. *T. fuana** (KV18); Pakistan: F.A.T.A., Kurram Valley; N 33°36′ E 70°20′; *Amin, 25332*; KUN; F680241; EF680261. *T. fuana** (KN8); Pakistan: Azad Kashmir, Neelam Valley; N 34°50′ E 74°22′; *Amin, 25068*; KUN; EF680238; EF680258. *T. fuana*; Pakistan: Azad Kashmir, Neelam Valley; N 34°50′ E 74°22′; *Amin, 25082*; KUN. *T. fuana*; Pakistan: Azad Kashmir, Dwarian; N 34°49′ E 74°23′; *Amin, 25102*; KUN. *T. fuana* (PK1); Pakistan: N.W.F.P, Khaira Gali; N 34°03′ E 73°20′; *Saboor & Ayaz, 71902A*; ISL. *T. fuana* (PK2); Pakistan: N.W.F.P., Kaghan Valley; N 35°10′ E 73°28′; *Zahoor M., HN6056A*; LAH. ***T. wallichiana*;** China: Yunnan, Dali; N 25°59′ E 100°23′; *Handel-Mazzetti, 6408*; E. *T. wallichiana*; China: Yunnan, Gongshan; N 27°48′ E 98°34′; *Gaoligongshan Exped., 9392*; E & KUN. *T. wallichiana*; China: Yunnan, Salween-Kiukiang; N 28°00′ E 98°20′; *Yu T.T., 21036*; E. *T. wallichiana*; China: Yunnan,Yangbi, Malutang; N 25°46′ E 100°01′; *Sino-Amer. Bot. Exped., 388*; E. *T. wallichiana** (GS1); China: Yunnan, Gongshan; N 27°43′ E98°34′; *GLM-2301*; KUN & E; EF680253; EF680273. *T. wallichiana** (YD4); China: Yunnan, Yongde; N 24°15′ E 99°30′; *GLM-2348*; KUN & E; EF680252; EF680272. *T. wallichiana*; China: Yunnan, Jingdong; N 24°21′ E 100°43′; GM-*24234*; KUN & E. *T. wallichiana** (DL25); China: Yunnan, Yangbi; N 25°40′ E 100°02′; GM-*2425*; KUN & E; EF680250; EF680270. *T. wallichiana** (XZ1); China: Xizang, Lalung, Pachakshiri; N 28°30′ E 96°10′; *Ludlow F. & al., 3719*; E; F680254; EF680274. *T. wallichiana*; India: West Bengal, Darjeeling

Appendix 1. Continued.

distr.; N 27°00′ E 88°10′; *Long D.G. & al., 1119*; E. *T. wallichiana*; India: West Bengal, Singalila Range; N 27°28′ E 88°04′; *Vos F. & Corbett E.G., 148*; E. *T. wallichiana*; India: Manipur, Sriobinfurar; N 25°00′ E 98°30′; *George W., 6493*; E. *T. wallichiana*; Bhutan: Thimphu Distr.; N 27°29′ E 89°45′; *Grierson & Long, 4417*; E. *T. wallichiana*; Bhutan: Tunle La; N 27°27′ E 90°37′; *Ludlow F. & al., 18672*; E. *T. wallichiana** (BT7); Bhutan: Upper Mo Chu distr.; N 27°55′ E 89°46′; *Sinclair & Long, 5028*; E; EF680251; EF680271. *T. wallichiana*; Nepal: Dhankuta distr.; N 27°10′ E 87°50′; *Ohba H. & al., 9154042*; E. *T. wallichiana*; Nepal: Sankhuwa Sabha distr.; N 27°35′ E 87°15′; *Minaki M. & al., 9060068*; E. *T. wallichiana*; Nepal: Sankhuwa Sabha distr.; N 27°44′ E 87°18′; *Long D.G. & al., 724*; E. *T. wallichiana** (ND14); Nepal: Solukhumbu distr.; N 27°41′ E 86°43′; *NEP014*; E; EF680255; F680275. ***T. baccata**** (EU2); Scotland: Aberlady; N 56°00′ W 2°52′; *Aberlady-4*; KUN & E; EF680248; 680268. *T. baccata*; Scotland: Aberlady; N 56°00′ W 2°52′; *Aberlady-5*; KUN & E. *T. baccata** (EU3); Spain: de Canencia; N 40°54′ W 3°73′; *AA-442-91*; KUN & E; EF680247; EF680267. *T. baccata*; Scotland (no details available); *RBGE 19699295*; KUN & E. *T. baccata* (IR1); Iran: Mazandaran: South of Babol; N 36°10′ E 52°38′; *Zarrei M., 476*; KUN & E. *T. baccata* (IR2); Iran: Gorgan; N 36°41′ E 54°35′; *Zarrei M., 477*; KUN & E. *T. baccata** (IR3); Iran: Gorgan; N 36°40′ E 54°34′; *Zarrei M., 478*; KUN & E; EF680249; EF680269. *T. baccata*; Germany: Weilh.-Sch; N 47°53′ E 11°12′; *546-89*; KUN & E. *T. baccata*; Bulgaria, Nila; N 43°00′ E 23°20′; *AA-370-35*; KUN & E. *T. baccata** (Bat1); Italy: Varese,Campagnano; N 46°03′ E 8°44′; *Fior S., 1*; KUN & E; EF680244; EF680264. *T. baccata*; Italy: Varese,Campagnano; N 46°03′ E 8°44′; *Fior S., 11*; KUN & E. *T. baccata**° (PO5); Portugal: Moncao rivulet; N 41°48′ E 8°08′; *Moncao-5*; E; EF680245; EF680265. *T. baccata**° (TU8); Turkey: Kumluca, Findicak Mevkii; N 41°22′ E 32°34′; *AAD 11812-8*; E; EF680246; EF680266.

*Samples used for sequencing of ITS and the *trnL-F* region.
°Samples used only for molecular analysis.

Appendix 2. Character ranks determined by product between character loading values and the proportion of variance (axis variances: 1 = 33.33, 2 = 17.94%).

Character no.	Axis 1	x%var	*rank*	Axis 2	x%var	*rank*	sum (%var)	*rank*
8	0.2771	0.0924	5	0.2059	0.0369	11	0.1293	1
25	0.2970	0.0990	2	0.1427	0.0256	15	0.1246	2
9	0.2640	0.0880	8	0.1971	0.0354	12	0.1234	3
7	0.2463	0.0821	11	0.2275	0.0408	8	0.1229	4
27	0.2966	0.0989	3	0.0669	0.0120	21	0.1109	5
11	0.2994	0.0998	1	0.0544	0.0098	22	0.1095	6
14	0.2721	0.0907	6	0.1013	0.0182	17	0.1089	7
1	0.2038	0.0679	12	0.2141	0.0384	10	0.1063	8
10	0.2779	0.0926	4	0.0684	0.0123	20	0.1049	9
13	0.1494	0.0498	14	0.2698	0.0484	6	0.0982	10
6	0.2653	0.0884	7	0.0208	0.0037	25	0.0922	11
19	0.0730	0.0243	20	0.3745	0.0672	1	0.0915	12
2	0.2556	0.0852	9	0.0350	0.0063	23	0.0915	13
4	0.1402	0.0467	15	0.2317	0.0416	7	0.0883	14
12	0.1626	0.0542	13	0.1870	0.0335	13	0.0877	15
5	0.2495	0.0832	10	0.0088	0.0016	27	0.0847	16
24	0.1349	0.0450	16	0.2195	0.0394	9	0.0843	17
16	0.0490	0.0163	24	0.3070	0.0551	2	0.0714	18
23	0.0439	0.0146	25	0.3045	0.0546	4	0.0693	19
3	0.0159	0.0053	26	0.3060	0.0549	3	0.0602	20
17	0.0725	0.0242	21	0.1810	0.0325	14	0.0566	21
26	0.1156	0.0385	17	0.0808	0.0145	18	0.0530	22
21	0.0006	0.0002	27	0.2890	0.0518	5	0.0520	23
22	0.0576	0.0192	23	0.1260	0.0226	16	0.0418	24
20	0.0648	0.0216	22	0.0802	0.0144	19	0.0360	25
15	0.0891	0.0297	18	0.0131	0.0024	26	0.0320	26
18	0.0748	0.0249	19	0.0303	0.0054	24	0.0304	27

Appendix 3. Variable nucleotide positions of the nrDNA ITS region in the 20 accessions of the three species, *Taxus wallichiana*, *T. baccata* and *T. fuana*. "–" = alignment gap, a dot (.) indicates that the character states are the same as the sequence of *T. baccata* Bat1.

		Position																								
Species	Code	35	144	190	192	226	268	282	354	380	392	448	489	529	537	541	552	654	729	1004	1077	1079	1082	1137	1138	1144
T. baccata	Bat1	G	A	C	G	T	T	–	C	T	T	C	C	A	C	T	T	T	C	C	T	G	T	G	C	G
	PO5	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	TU8	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	EU3	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	EU2	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	IR3	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
T. fuana	Fu1	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	Fu2	C	T	.	.	.	G	–	.	C	C	.	T	.	A	G	.	.	A	T	.	A	G	T	T	C
	KN8	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	JK12	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	SW12	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	CH8	C	T	.	T	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	KV18	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
	TH1	C	T	.	.	.	G	–	.	C	C	.	T	.	A	.	.	.	A	T	.	A	G	T	T	C
T. wallichiana	ND14	C	.	G	.	.	G	T	A	.	.	T	T	C	.	.	C	.	.	T	C	A	.	.	T	C
	BT7	C	.	G	.	.	G	T	A	.	.	T	T	C	.	.	C	.	.	T	C	A	.	.	T	C
	XZ1	C	.	G	.	.	G	T	A	.	.	T	T	C	.	.	C	.	.	T	C	A	.	.	T	C
	GS1	C	.	G	.	.	G	T	A	.	.	T	T	C	.	.	C	.	.	T	C	A	.	.	T	C
	YD4	C	.	G	.	.	G	T	A	.	.	T	T	C	.	.	C	.	.	T	C	A	.	.	T	C
	DL25	C	.	.	.	C	G	T	A	.	.	T	T	C	.	.	C	C	.	T	C	A	K	.	T	C

Appendix 4. Variable nucleotide positions of the chloroplast *trn*L-F region in the 20 accessions of the three species, *Taxus wallichiana*, *T. baccata* and *T. fuana*. A dot (.) indicates that the character states are the same as the sequence of *T. baccata* Bat1.

		Position																		
Species	Code	037	131	326	357	367	374	477	535	538	582	608	612	719	737	785	795	801	806
T. baccata	Bat1	T	C	T	T	C	T	G	C	0	A	T	T	A	G	1[d]	G	C	G
	PO5	.	.	.	.	.	.	.	.	0	.	.	.	.	.	1[d]	.	.	.
	TU8	.	.	.	.	.	.	.	.	0	.	.	.	.	.	1[d]	.	.	.
	EU3	.	.	.	.	.	.	.	.	0	.	.	.	.	.	1[d]	.	.	.
	EU2	.	.	.	.	.	.	.	.	0	.	.	.	.	.	1[d]	.	.	.
	IR3	C	.	.	.	.	.	.	.	0	.	G	.	.	.	1[c]	.	.	.
T. fuana	Fu1	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	Fu2	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	KN8	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	JK12	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	SW12	.	A	G	.	.	G	.	A	0	C	G	A	C	.	0	T	.	.
	CH8	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	KV18	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
	TH1	.	.	G	.	.	G	.	A	0	C	G	A	C	.	0	.	.	.
T. wallichiana	ND14	.	.	.	G	T	G	.	A	1[b]	C	G	A	.	T	1[e]	.	A	T
	BT7	.	.	.	G	T	G	.	A	1[b]	C	G	A	.	T	1[e]	.	A	T
	XZ1	.	.	.	G	T	G	T	A	1[a]	C	G	A	.	T	1[e]	.	A	T
	GS1	.	.	.	G	T	G	.	A	1[b]	C	G	A	.	T	1[e]	.	A	T
	YD4	.	.	.	G	T	G	.	A	1[a]	C	G	A	.	T	1[e]	.	A	T
	DL25	.	.	.	G	T	G	.	A	0	C	G	A	.	T	1[e]	.	A	T

0 = absent; 1[a] = ATTAAGAAAGATCAAATATT; 1[b] = ATTAAGAAAGATCAAATATTATTAAGAAAGATCAAATATT; 1[c] = AAAAAAAAAG; 1[d] = AAAAAAAAG; 1[e] = AAAAAAAG

Note: Since this paper went to press, a revision of *Taxus* has been published by Spjut [Spjut, R.W. 2007. Taxonomy and nomenclature of *Taxus* (Taxaceae). *J. Bot. Res. Inst. Texas* 1: 203–289] that recognizes 21 named species and 3 other unnamed species, 12 of them occurring in China. The name *Taxus fuana* Nan Li & R.R. Mill is replaced by the earlier but long overlooked name, *T. contorta* Griffith, published in 1854.