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Research Article

DNA barcoding of East Asian *Amentotaxus* (Taxaceae): Potential new species and implications for conservation

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Abstract DNA barcoding is a useful tool for species identification using standardized genomic DNA fragments. The genus *Amentotaxus*, consisting of five or six species, is confined to South China, Northeast India, Laos, and Vietnam. All species have been assessed as globally or nationally threatened. However, there is uncertainty about the number of species involved, especially in the border areas of southern China, northern Vietnam, and Laos. We selected five DNA barcodes (*rbcL*, *matK*, *trnH-psbA*, *trnL-F*, and internal transcribed spacer (ITS)) to evaluate their discrimination ability in this genus, and to investigate the current taxonomy of *Amentotaxus*. Our results indicate that all the selected barcoding regions showed a high level of universality for PCR and sequencing. When six species are recognized, the nuclear ribosomal DNA region ITS and the chloroplast DNA region *trnL-F* used on their own provided the highest identification success (60%). Two barcode combinations that included either ITS or *trnL-F* had the same species discrimination ability. Combinations using additional barcodes did not improve the species identification success. When only five species are recognized, with *A. hatuyenensis* T. H. Nguyen treated as a synonym of *A. yunnanensis* H. L. Li, the discrimination rate rises to 100%. Our results also indicate that recent collections from Yunnan province, China, Lao Cai province, Vietnam, and Laos may represent a potential new species. The findings from this study will be very useful for the formulation of appropriate conservation strategies for threatened *Amentotaxus* species in national and trans-boundary regions.

Key words: *Amentotaxus*, biodiversity conservation, DNA barcoding, new species discovery, species discrimination.

DNA barcoding is a technique for species discrimination and identification using short and standard DNA regions (Hebert et al., 2003; Hollingsworth, 2011). It has been successfully used in a variety of biological applications (Joly et al., 2014; Kress et al., 2015), such as the discovery of new or cryptic species (Hebert et al., 2004; Newmaster et al., 2008; Liu et al., 2011), in biodiversity inventory and assessments (Valentini et al., 2009; Ji et al., 2013), the detection of invasive species (Steinke et al., 2012), the identification of medicinal plants in mixtures (Kool et al., 2012; Newmaster et al., 2013) and in ecological investigations (Kress et al., 2009; Costion et al., 2011; Erickson et al., 2014).

As no single barcode performs as well in plants as *COI* does in animals, several combinations of candidate DNA regions have been proposed (e.g., Chase et al., 2007; Kress & Erickson, 2007; CBOL Plant Working Group, 2009). Based on a comprehensive evaluation, the combination of *matK* + *rbcL* was recommended by the Consortium for the Barcode of Life (CBOL) as a core barcode for land plants (CBOL Plant Working Group, 2009). However, this combination may perform poorly in many complex groups with low species discrimination (e.g. *Ficus*: Li et al., 2012; *Salix*: Percy et al., 2014; *Rhododendron*: Yan et al., 2015). The internal transcribed spacer (ITS) of nuclear ribosomal DNA and the chloroplast *psbA-trnH* region were additionally recommended as supplementary barcodes (Yao et al., 2010; Hollingsworth et al., 2011). Based on a large-scale sampling evaluation, ITS was proposed to be incorporated into the core barcode for seed plants (China Plant BOL Group, 2011). Several studies proposed to include chloroplast DNA *trnL-F* as a DNA barcode owing to its high sequence variation and high species resolution (Liu et al., 2011; Chen et al., 2013).

Amentotaxus Pilg. (Taxaceae), a small genus of gymnosperms, is confined to southern China (including Taiwan),

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small areas of the eastern Himalayas, and parts of Indo-China (Farjon, 2010). Its fossil record dates back to at least the Paleocene but most are from the Miocene from Europe and western North America. Throughout its history, the morphology of *Amentotaxus* appears to have been relatively stable, with most records referring to a single species, *A. gladifolia* (Ludwig) Ferguson, Jähnichen & Alvin. Marked geological changes such as the uplift of the Tibetan plateau combined with changing climates and repeated glacial cycles since the start of the Pliocene may have led to an increase in speciation in *Amentotaxus* with currently up to six species recognized (Ferguson, 1992; Farjon, 2010).

All species are dioecious, evergreen trees or large shrubs that occur in the understory of moist submontane and montane semideciduous or evergreen forests. *Amentotaxus argotaenia* (Hance) Pilg. is the most widespread species, recorded from most provinces of southern China as well as Vietnam and Laos (Fu et al., 1999; Nguyen et al., 2004; Farjon, 2010; Averyanov et al., 2014). In Taiwan, *A. formosana* H. L. Li is restricted to a few localities in the south, whereas *A. yunnanensis* H. L. Li is generally confined to the evergreen forests in the karst formations of southeast Yunnan and southwest Guizhou, adjacent to Vietnam and Laos (Fu et al., 1999; Farjon, 2010; Averyanov et al., 2014). In 1996, *A. hatuyenensis* T. H. Nguyen was described from a single locality in what is now Hagiang Province in northern Vietnam (Nguyen & Vidal, 1996). The remaining two species have markedly disjunct distributions in southern Vietnam (*A. poilanei* (De Ferré & Rouane) D. K. Ferguson; Nguyen et al., 2004) and northeastern India (*A. assamica* D. K. Ferguson; Ferguson, 1985; Gajurel et al., 2006; Das et al., 2008). All species of *Amentotaxus* are currently assessed as threatened at either the national or global level, primarily as a result of deforestation and habitat conversions (Wang & Xie, 2004; IUCN, 2016).

Historically, the identification of *Amentotaxus* species has mainly been based on the geographical origin of the specimen and a few morphological characters, relating to leaf structure and the color and width of stomatal bands (Li, 1952; Ferguson, 1992; Nguyen & Vidal, 1996; Fu et al., 1999). The geographic location is a good indicator for the disjunctly occurring *A. assamica* and *A. poilanei* as well as for the insular species *A. formosana*, despite a disputed historical record of *A. argotaenia* from Taiwan (Farjon, 2010). In southern China *A. argotaenia* and *A. yunnanensis* have had an allopatric, edaphically distinct distribution prior to 1990. Since then, the description of new taxa and the discovery of numerous, occasionally intermingled populations of *A. argotaenia* and *A. yunnanensis* in Vietnam and Laos has decreased the utility of geographic origin as a guide to their identification (Nguyen et al., 2004; Thomas et al., 2007; Averyanov et al., 2014). In addition, several populations whose morphology differs from all the extant species have recently been discovered in Hekou (China), Lao Cai (Vietnam), and Xiang Khouang (Laos) by the first author of this paper.

In *Amentotaxus*, female structures are broadly similar between species, and although there are differences in numbers of pollen sacs and the length of the male racemes, male organs are transitory and rarely collected. Most identifications and even new taxa are based on vegetative characters such as color and width of the stomatal bands. For example, *A. hatuyenensis* was described as new species based on a single collection that showed differences in the coloring of the stomatal bands compared to *A. yunnanensis* and *A. argotaenia*. However, such vegetative characters are quite variable and may not always be reliable as diagnostic characters (Fu et al., 1999; Farjon, 2010). A recent study, focusing on collections made from the type locality of *A. hatuyenensis* and all surrounding localities where either that species or *A. yunnanensis* has been recorded, failed to find any plants in the field with these distinctive stomatal bands. The study further found that stomatal color varied according to the age of the leaf and the method of drying. In addition, preliminary analysis using ITS1 failed to discriminate between the two taxa (Phan et al., 2014).

Correct species identification is one of the keys to improved species management and conservation (Trias-Blasi & Vorontsova, 2015). Where morphology may be confusing, DNA barcoding can provide an alternative method for rapidly and accurately identifying species. In the present study, we selected five candidate DNA barcodes (*rbcL*, *matK*, *trnH-psbA*, *trnL-F*, and ITS). We aimed to: (i) evaluate the species discrimination ability of those five barcodes on their own and in combinations for *Amentotaxus*; (ii) select the most suitable DNA barcodes for distinguishing the species; and (iii) determine whether the recent collections from Hekou, Lao Cai, and Xiang Khoang differ from other *Amentotaxus* species and represents a new species. The findings from this study will be valuable to support the developing conservation plans and management of threatened species of *Amentotaxus*.

Material and Methods

Taxon sampling

We used Farjon (2010) as an initial taxonomic framework for this study; this work accepts six species as detailed in the introduction. A total of 23 individuals of four *Amentotaxus* species were collected from China, Vietnam, and Laos, including five individuals of a putative new species. Samples of *A. poilanei* and *A. assamica* were not available. To cover the maximum diversity within the species, 3–7 individuals per species were sampled covering the majority of their respective distribution ranges. Young, healthy leaves were collected from the wild populations and dried immediately in silica gel for DNA extraction. Vouchers specimens of sampled species were collected and deposited in the Herbaria of Kunming Institute of Botany (KUN), Chinese Academy of Sciences (Kunming, China), or in the Vietnam National Museum of Nature (Hanoi, Vietnam) and at the Herbarium of Vietnam National University – University of Science (HNU) (Hanoi, Vietnam). Detailed information about the samples is provided in Table S1.

Polymerase chain reaction and sequencing

Genomic DNA was isolated from silica gel-dried leaves using a modified CTAB protocol (Doyle & Doyle, 1987). The DNA was diluted with TE buffer (10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L ethylenediaminetetraacetic acid) to a final concentration of 30–60 ng/μL for polymerase chain reaction (PCR) amplification. Five plant DNA barcodes, *rbcL*, *matK*, *psbA-trnH*, *trnL-F*, and ITS, were amplified separately in 25-μL reactions with

30–60 ng template DNA, 0.625 U AmpliTaq DNA polymerase, and final concentrations of 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, and 0.3 μmol/L each primer. The PCR amplification was carried out on a GeneAmp PCR System 9700 thermal cycler (PerkinElmer, Foster City, CA, USA). Primers for PCR and sequencing, as well as PCR cycling conditions, followed Liu et al. (2011), except for *matK* which followed the proposed protocol for Gymnosperms of Li et al. (2011). Polymerase chain reaction products were purified using ExoSAP-IT (GE Healthcare, Cleveland, OH, USA). Purified PCR products were sequenced in both directions with the same primers used for PCR and then analyzed on an ABI 3730xl DNA Sequencer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. As the whole sequence of ITS could not be attained by direct sequencing for samples Am32 and Am34 due to the presence of some "messy" sequence regions in direct sequencing, the ITS region was cloned for these two samples. All newly generated sequences were deposited in GenBank (Table S1).

Data analysis

The original sequence trace files for each region were assembled and edited using Sequencher 5.5 (Gene Codes Co., Ann Arbor, MI, USA). The edited sequences were then aligned in ClustalX 2.0 (Larkin et al., 2007) with a final manual adjustment in BioEdit version 7 (Hall, 1999). All variable sites were rechecked on the original trace files for final confirmation. As low interspecific variation of *rbcL* was detected in 13 individuals of the four species of *Amentotaxus* (Table 1), PCR and sequencing for *rbcL* of the remaining 10 samples was not carried out. Therefore, *rbcL* was excluded from further analysis of species discrimination using combinations of DNA barcodes.

Genetic divergences for the five markers were calculated using MEGA 6.0 (Tamura et al., 2013) according to the P-distance model. The tree-based method was used to evaluate species discrimination success for the five single markers and all combinations of the four barcodes except for *rbcL*. Neighbor-joining trees were constructed using the software MEGA 6.0 with pairwise deletion and the P-distance model according to the published protocols for species-level discrimination in closely related groups (Srivathsan & Meier, 2012). Node support was assessed by 1000 bootstrap replicates in MEGA 6.0. Species discrimination was considered successful only when all the conspecific individuals formed a single clade (Yan et al., 2015) with a bootstrap value above 50%. In addition, the functions of the "best match" and the "best close match" based on similarity in TaxonDNA were also used to test the individual-level discrimination rates for each single marker and all combinations under the P-distance model (Meyer & Paulay, 2005). To assess the barcoding gap, the relative distribution of pairwise genetic distances was calculated using TaxonDNA (Meier et al., 2006) under the P-distance model.

Results

Barcode universality and sequence characteristics

Information for universality, sequence length, number of indels and variable sites, and intra- and interspecific divergence for the five barcodes are summarized in Table 2. All five barcodes had a 100% success rate for PCR and

Table 1 Success of sequence recoverability in *Amentotaxus* among five barcodes

Barcode	No. of samples	PCR success	Sequencing success	Sequence length	Aligned length	G+C ratio, %	No. variable sites, %	No. informative sites, %	No. indels (length, bp)	Intraspecific distances (mean), %	Interspecific distances (mean), %
rbcL	13	13	13	696	696	43	1	1	0	0	0–0.14 (0.07)
matK	23	23	23	786	786	33.2	2	2	0	0	0–0.25 (0.13)
trnH-psbA	23	23	23	549–563	565	36.7	8	5	2 (1–3, 13)	0–0.12 (0.05)	0–0.91 (0.45)
trnL-F	23	23	23	924–926	926	32.7	12	12	2 (1)	0–0.09 (0.02)	0–1.06 (0.61)
ITS	23	23	23	1049–1052	1052	63.7	25	23	3 (1)	0–0.06 (0.01)	0–1.05 (0.60)

ITS, internal transcribed sequence.

sequencing in the *Amentotaxus* species included in this study (Table 2). The five clone sequences of Am32 could be divided into two different ITS types, with one type (C2) being identical to the ITS sequences of *A. yunnanensis*, and the other type (C1) being genetically close to that of the putative new species with one nucleotide site variation. All 10 cloned sequences of Am34 had identical ITS sequences.

Due to the presence of indels, the sequences varied in length in *trnH-psbA*, *trnL-F*, and ITS; *trnH-psbA* had the biggest length variation (549–563 bp) stemming from two indels, and the highest mean intraspecific genetic distance (0.05%), followed by *trnL-F* (0.02%) and ITS (0.01%) with only one indel each. No length variation and zero intraspecific genetic distance was present in *rbcL* and *matK*. Among the five barcodes, *trnL-F* showed the highest mean interspecific divergence (0.61%), followed by ITS (0.60%), *trnH-psbA* (0.45%), and *matK* (0.13%), while *rbcL* had the lowest variation (0.07%) (Table 1).

DNA barcoding gap assessment

An ideal barcode should possess a barcoding gap with higher interspecific variation than intraspecific variation to distinguish species. In this study, we did not find any distinct barcoding gap in the distributions of divergences for any single marker (Fig. 1). Among the five single barcodes, *trnH-psbA* showed the highest variation in interspecific divergence and range of intraspecific distances (Table 1).

Species discrimination

Tree-based, "best match", and "best close match" methods were applied to evaluate the discriminatory power of the five barcodes in *Amentotaxus*. For the tree-based method for the four accepted and one putative new species, the highest species discrimination success was obtained by ITS and *trnL-F* (both 60%) among the five single barcodes, whereas the other three plastid DNA barcodes had only a 20% species identification rate (Table 2; Figs. 2, 3). When combining two to four barcodes, the highest discrimination rate (60%) was achieved by all combinations, except *matK* + *trnH-psbA* which showed the same discriminatory ability (20%) as each single barcode (Table 2). The support values of all species clades based on the four-barcode combinations showed distinct higher values than the single barcodes (Figs. 2–4). However, when an alternative taxonomy is used, with *A. hatuyenensis* treated as a synonym of *A. yunnanensis*, the species discrimination success was improved, with all species including the putative new species sampled here being successfully distinguished by ITS and *trnL-F* on their own, or in any combination, except for *matK* + *trnH-psbA* (Table 2).

Both the "best match" and "best close match" methods provided the same species discrimination success (Table 2). For the single barcode, ITS had the highest species identification success rate at the individual level (52.0%), followed by *trnL-F* (29.2%), while *matK* had the lowest (12.5%). When two barcodes are used, combinations of *trnH-psbA* + ITS, *matK* + ITS, and *trnL-F* + ITS showed the highest species discrimination success with 56.5%. This is the same as observed for three-barcode and four-barcode combinations (Fig. 4), with the exception of *matK* + *trnH-psbA* + *trnL-F* which resulted in a 29.2% species resolution. The combination of *matK* + *trnH-psbA* had the lowest success in species identification with 12.5% amongst all two-barcode combinations (Table 2).

Table 2 Species resolution of *Amentotaxus* based on methods of genetic distance with "best match", "best close match", and neighbor-joining (NJ) tree-based methods of five barcodes and their combinations. Based on the taxonomy of Farjon (2010), four accepted species and a putative new species were included in the present study

	Best match, *n* (%)			Best close match, *n* (%)			Threshold (%)	NJ trees, %	NJ trees, †%
	Correct	Ambiguous	Incorrect	Correct	Ambiguous	Incorrect			
rbcL	3 (21.4)	11 (78.6)	0 (0.0)	3 (21.4)	11 (78.6)	0 (0.0)	0	20	25
matK	3 (12.5)	21 (87.5)	0 (0.0)	3 (12.5)	21 (87.5)	0 (0.0)	0	20	50
trnH-psbA	5 (20.8)	19 (79.2)	0 (0.0)	5 (20.8)	18 (75.0)	0 (0.0)	0.18	20	50
trnL-F	7 (29.2)	16 (66.7)	1 (4.2)	7 (29.2)	16 (66.7)	1 (4.2)	0.21	60	100
ITS	13 (52.0)	11 (44.0)	1 (4.0)	13 (52.0)	11 (44.0)	1 (4.0)	1.64	60	100
matK + *trnH-psbA*	3 (12.5)	21 (87.5)	0 (0.0)	3 (12.5)	18 (75.0)	0 (0.0)	0.07	20	50
matK + *trnL-F*	7 (29.2)	16 (66.7)	1 (4.2)	7 (29.2)	16 (66.7)	1 (4.2)	0.11	60	100
matK + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.93	60	100
trnH-psbA + ITS	14 (56.5)	9 (34.8)	3 (8.7)	14 (56.5)	9 (34.8)	3 (8.7)	1.06	60	100
trnH-psbA + *trnL-F*	7 (29.2)	16 (66.7)	1 (4.2)	7 (29.2)	16 (66.7)	1 (4.2)	0.13	60	100
trnL-F + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.86	60	100
matK + *trnH-psbA* + *trnL-F*	7 (29.2)	16 (66.7)	1 (4.2)	7 (29.2)	16 (66.7)	1 (4.2)	0.08	60	100
matK + *trnH-psbA* + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.71	60	100
matK + *trnL-F* + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.61	60	100
trnH-psbA + *trnL-F* + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.67	60	100
matK + *trnH-psbA* + *trnL-F* + ITS	13 (56.5)	8 (34.8)	2 (8.7)	13 (56.5)	8 (34.8)	2 (8.7)	0.51	60	100

†*Amentotaxus hatuyenensis* is treated as a synonym of *A. yunnanensis*. ITS, internal transcribed spacer; *n*, number of individuals.

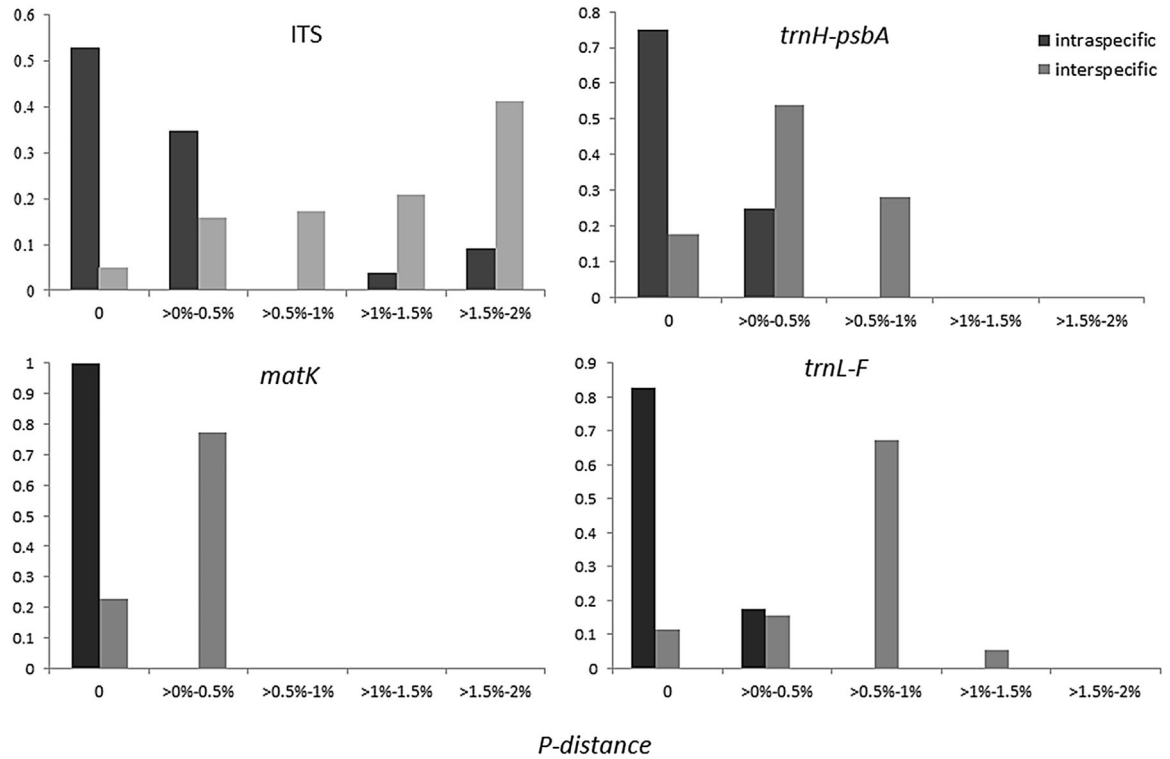

Fig. 1. Relative distribution of intraspecific and interspecific distances for four DNA barcodes in *Amentotaxus*. *X*-axes relate to P-distances arranged in intervals, and *y*-axes correspond to the percentage of occurrences. ITS, internal transcribed spacer.

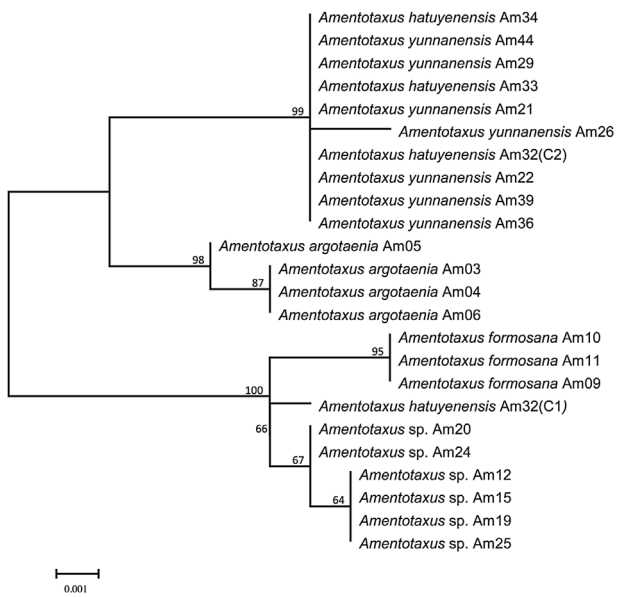

Fig. 2. Neighbor-joining tree of internal transcribed spacer of *Amentotaxus* based on P-distance with bootstrap values above 50%. Each sample (Am) is numbered. Two internal transcribed spacer (ITS) types (C1 and C2) of cloning sequences of sample Am32 were used in the ITS analysis.

Discussion

Performance of DNA barcodes in *Amentotaxus*

A high universality of a primer is an important criterion for an ideal DNA barcode (Kress & Erickson, 2007; Hollingsworth et al., 2009) and should be routinely retrievable with a single primer pair (CBOL Plant Working Group, 2009). In the present study, all five barcodes showed 100% success of PCR and sequencing for *Amentotaxus* (Table 1), meeting the criterion of high universality. An ideal DNA barcode should also provide a high rate of success for species discrimination and identification (Kress et al., 2005; Lahaye et al., 2008; Hollingsworth et al., 2011) and have a distinct "barcode gap" among species (Meyer & Paulay, 2005). However, no obvious barcode gap was detected for the barcodes used in this study (Fig. 1).

Internal transcribed spacer was proposed as a DNA barcode for seed plants because of its high species identification ability (Kress et al., 2005; China Plant BOL Group, 2011), although its utility may be limited by incomplete lineage sorting and the presence of divergent paralogous copies or pseudogenes within individuals despite concerted evolution (Alvarez & Wendel, 2003; Starr et al., 2009; Xiao et al., 2010; Hollingsworth et al., 2011). Due to its high sequence variation and high species resolution, *trnL-F* was also proposed as a DNA barcode (Taberlet et al., 2007; Liu et al., 2011). Both barcode regions have already been successfully used in discriminating conifers and Eurasian yews, another taxonomically complex group that is related to *Amentotaxus* (Liu et al., 2011). Our results here indicated that ITS and *trnL-F* provided the highest species resolution, and also showed a higher

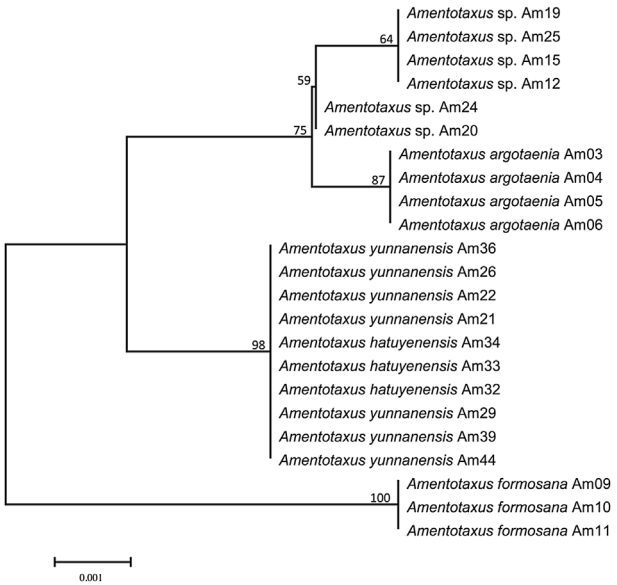

Fig. 3. Neighbor-joining tree of *trnL-F* of *Amentotaxus* based on P-distance with bootstrap values above 50%. Each sample (Am) is numbered.

sequence divergence compared to other barcodes. In this study, the three additional barcoding regions (*rbcL*, *matK*, and *trnH-psbA*), proposed as core or supplementary regions for barcoding plants (Kress et al., 2005; CBOL Plant Working Group, 2009; Chen et al., 2010; Hollingsworth et al., 2011), showed the lowest species resolution, and only *A. formosana* was distinguished from the other species.

A combination of DNA barcodes can improve species discrimination (CBOL Plant Working Group, 2009; China Plant BOL Group, 2011; Yang et al., 2012; Liu et al., 2015). In this study, the species discrimination rate increased for all two-barcode combinations, except for *matK* + *trnH-psbA*. Any combination that included either ITS or *trnL-F* achieved the same species resolution (60%) as when ITS or *trnL-F* were used alone. Combinations of more than two barcodes did not increase the species discrimination success any further. Overall, using ITS or *trnL-F* as single barcodes, a two-barcode combination of *trnL-F* + ITS, or a combination that included one of those two, are proposed as the most suitable for barcoding *Amentotaxus* species.

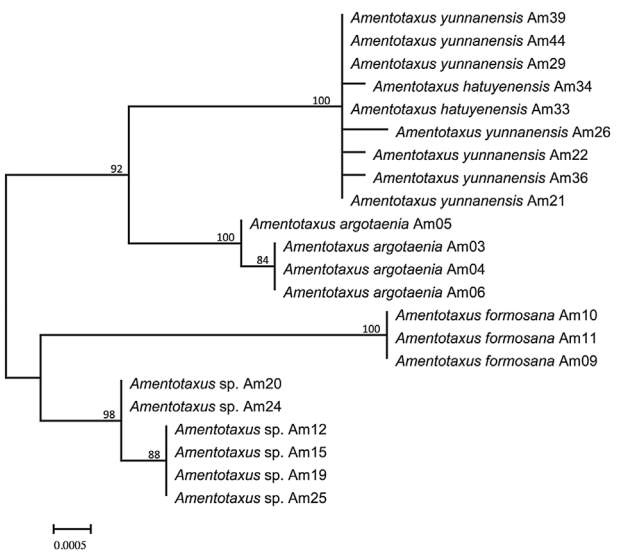

Fig. 4. Neighbor-joining tree of the combination of *rbcL* + *matK* + internal transcribed spacer + *trnH-psbA* of *Amentotaxus* based on P-distance with bootstrap values above 50%. Each sample (Am) is numbered.

Species identification and a new species discovery in *Amentotaxus*

The taxonomy of *Amentotaxus* species has been mainly based on morphological characters, such as width of stomatal band, color of stomatal band, and leaf shape and size. However, these characters are often variable and their states can overlap between species (Fu et al., 1999; Phan et al., 2014). Three species, *A. argotaenia*, *A. formosana*, and *A. yunnanensis*, have even been regarded as a species complex due to their morphological similarities (Zhou, 2001; Ge et al., 2005). In the present study, these species could be well differentiated using the DNA barcoding approach. Our results suggest that they are three distinct species corresponding to three different lineages, rather than a species complex. This was also supported by a recent population genetic analysis (Ge et al., 2014). In the present study, we collected three individuals of *A. hatuyenensis* from different locations near to the type locality. Sequences from all plastid barcodes for each individual and the ITS sequences of the individuals Am33 and Am34 were identical to those from *A. yunnanensis*. Our results support the treatment of *A. hatuyenensis* as a synonym of *A. yunnanensis* (Phan et al., 2013, 2014). When considering this synonymy, the discrimination rate using the barcodes described above (excluding sample Am32) (Figs. 2, 3) rises to 100% (Table 2).

Specimens from Hekou, (Yunnan), Lao Cai (Vietnam), and Xiang Khoang (Laos) formed a distinct clade, indicating that they represent a separate evolutionary lineage, and possibly a new species. In addition, the voucher specimens show differences from other recognized species in their morphology. Differences include the length and width of the leaves, the shape of the leaf apex, recurvature of the leaf margin, stomatal band width, and ratio to the leaf margin bands. However, no fertile specimens have been collected yet so further fieldwork is required before a formal description can be published. In addition, its distribution and habitat preferences compared to *A. yunnanensis* need to be clarified; currently it appears to be sympatric with *A. yunnanensis* in a small area at the boundary between China and Vietnam.

Interestingly, the ITS clone sequences of individual Am32 (*A. 'hatuyenensis'*) suggested that this individual may be of hybrid origin between *A. yunnanensis* and the putative new species. Considering that *Amentotaxus* are dioecious and wind pollinated, and that the distributions of these two taxa overlap at the edges of their ranges, hybridization could have occurred. This is the first indication of an interspecific hybridization in natural populations of *Amentotaxus*, although it has been observed in cultivation (Gosling et al., 2009) and hybrid individuals with intermediate morphological and molecular characters of both parental species have been observed in *Taxus* (Poudel et al., 2012). Thus, DNA barcoding is

an effective tool for new species discovery, even identifying the hybrid individuals, which will be of importance for conservation of the endangered *Amentotaxus* species in the future.

Implications for conservation of *Amentotaxus*

All *Amentotaxus* species are currently listed as either globally or nationally threatened (Wang & Xie 2004; IUCN, 2016) on the global IUCN Red List (http://www.iucnredlist.org/search). *Amentotaxus assamica* and *A. hatuyenensis* are listed as endangered, *A. argotaenia* as near threatened, and the other three species are categorized as vulnerable. The results of our study, combined with those of Phan et al. (2014) indicate that *A. hatuyenensis* is synonymous with *A. yunnanensis* and therefore does not warrant listing as a distinct species. Such a change would not impact the conservation status of *A. yunnanensis*. Our results also indicate the presence of a potentially new taxon, although further fieldwork, particularly aimed at collecting fertile specimens, is required before this can be confirmed. If it is, then it is likely that it would be assessed as threatened due to ongoing habitat degradation from surrounding agriculture and forest clearance.

Our identification of the most effective barcodes and barcode combinations should facilitate the accurate identification of recently discovered populations and those whose identity may have been uncertain or disputed. This should aid their conservation through a better understanding of each species' distribution and population size. The findings from this study are highly relevant for the formulation of appropriate conservation strategies for threatened *Amentotaxus* species in national and transboundary regions.

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Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12207/suppinfo:

Table S1. Species information and GenBank accession numbers in the genus *Amentotaxus* used in this study (species concept followed Farjon, 2010).