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A New Species and Introgression in Eastern Asian Hemlocks (Pinaceae: *Tsuga*)

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Abstract—Species delimitation in Pinaceae is often challenged by limited morphological differentiation and introgression. In *Tsuga* (hemlocks), species delimitation has been most challenging among northeastern Asian taxa, where the species are weakly marked morphologically and range in number from three to five in previous studies. Two low-copy nuclear four-coumarase-ligase (*4CL*) genes and morphology strongly support a clade of the Japanese endemic *T. diversifolia* and *T. sieboldii* from Japan and Ulleung island (Ulleungdo) in Korea. This clade is here referred to as the oceanic hemlocks. *4CL* strongly supports a sister-group relationship of the widespread northeastern Asian *T. chinensis* and eastern North American *T. caroliniana*. In contrast, chloroplast genomes, which are markedly reduced in *Tsuga* and relatives, strongly support Japanese *T. sieboldii* as sister to *T. chinensis* and moderately support *T. caroliniana* as sister to a clade of *T. diversifolia* and hemlocks from Ulleungdo. These divergent topologies suggest chloroplast capture of *T. chinensis* by Japanese *T. sieboldii*. Ulleungdo hemlocks are distinct from other northeastern Asian species in leaf and cone morphology and phenologically in common-garden observations. We therefore describe these hemlocks as a new species, ***T. ulleungensis***.

Keywords—Chloroplast capture, island endemic, plastid genomes, quantitative morphology, Ulleungdo.

Pinaceae species are often weakly differentiated using DNA and morphological inferences (Campbell et al. 2005; Syring et al. 2005, 2007; Havill et al. 2008; Xiang et al. 2009). Individuals of Pinaceae typically form large populations, are long-lived, and readily cross with congeners (Tsutsui et al. 2009; Willyard et al. 2009; Zhang et al. 2010). These conditions retard evolutionary divergence by prolonging retention of ancestral polymorphisms and admixing parental genomes, resulting in morphologically similar species (Syring et al. 2005, 2007; Cun and Wang 2014). Difficulties in species delimitation in Pinaceae may also result from recent diversification (Aguirre-Planter et al. 2000, 2012; Bouillé et al. 2011), convergent evolution (Wang and Ran 2014), and hybrid speciation (Havill et al. 2008; Abbott et al. 2010; Ren et al. 2012; Xiang et al. 2015).

Delimiting *Tsuga* species is difficult only in eastern Asia (Table 1), where the number of recognized species is three (Eckenwalder 2009), four (Farjon 1990, 2010), or five (Fu et al. 1999). Long-accepted species, such as northern Japanese hemlock (*T. diversifolia*) and southern Japanese hemlock (*T. sieboldii*), differ subtly by presence of twig pubescence, stomatal band color, and seed (ovulate) cone scale texture (Farjon 1990; Baker 2009). These species also differ in the shape and margins of the ovulate cone-scale bracts, characters that are useful across living and fossil *Tsuga* (LePage 2003; Xing et al. 2013).

Although widespread across northern temperate continents during the Neogene, *Tsuga* contracted during the Quaternary to eastern Asia, and eastern and western North America, perhaps due to its dependence on moist environments (LePage 2003; Tsuyama et al. 2014). The two western North American hemlocks, mountain hemlock (*T. mertensiana*) and western hemlock (*T. heterophylla*), form a clade that is sister to the remainder of the genus (Vining 1999; Havill et al. 2008). Eastern hemlock (*T. canadensis*) is sister to a well-supported clade consisting of the southern Appalachian endemic Carolina hemlock (*T. caroliniana*) plus Asian hemlocks (Havill et al. 2008). Central Asian *T. dumosa* is morphologically distinct from northeastern Asian hemlocks, and *T. chinensis*, which is widespread in China plus a small region of Vietnam (Farjon 2010), is morphologically diverse. The Japanese endemic *T. diversifolia* is morphologically most similar to *T. sieboldii*, which is native to Japan and the small Korean island, Ulleungdo. Havill et al.'s (2008) ITS data did not resolve relationships within the clade of *T. caroliniana* and Asian hemlocks. Their cpDNA did find a sister-group relationship of *T. chinensis* and Japanese *T. sieboldii* and weakly supported *T. caroliniana* as sister to a clade of *T. diversifolia* and Korean *T. sieboldii*. The close relationship between northeastern Asian *Tsuga* and eastern North American *T. caroliniana* is a common pattern in the boreotropical flora (Tiffney 1985; Manos and Meireles 2015).

Ulleungdo is a volcanic island about 130 km east of the Korean peninsula and separated from the closest point in Japan by about 284 km (Fig. 1). Ulleungdo is approximately 73 km^2, reaches an altitude of 983 m above sea level, and originated 2.7 ± 0.9 million years ago (Kim and Lee 1983). Ulleungdo has 30–40 endemic angiosperms (Stuessy et al. 2006; Jung et al. 2013), which make up 4–6% of the vascular plant species on the island (Jung et al. 2013). The majority of Ulleungdo endemics are hypothesized to have evolved by anagenesis (Stuessy et al. 2006). Ulleungdo has eight gymnosperm species, all of which are native, and Ulleungdo hemlocks are the first endemic gymnosperm to be described from the island. In Korea, *Tsuga* is only found on Ulleungdo (Jung et al. 2013), and has been treated as *T. sieboldii* (Wilson 1918; Nakai 1919; Kim 1988; Lee 1993; Lee 2001; Sun 2007).

The goal of this paper is to resolve the evolutionary relationships of Ulleungdo hemlocks. We assess their phylogenetic relationships to other hemlocks with DNA

Table 1. Comparison of three recent species classifications of *Tsuga* and *Nothotsuga*. NA = Not Applicable.

Eckenwalder 2009	Farjon 2010	Fu et al. 1999
Nothotsuga longibracteata (W. C. Cheng) H. H. Hu ex C. N. Page	*N. longibracteata*	*Tsuga longibracteata* W. C. Cheng
T. canadensis Carrière	*T. canadensis*	NA
T. caroliniana Engelm.	*T. caroliniana*	NA
T. chinensis (Franch.) E. Pritz. in Diels	*T. chinensis* var. *chinensis*	*T. chinensis* var. *chinensis*
T. chinensis	*T. chinensis* var. *chinensis*	*T. chinensis* var. *formosana* (Hayata) H. L. Li & H. Keng
T. chinensis	*T. chinensis* var. *oblongisquamata* W. C. Cheng & L. K. Fu	*T. oblongisquamata* (W. C. Cheng & L. K. Fu) L. K. Fu & Nan Li
T. × *forrestii* Downie	*T. forrestii* Downie	*T. chinensis* var. *forrestii* (Downie) Silba
T. chinensis	*T. chinensis* var. *robusta* W. C. Cheng & L. K. Fu	*T. chinensis* var. *robusta*
T. chinensis	*T. chinensis* var. *chinensis*	*T. chinensis* var. *patens* (Downie) L. K. Fu & Nan Li
T. diversifolia (Maxim.) Mast.	*T. diversifolia*	NA
T. dumosa Eichl.	*T. dumosa*	*T. dumosa*
T. heterophylla Sarg.	*T. heterophylla*	NA
T. × *jeffreyi* (A. Henry) A. Henry	*T.* × *jeffreyi*	NA
T. mertensiana (Bong.) Carrière	*T. mertensiana*	NA
T. mertensiana	*T. mertensiana* var. *grandicona* Farjon	NA
T. sieboldii Carrière	*T. sieboldii*	*T. sieboldii*

sequences from nearly complete chloroplast genomes and the low-copy nuclear gene *4CL*. We test their phenotypic distinctness with quantitative morphology and their phenological distinctness in a common garden.

Materials and Methods

Plant Samples—We follow Eckenwalder (2009; Table 1) in recognizing three northeastern Asian hemlocks: *T. chinensis*, *T. diversifolia*, and *T. sieboldii*. We collected plant tissue for DNA extraction from 14 hemlock individuals (Appendix 1), including Ulleungdo hemlock and multiple accessions of each northeastern Asian hemlock species. For quantitative morphology, we studied 78 Gray Herbarium specimens of wild-collected Asian hemlocks from across their range (Fig. 1; Table 2; Appendix 1). We studied the morphology and phenology of 18 individuals of northeastern Asian taxa growing in the Arnold Arboretum, Boston, Massachusetts, U. S. A.

DNA Extraction and Sequencing—DNA was extracted from silica-preserved leaf material with a DNeasy plant mini kit (Qiagen, Valencia, California). Leaf material was frozen in liquid nitrogen for 30 sec before grinding. For mature leaf material the lysis buffer step was extended by several hours, up to an overnight incubation at 65°C.

The *4CL* gene was amplified following primers and PCR conditions from Wang et al. (2000), using an Eppendorf Mastercycler ep (Eppendorf AG, Hamburg, Germany). We cloned PCR amplicons with a TOPO TA kit (Invitrogen, San Diego, California), and screened for the correct insert using an *Eco*R1 digest. To assess intra-individual variation, we sequenced 10–16 colonies for each accession, including all detected variants in *Eco*R1 digest profiles. *4CL* sequencing was performed with PCR primers at the University of Maine DNA Sequencing Facility using an ABI 3730 sequencer and an ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster, California). We sampled three individuals of *T. diversifolia*; two each of *T. caroliniana, T. chinensis,* Japanese *T. sieboldii,* and Ulleungdo hemlocks; and one *T. canadensis* (Appendix 1). Published sequences of *4CL* from *T. canadensis* (AF144525, AF144526), *T. mertensiana* (AF144524), and *N. longibracteata* (AF144523) were used as outgroups.

We generated nearly complete plastome sequences of one individual each of *T. canadensis*, *T. caroliniana*, *T. chinensis*, *T. diversifolia*, *T. heterophylla*, Japanese *T. sieboldii*, Ulleungdo hemlocks, and *Nothotsuga longibracteata* (Appendix 1) with Illumina second-generation sequencing technology, following techniques of Cronn et al. (2008). Sequencing was conducted on an Illumina Genome Analyzer IIx at the FAS Center for Systems Biology at Harvard University. Read length varied between 33 and 100 base pairs, and assembly coverage depth averaged 1,000×, with a range of 100–2,000× (Appendix 2). We sorted raw short-read sequences by barcode using perl scripts (Knaus 2014) and assembled to a *Keteleeria davidiana* (NC011930) reference using the "align-reads" pipeline (Straub et al. 2011). We conducted chloroplast genome annotation using DOGMA (Wyman et al. 2004). As an outgroup, we analyzed *Nothotsuga longibracteata,* of southeastern Asia, the closest relative of *Tsuga* (Wang et al. 2000).

Phylogenetic Analyses—We manipulated DNA sequences in Geneious version 9.0.5 (http://www.geneious.com, Kearse et al. 2012). We aligned DNA sequences with MAFFT (Katoh et al. 2009), and sequence alignments were then analyzed with maximum parsimony (MP) and maximum likelihood (ML). DNA alignments are available from Dryad Digital Repository (Holman et al. 2017). Maximum parsimony analyses utilized PAUP* version 4.0b10 (Swofford 2002). Parsimony heuristic searches were performed in two steps, first to find the most parsimonious tree(s) using 1,000 random addition sequence replicates with tree-bisection-reconnection (TBR). Subsequent MP bootstraps analyses utilized the same settings as above, retaining 10 trees per replicate. Selection of models for use in maximum likelihood was conducted in jModelTest version 2.1.4, using the Akaike information criterion (Guindon and Gascuel 2003; Darriba et al. 2012). Maximum likelihood analyses were conducted with Garli 2.0, with an initial run to find the best tree, followed by 1,000 bootstrap replicates (Zwickl 2006).

Consensus trees from multiple runs of Garli were constructed using Sumtrees v.3.3.1 (Sukumaran and Holder 2010). Incongruence between phylogenetic markers was analyzed by visually comparing topologies and the incongruence-length distance (ILD) test (Farris et al. 1994) implemented in PAUP* (Swofford 2002). We delimit species as phenotypic clusters (Hausdorf 2011).

Morphology—Morphological analysis of continuous and qualitative characters was conducted to assess variance within and between northeastern Asian hemlocks and test the null hypothesis of no distinguishable groups. A scoop of eight milliliters of leaves from fragment packets of herbarium specimens were numbered and randomly selected (www.random.org, Haahr 2015). We measured 11 quantitative characters for each leaf, including width at 10, 50, and 90% of leaf length, leaf length, petiole length, petiole width at point of connection with branch, width and depth of emarginate leaf apex notch, width of stomata bands, number of rows per stomatal band at midpoint of leaf, and width of resin canals (Fig. 2). Distribution of values for each character was screened using histograms and boxplots in R version 3.1.3 (R Core Team 2015), and extreme outliers were removed. We used analysis of variance to test the significance of all characters in discriminating between taxonomic groups. Of the 11 characters we assessed, six were significant (Table 2) and included in further analyses. Principal components analyses (PCA) and boxplots of individual characters were implemented in R version 3.1.3 (R Core Team 2015). Significance of PCA axes was assessed using the broken-stick model. To conduct Tukey's post hoc test for multiple comparisons of means, we used the glht function in the multcomp package in R (Herberich et al. 2010), which is robust for comparison of means from unbalanced data. Morphological data matrix is available from Dryad Digital Repository (Holman et al. 2017).

Common Garden Leaf Morphology—We studied morphological variation in three mature individuals each of *T. diversifolia* and Japanese

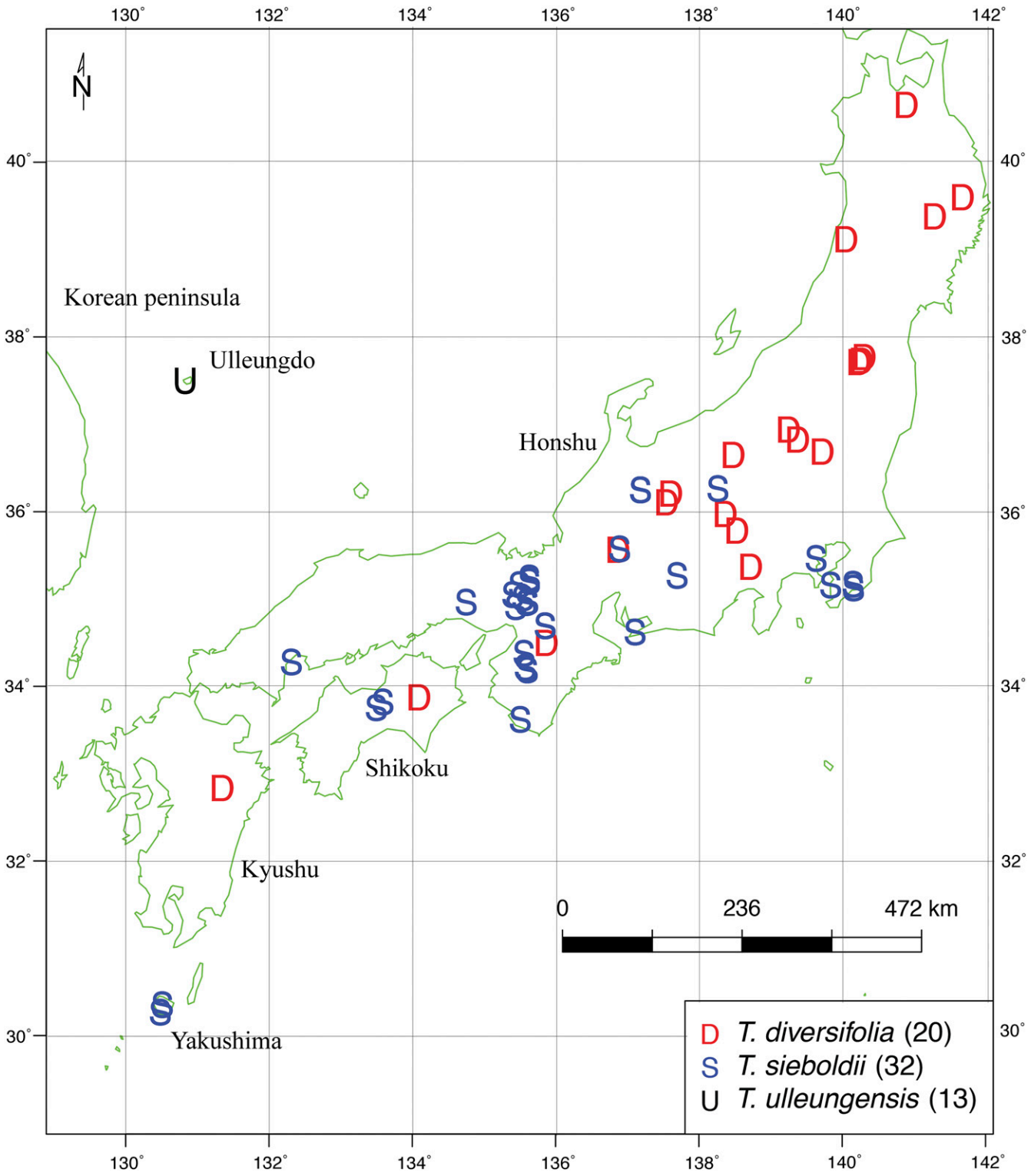

FIG. 1. Sampling of oceanic hemlock species.

T. sieboldii plus two Ulleungdo hemlocks at the Arnold Arboretum. We used four branches from each individual from the same aspect, exposure, and developmental stage.

Phenology—We recorded bud burst and leaf maturation on reproductively mature plants of Ulleungdo hemlocks, *T. chinensis*, *T. diversifolia*, and Japanese *T. sieboldii* growing out-of-doors at the Arnold Arboretum in 2010, 2011, and 2012. We measured one branch on the north, east, south, and west sides of each specimen weekly from April 1 through September 1.

RESULTS

Alignments of *4CL* were 1,049 base pairs in length. Preliminary phylogenetic analysis of *4CL* revealed two distinct copies of the gene in *Tsuga*, as in *Larix* (Semerikov and Lascoux 2003). We analyzed alleles from these two *4CL* copies to better understand patterns that may be attributed to retention of ancestral polymorphisms, a phenomenon of non-coalesence

TABLE 2. Morphological differentiation of northeastern Asian *Tsuga* in six quantitative leaf characters, and three qualitative characters. Sample sizes are given in parentheses following taxon name. All measurements are in millimeters. Means differing significantly between *T. ulleungensis* and other taxa are indicated by different letters (ANOVA with post hoc Tukey HSD test). a = $p < 0.001$; b = $p < 0.01$; c = $p < 0.05$; d = not significant, $p > 0.05$; and e = irrelevant comparisons. See text for p values of quantitative characters for *T. sieboldii* and *T. ulleungensis*.

	T. chinensis (10)	*T. diversifolia* (20)	*T. sieboldii* (32)	*T. ulleungensis* (13)
Leaf blade length	9.73 ± 2.03 [d]	7.35 ± 1.23 [a]	9.25 ± 1.76 [c]	10.87 ± 1.06 [e]
Petiole width	0.41 ± 0.05 [d]	0.39 ± 0.07 [a]	0.40 ± 0.03 [b]	0.44 ± 0.02 [e]
Leaf width at apex	1.53 ± 0.17 [c]	1.56 ± 0.11 [b]	1.64 ± 0.15 [d]	1.74 ± 0.10 [e]
Leaf notch depth	0.07 ± 0.02 [a]	0.105 ± 0.02 [c]	0.11 ± 0.03 [d]	0.133 ± 0.02 [e]
Leaf notch width	0.19 ± 0.08 [a]	0.33 ± 0.04 [b]	0.33 ± 0.06 [a]	0.41 ± 0.04 [e]
Resin canal width	0.11 ± 0.03 [a]	0.14 ± 0.02 [a]	0.13 ± 0.02 [a]	0.06 ± 0.01 [e]
Twig hairiness	none to sparse	moderately dense	none	none
Cone-scale bract margins	erose	entire to crenulate	erose	crenulate

that is inferred in nuclear phylogenies in *Pinus* (Syring et al. 2007; Willyard et al. 2009) and organellar markers in *Tsuga* (Cun and Wang 2014). *Tsuga canadensis* was selected as an outgroup given its phylogenetic position as sister to Asian hemlocks plus *T. caroliniana* (Havill et al. 2008). The ILD tests revealed no significant differences in phylogenetic signal between the two copies, which were combined for further analysis. The combined, two-copy *4CL* alignment was 2,098 base pairs in length, with 46 parsimony-informative characters, and the best-fitting model, HKY + I, was selected. The strongly supported clade of *T. caroliniana* and *T. chinensis* is sister to the strongly supported oceanic hemlock clade of *T. diversifolia*, *T. sieboldii*, and Ulleungdo hemlocks (Fig. 3).

The abietoid clade (*Abies*, *Cedrus*, *Keteleeria*, *Nothotsuga*, *Pseudolarix*, and *Tsuga*) has a synapomorphic reduction in the chloroplast inverted repeat (IR; Lin et al. 2010). Like *Keteleeria*, *Nothotsuga* and *Tsuga* chloroplast genomes are also compact, sharing intron reductions documented by Wu et al. (2009). Out of 68 protein-coding genes in the chloroplast, we document 13 genes (*accD*, *cemA*, *chlN*, *matK*, *psbM*, *rpl2*, *rpl20*, *rpl22*, *rpoB*, *rpoC2*, *rps2*, and *rps4*) that are shorter in *Tsuga* than in *Keteleeria*, typically by a one- or two-codon deletion near the 3′ end of the gene. On the other hand, insertions near the 5′ end of *psaI* and *psaJ* make these two genes 24 total base pairs longer in *Nothotsuga* and *Tsuga* than in *Keteleeria*. A nine-base-pair insertion at the 5′ end of *psbH* is shared by Asian hemlocks and *T. caroliniana*. Numerous small (3–24 base pairs) indels are found in the giant open reading frame *ycf*2.

The cpDNA alignment was 122,525 bp, with 1,574 parsimony informative characters. For the cpDNA dataset, the best fitting model, GTR + G, was selected, with four separate rate categories. With one exception, branches in the cpDNA phylogeny (Fig. 4) have 100% bootstrap (BS) support from MP and ML. The cpDNA phylogeny resolves *T. caroliniana* as sister to a clade of *T. diversifolia* and Ulleungdo hemlocks with moderate support (80 MPBS/77% MLBS), and these three taxa are in turn sister to a clade of Japanese *T. sieboldii* and *T. chinensis.* The *4CL* and cpDNA phylogenies are therefore not congruent. Sequence divergence (Table 3) between Ulleungdo hemlocks and *T. diversifolia* (0.0015) is similar to that between Japanese *T. sieboldii* and *T. chinensis* (0.00168).

Morphology—Ulleungdo hemlocks resemble Japanese *T. sieboldii* in having glabrous branches, but Ulleungdo hemlocks have larger leaves (Figs. 5, 6), wider petioles, larger leaf notches, buff-colored stomatal bands, and smaller resin canals. The margins of Ulleungdo hemlocks cone-scale bracts are crenulate, with large, wave-like teeth, especially evident on the proximal-most one to three cone-scale bract margins, whereas bracts of Japanese *T. sieboldii* have finely serrate teeth (erose margins, Table 2, Fig. 7). The taxonomic utility of cone-scale bract morphology is limited by the absence of cones from many herbarium specimens. The diameter of Japanese *T. sieboldii* and *T. diversifolia* resin canals averages more than twice that of Ulleungdo hemlocks' (Table 2) resin canals. We did not record any overlap in resin canal diameters between Japanese *T. sieboldii* plus *T. diversifolia* and Ulleungdo hemlocks. Plants in a common garden setting at the Arnold Arboretum have larger leaves than those observed on wild-collected specimens. Differences between species are similar in the common garden and the wild; just as we observe in wild-collected specimens, the common garden Ulleungdo hemlocks have larger leaves on average than *T. sieboldii*, which are in turn larger than *T. diversifolia*.

Four quantitative characters (Table 2) differed significantly between Ulleungdo hemlocks and Japanese *T. sieboldii* (with ANOVA and post hoc Tukey HSD test): leaf length ($p = 0.030$), petiole width ($p = 0.0029$), leaf notch width ($p = 0.0013$), and resin canal width ($p = 0.001$) (Fig. 6). Two additional quantitative characters were included in the PCA, as they significantly differed between *T. chinensis* and Ulleungdo hemlocks: leaf width at apex ($p = 0.027$), and leaf notch depth ($p = 0.0001$) (Table 2). The broken-stick model indicates that only two principal components were significant. PCA reveals that Ulleungdo hemlocks form a cluster that is mostly separate from a broadly overlapping grouping of *T. diversifolia* and Japanese *T. sieboldii* (Fig. 5). The two Japanese *T. sieboldii* herbarium samples (*Kobayashi 1731*; *Wilson 6037*; Appendix 1) that are close or adjacent to the Ulleungdo hemlocks cluster have large leaves and are from Yakushima, the southernmost occurrence of Japanese *T. sieboldii* (Fig. 1). Yakushima hemlocks, however, have large resin canals and finely-toothed ovulate cone-scale bracts typical of Japanese *T. sieboldii*. Two *T. chinensis* herbarium samples from Anhui Province (Steward 7194; Wilson 2100; Appendix 1) are also close or adjacent to the Ulleungdo hemlocks cluster. Like Ulleungdo hemlocks and Yakushima *T. sieboldii*, these two *T. chinensis* have large leaves with short petioles, but the diameter of their resin canals (0.074, 0.069 mm, respectively) approach that of Ulleungdo hemlocks (Table 2). Anhui *T. chinensis* have, however, smaller leaf notches and rhomboid shaped ovulate

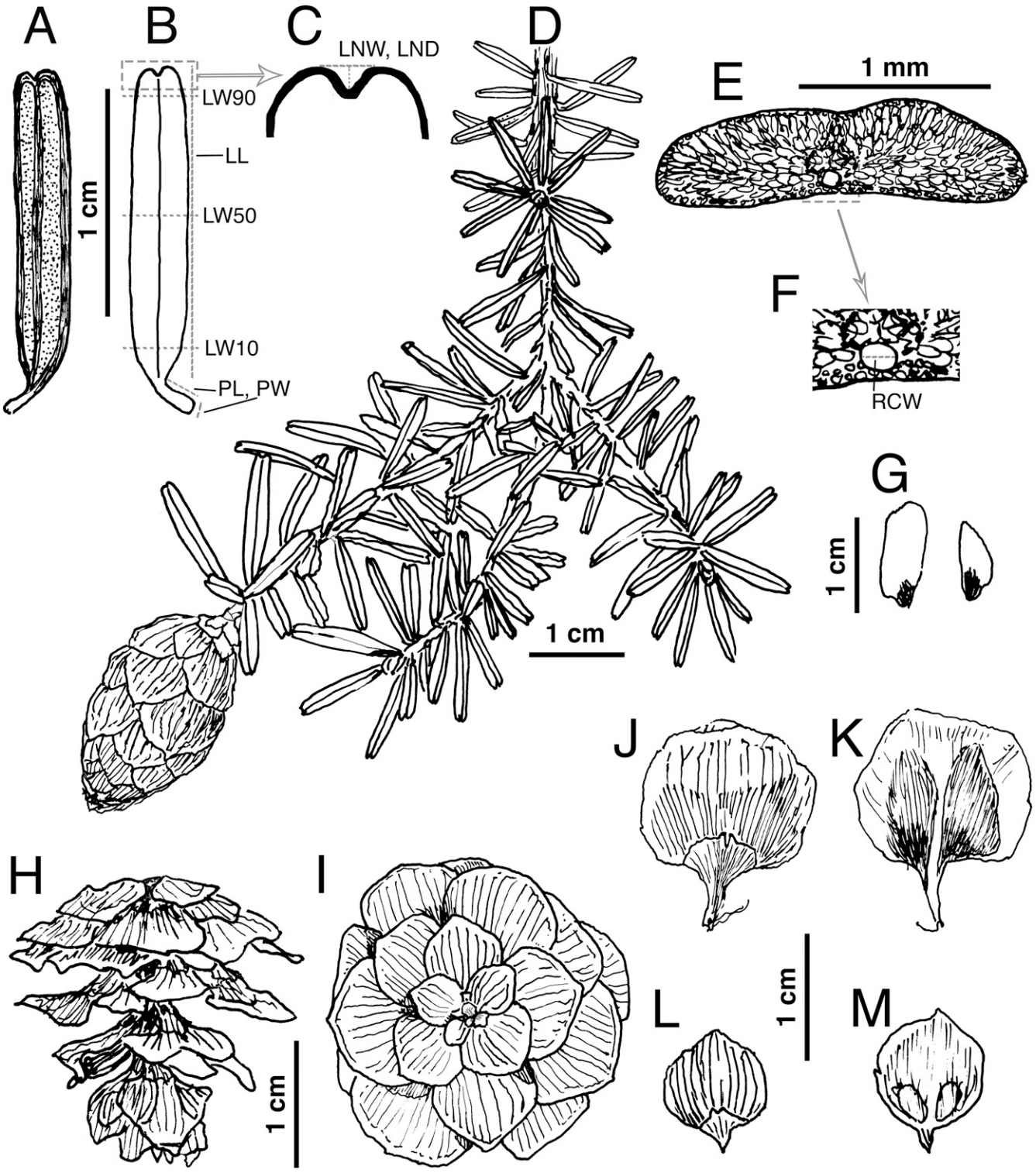

Fig. 2. *Tsuga ulleungensis*. A. Abaxial leaf surface. B. Adaxial leaf surface. LW90, LW50, LW10 = leaf width at 90, 50, and 10% of leaf length, respectively. LL = leaf length. PL = petiole length. PW = petiole width. C. Enlarged view of portion of leaf tip, adaxial surface. LNW = leaf notch width. LND = leaf notch depth. D. Foliated branch, with ovulate cone. E. Leaf cross section at leaf midpoint, with central resin canal near abaxial surface. F. Enlarged view of portion of leaf at midpoint, adaxial surface. RCW = resin canal width at transverse section of leaf at midpoint. G. Seeds. H. Ovulate cone, side view. I. Ovulate cone, proximal view. J. Large ovulate scale, abaxial view with bract. K. Large ovulate scale, adaxial view with impressions of winged seeds. L. Small ovulate scale, abaxial view with bract. M. Small ovulate scale, adaxial view with impression of seed body.

cone-scale bracts with irregularly serrate margins typical of *T. chinensis*.

Phenology—At the Arnold Arboretum, Ulleungdo hemlocks and *T. chinensis* break bud at nearly the same time, but *T. chinensis* ceases its annual growth about four weeks later than Ulleungdo hemlocks (Fig. 8). *Tsuga diversifolia* has a relatively short period of growth; bud break occurs just over a week after *T. chinensis* and Ulleungdo hemlocks, and growth

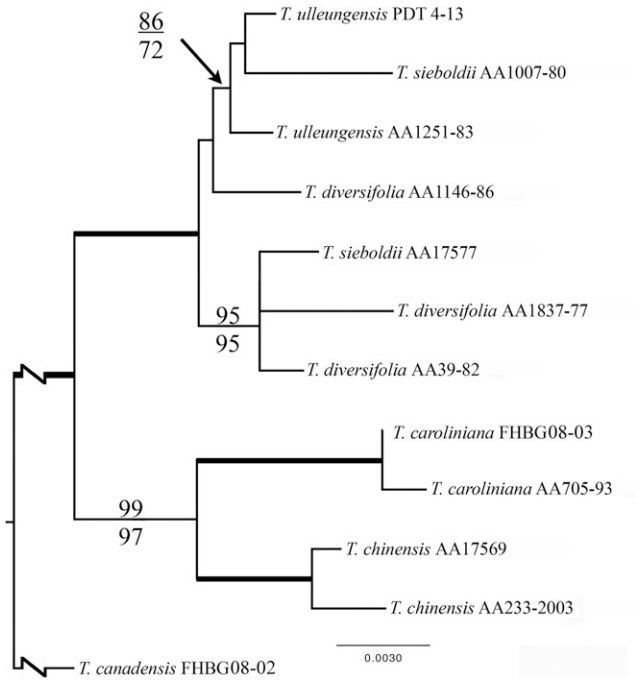

FIG. 3. Phylogram of *Tsuga* from two concatenated *4CL* paralogs, with *T. canadensis* as outgroup. Heavy lines indicate 100% MP and ML bootstrap support. Nodes with 70–99% support are labeled with MPBS above and MLBS below. Nodes with less than 70% are unlabeled. Sideways Z-shaped lines indicate branch lengths that have been truncated for display. Arnold Arboretum accession numbers follow species names.

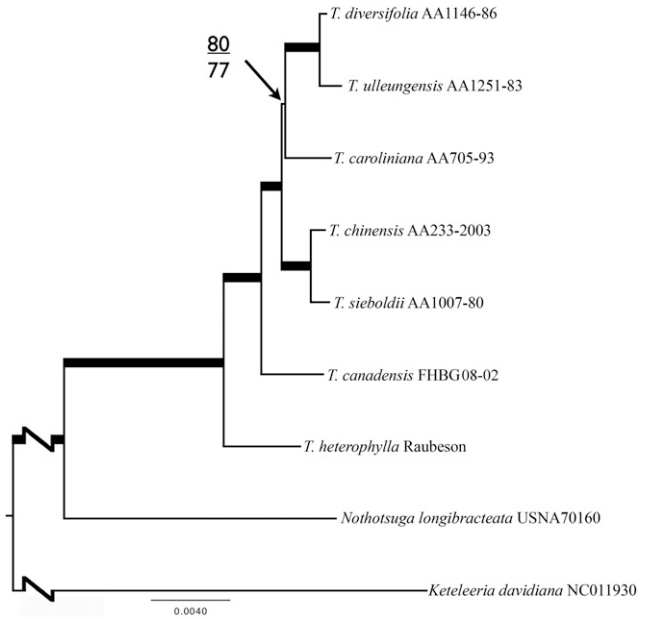

FIG. 4. cpDNA phylogram, using *Nothotsuga longibracteata* as outgroup. Heavy lines indicate 100% bootstrap support from maximum parsimony and likelihood. Sideways Z-shaped lines indicate branch lengths that have been truncated for display.

ceases about two and a half weeks earlier than Ulleungdo hemlocks. Japanese *T. sieboldii* initiates growth almost 40 d later (Fig. 9) than *T. chinensis* and Ulleungdo hemlocks, but concludes its growth a week earlier than *T. chinensis.* Earlier bud break in Ulleungdo hemlocks than Japanese *T. sieboldii* was observed in native habitats of both species during fieldwork in May 2008.

DISCUSSION

The Species Problem in Pinaceae—Delimitation of species has been challenging in all Pinaceae genera with more than one species. This challenge is shown by Farjon's (2010) recognition of 18% more species in the family than Eckenwalder (2009). Species delimitation is difficult in Pinaceae because speciation may be slowed by long lifespans, large population sizes, extensive gene flow among populations, and large genomes (Knight et al. 2005; Syring et al. 2005, 2007). During such protracted speciation, criteria for species status (such as morphological distinctness, allelic coalescence (distinct lineage status), and reproductive isolation) may occur at different times. Complete allelic coalescence of nuclear markers, for example, could take up to 7.6 million years in *Pinus* (Syring et al. 2007) and has lagged behind morphological distinctness in many conifer species (Syring et al. 2005). Ulleungdo hemlocks, which have not achieved allelic monophylly in *4CL* (Fig. 3) but are morphologically distinct, fit this pattern. Intergradation of taxa, such as some western North American *Abies* (Aguirre-Planter et al. 2012) and some members of *Pinus* (Wang and Szmidt 1994; Jasińska et al. 2010) may represent ecogeographic but not morphological separation.

Although hybridization is generally not a source of difficulty in delimiting species (Rieseberg et al. 2006), it obscures some species boundaries in Pinaceae. Frequent hybridization between *Picea mariana* and *P. rubens*, for example, creates numerous intermediate forms that are difficult to distinguish from parental species (Bouillé et al. 2011). Hybridization has been reported as frequent in some genera of Pinaceae (Lanner and Van Devender 1998; Semerikov and Lascoux 2003; Kormatak et al. 2004; Bouillé et al. 2011; Xiang et al. 2015), but there is little evidence for current hybridization in *Tsuga*. *Tsuga caroliniana* and *T. canadensis* are partially sympatric, but hybrids have not been documented. *Tsuga canadensis* does not hybridize with northeastern Asian hemlocks in cross-pollination trials, but *T. caroliniana* will produce fertile hybrids with northeastern Asian hemlocks (Bentz et al. 2002). Morphological intermediates between *T. heterophylla* and *T. mertensiana* have been attributed to hybridization. Phytochemical analysis showed that hybridization between the two species is rare, however, and morphological intermediacy was attributed to phenotypic plasticity (Taylor 1972). Support for a sister-group relationship of *T. heterophylla* and *T. mertensiana* is unequivocal from ITS, but only moderately supported by cpDNA, which may be evidence of past hybridization (Havill et al. 2008). *Tsuga chinensis* var. *forrestii* is morphologically intermediate between *T. dumosa* and *T. chinensis* and has been hypothesized to be their hybrid (Eckenwalder 2009). In contrast, ITS, cpDNA, and mtDNA place *T. chinensis* var. *forrestii* close to *T. chinensis* and far from *T. dumosa* (Havill et al. 2008, Cun and Wang 2014). A close relationship between *T. chinensis* var. *chinensis* and *T. chinensis* var. *forrestii* was hypothesized to result from their progenitor-derivative relationship and subsequent introgression from *T. chinensis* var. *forrestii* to *T. chinensis* var. *chinensis* (Cun and Wang 2014).

Ancient hybridization has been suggested in many conifers (Wei and Wang 2004). ITS phylogenies divide *Larix* into Eurasian and North American clades (Semerikov and Lascoux

Table 3. Kimura 2-parameter cpDNA sequence divergence.

	K. davidiana	*N. longibracteata*	*T. heterophylla*	*T. canadensis*	*T. chinensis*	*T. sieboldii*	*T. ulleungensis*	*T. diversifolia*
Keteleeria davidiana								
Nothotsuga longibracteata	0.04502							
Tsuga heterophylla	0.04288	0.02284						
T. canadensis	0.04328	0.02327	0.00837					
T. chinensis	0.04395	0.02346	0.00807	0.00594				
T. sieboldii	0.04343	0.02374	0.00818	0.00615	0.00168			
T. ulleungensis	0.04462	0.02448	0.00884	0.00653	0.00476	0.00501		
T. diversifolia	0.04371	0.02314	0.00791	0.00613	0.00418	0.00416	0.0015	
T. caroliniana	0.04369	0.02359	0.00833	0.00601	0.00435	0.00457	0.00507	0.00426

2003). Chloroplast phylogenies, on the other hand, resolved Eurasian *L. sibirica* as sister to all other *Larix*. Semerikov and Lascoux (2003) suggested that this incongruence arose through ancient hybridization between a member of the Eurasian clade *Larix* and an extinct, stem-lineage *Larix*. Taiwanese endemic *Pseudotsuga wilsoniana* retains divergent copies of *LEAFY* that may be a signal of introgression of ancestors of *P. brevifolia* and the *P. gaussenii-P. sinensis-P. japonica*

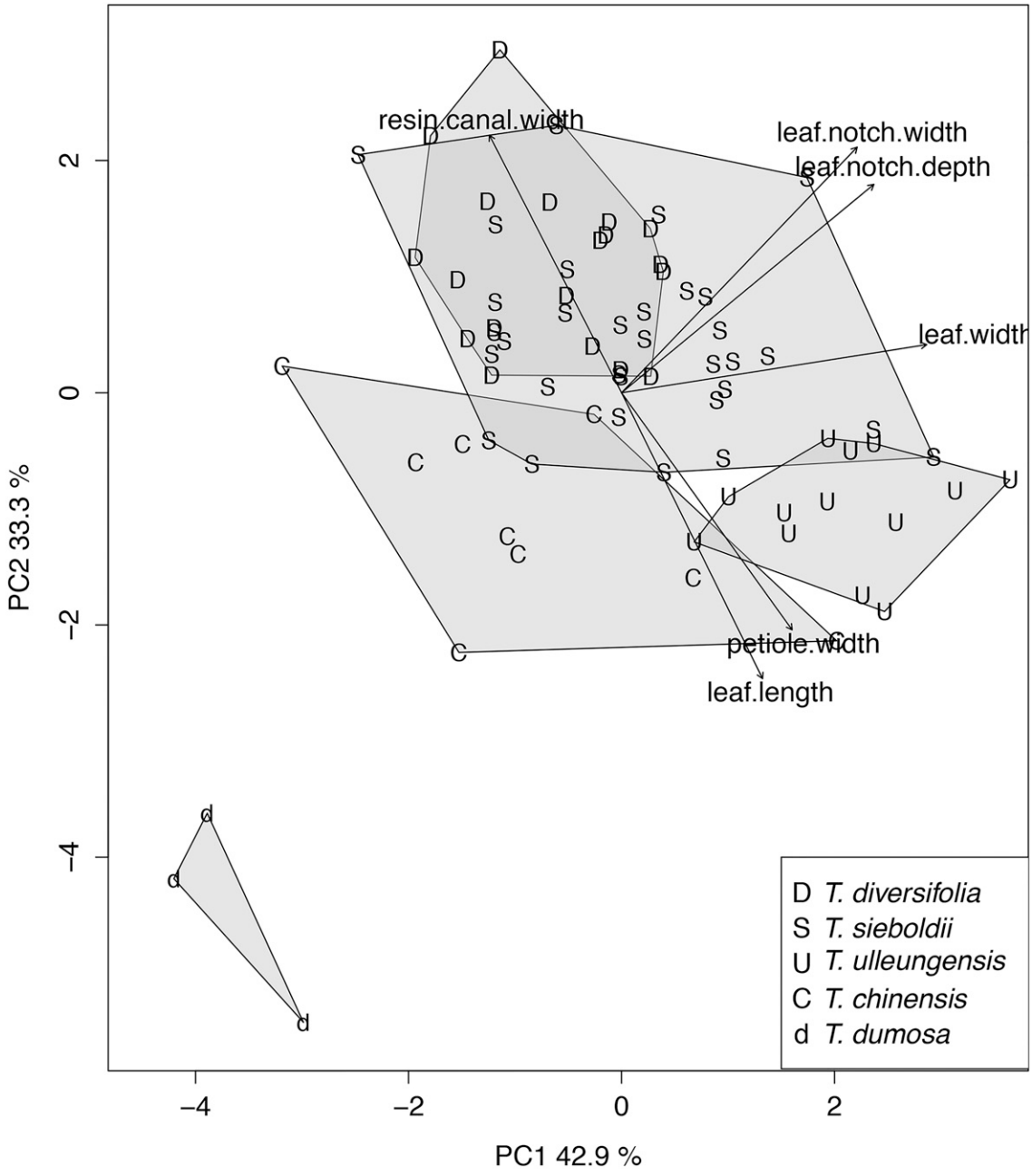

Fig. 5. PCA biplots of morphological analyses for 78 accessions of Asian *Tsuga*. The percentage of variation represented in each PCA axis is displayed. Vectors depict contributions of quantitative characters. See text for a fuller description of leaf morphological characters.

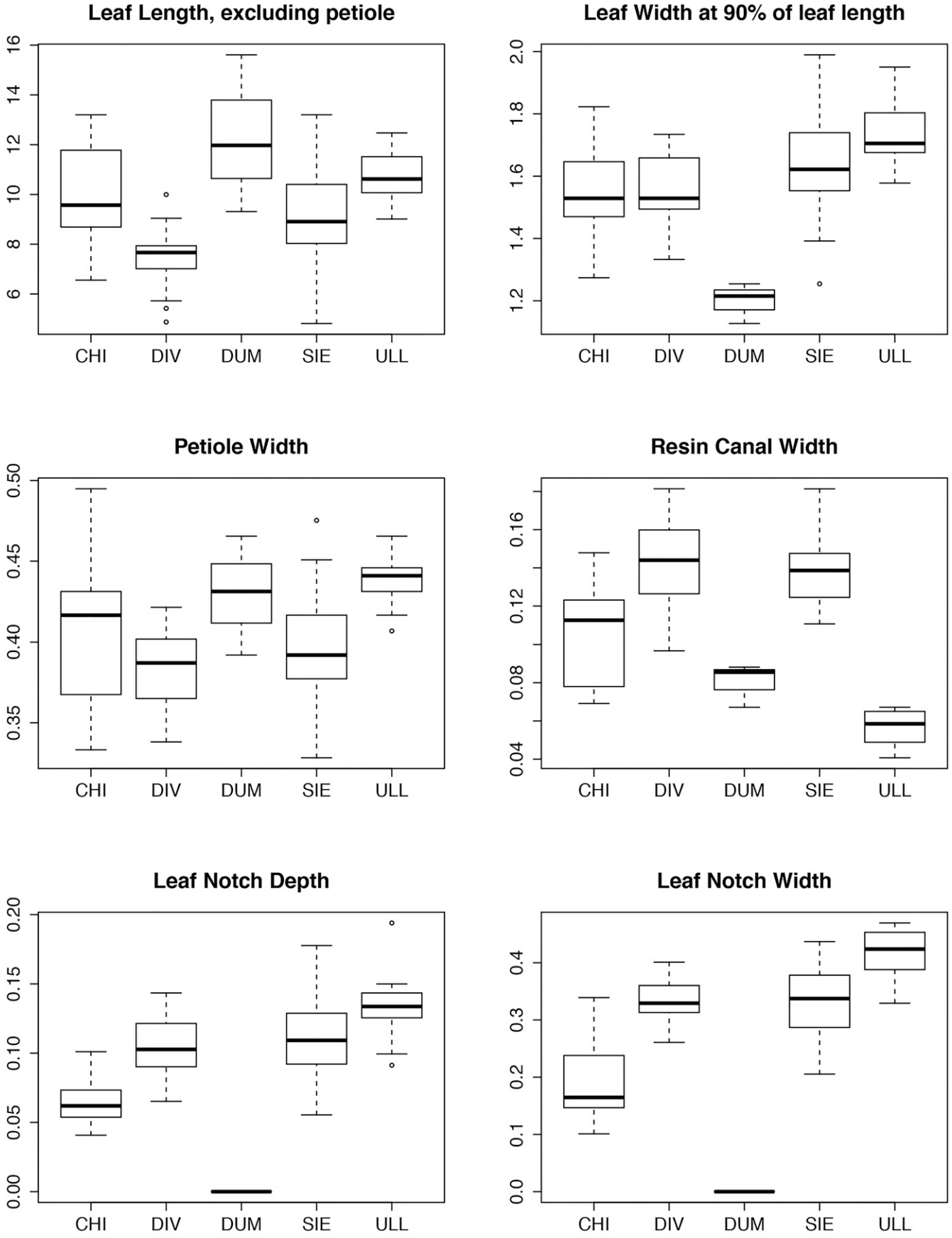

FIG. 6. Boxplots of Asian *Tsuga* leaf morphology. Y-axis is millimeters. CHI = *T. chinensis* (10 accessions); DIV = *T. diversifolia* (20 accessions); DUM = *T. dumosa* (3 accessions); SIE = *T. sieboldii* (32 accessions); ULL = *T. ulleungensis* (13 accessions).

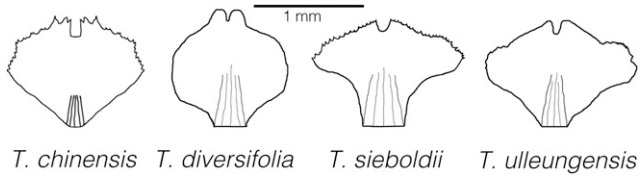

Fig. 7. Bract margins and shapes of northeastern Asian *Tsuga*, drawn from proximal (closest to the peduncle) 2–3-ovulate cone scales.

clade (Wei et al. 2010). Eastern Asian *Thuja koraiensis* apparently acquired an eastern North American *T. occidentalis* chloroplast (Peng and Wang 2008).

In *Abies*, Asian-North American disjunct section *Balsamea* is monophyletic in ITS, but paraphyletic in cpDNA, with the North American *Balsamea* species sister to a clade of sections *Momi*, *Pseudopicea*, and Asian *Balsamea* species (Xiang et al. 2015). Xiang et al. (2015) posited that section *Balsamea* originated by ancient hybridization with northeastern Asian firs contributing pollen (cpDNA) and North American firs as the seed parent (mitochondrial DNA), followed by backcrossing between Asian *Balsamea* and sections *Momi* and *Pseudopicea* (or their ancestors). CpDNA placed *Tsuga dumosa* in a clade containing *T. chinensis* and *T. sieboldii*, whereas ITS supported *T. dumosa* as sister to a clade of *T. caroliniana* and all other Asian hemlocks (Havill et al. 2008). *Tsuga dumosa* may be a hybrid between an extinct Eurasian hemlock that was similar to *T. canadensis* and an Asian hemlock (Havill et al. 2008). Finally, conflict between *4CL*, which has *T. caroliniana* and *T. chinensis* as sister species (Fig. 3), and cpDNA, which has *T. caroliniana* as sister to the clade of *T. diversifolia* and Ulleungdo hemlocks may be evidence for an ancient hybridization involving *T. caroliniana*.

Evolution of Ulleungdo Hemlocks—The *4CL* phylogeny defines two clades of northeastern Asian hemlocks: the *T. caroliniana-T. chinensis* clade and the oceanic clade (Fig. 3). The *T. caroliniana-T. chinensis* clade has smaller leaf notches and shorter petioles than oceanic hemlocks (Nan and Li-kuo 1997), and morphology shows strong similarities among hemlocks of the oceanic clade (Figs. 5, 6). Because Ulleungdo hemlocks are genetically most closely related to, but morphologically and phenologically distinct from the Japanese hemlocks, we describe them as the new species, *T. ulleungensis*.

Of the 30–40 endemic vascular plants on Ulleungdo, most are assumed to result from progenitor-derivative relationships because they are the only species in their genera on the island

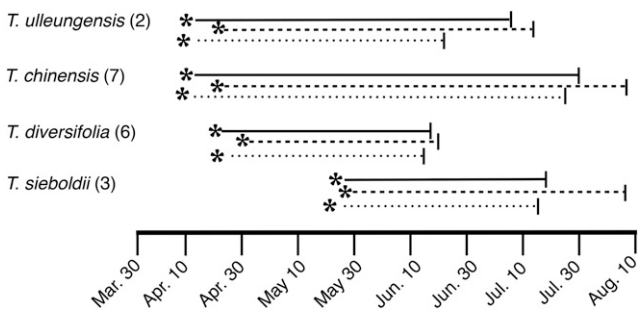

Fig. 8. Phenology of Asian *Tsuga* at the Arnold Arboretum from bud break (asterisk) to growth cessation (vertical line). Numbers of accessions are in parentheses following species. Solid lines indicate 2010, dashed lines 2011, and dotted lines 2012.

(Stuessy et al. 2006). Lack of resolution within the *4CL* oceanic clade blocks identification of a progenitor of *T. ulleungensis*. Our two *4CL* loci together moderately support a sister-group relationship of our two accessions of *T. ulleungensis* and one of our two accessions of *T. sieboldii* (Fig. 3). However, our other accession of *T. sieboldii* nests with *T. diversifolia* in a well-supported clade (Fig. 3). While *T. diversifolia* and *T. sieboldii* are the most morphologically similar Asian hemlock species, *T. ulleungensis* is morphologically closest to *T. sieboldii* and relatively distinct from *T. diversifolia* (Figs. 5, 6). It is therefore plausible that *T. ulleungensis* evolved anagenetically from *T. sieboldii* prior to the latter's putative capture of the *T. chinensis* chloroplast.

Hybridization may have been involved in the ancestry of the Ulleungdo endemic *Fagus multinervis* based on incongruence between cpDNA and *LEAFY* (Oh et al. 2016). In addition, it appears to be distinct from but closely related to the Japanese endemic *F. japonica* and the Chinese *F. engleriana*. The prevalence of hybridization in conifers suggests its potential role in the ancestry of *T. ulleungensis*, but there is no clear evidence for this possibility.

Use of cpDNA as a molecular clock would be misleading for *T. sieboldii* because of putative chloroplast capture. If *T. sieboldii* and *T. ulleungensis* are indeed sister taxa, then their divergence would have been more recent than Havill et al.'s (2008) estimate of about 10 MYA for the split between *T. ulleungensis* and *T. diversifolia*. Volcanic analyses indicate that Ulleungdo originated about 2.7 MYA (Kim and Lee 1983) and has likely been occupied by vegetation only since 1.7 MYA (Kim 1985). While the oceanic clade does not currently occur on mainland Asia, fossils similar to *T. diversifolia* were widely distributed in North America and Eurasia in the Eocene (Matsumoto et al. 1995; LePage 2003). Miocene fossils from Japan resemble *T. caroliniana*, *T. diversifolia*, and *T. heterophylla* (LePage 2003), and *Tsuga* fossils from the Pliocene have been discovered on the Korean mainland (Kong 2000). Despite the presence of suitable habitat, *T. diversifolia* is currently absent from Hokkaido, but was present there in the late Quaternary (Tsuyama et al. 2014). Tsuyama et al. (2014) hypothesized that *T. diversifolia* was extirpated from Hokkaido by the colder, drier climate during the last glacial period, and has not yet been able to recolonize. A similar climate in Korea in the late Quaternary may have restricted Korean *Tsuga* to the climatically milder Ulleungdo. This glacial refuge may have been the place of origin of *T. ulleungensis*.

Taxonomic Treatment

Tsuga ulleungensis G. P. Holman, Del Tredici, Havill, N. S. Lee, and C. S. Campb., sp. nov.—TYPE: SOUTH KOREA. Ulleungdo. 2 June 1917, *E. H. Wilson 8529* (GH!).

Korean name: Ul-leung-sol-song-na-mu

Trees to 22 m, trunk 0.5–0.8 m diam. Branchlets glabrous, 1–1.5 mm in diam. Buds: 2–3(–4.1) mm long, 1.8–2.4(–3.8) mm wide; globular or obovoid; glabrous; scales orbicular, dark brown, with crenate margins. Mature leaves: petioles 0.9–1.6 mm long, 0.3–0.6 mm wide; blades (4–)8–14(–22) mm long, (1.0–)1.7–2.1(–2.3) mm wide; base attenuate; margins entire and slightly thickened; apex emarginate; hypostomatic, stomata in two broad, dull white to buff bands on either side of midrib, each band with mostly 8 rows of stomata, decreasing to 2–3 proximally, and 4–5 distally; with a single resin canal,

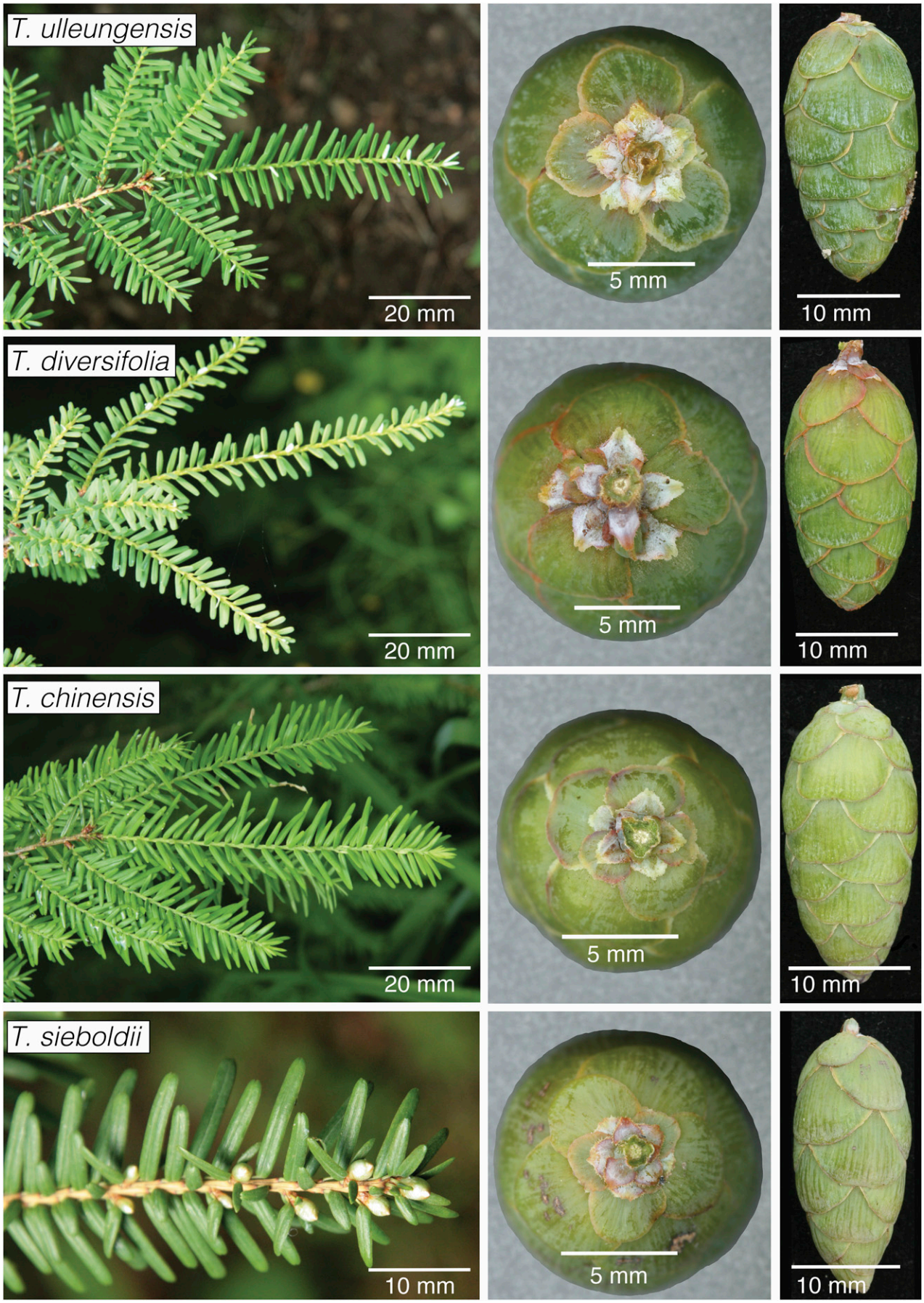

FIG. 9. Comparison of northeastern Asian *Tsuga*. From left to right: foliated branches on 23 May 2012, showing phenological state; proximal view of ovulate cone, showing ovulate cone-scale bracts near the peduncle; side view of ovulate cone.

Fig. 10. Mature tree of *T. ulleungensis*, approximately 30 m tall, 450 m elevation, 11 May 2008, at Namseo, Taeha Ryeong Protected Reserve (Natural Monument #50) on Ulleungdo. Photo credit: Peter Del Tredici.

0.4–0.7 mm in diam at midpoint of leaf. Pollen cones 4–8 mm long, reddish-purple. Young ovulate cones purple; bracts broader than long, with more or less crenulate margins, apex with a bifid apical projection 0.5 times as long as bract body. Mature ovulate cones globular to ovoid; 19–30 mm long, 18–26 mm wide when open; on 1–2 mm long peduncles; cone scales number 20–26, 9–15 mm long, 10–15 mm wide. Seeds 4–5.5 mm long, 2–2.5 mm wide; with wings 4.5–8.5 mm long, 3.4–4.2 mm wide. Figure 2.

Additional Specimens Examined—All specimens are from Korea, North Gyeongsang Province. Specimen information is organized by locality, date, *collector, collection number* (herbarium). Specimens from botanical gardens also include the garden, accession number, and wild collection locality, if known. Plants included in the common garden phenology study are noted as CG #. GenBank accession numbers for specimens used in molecular analyses indicated as: (cpDNA; *4CL* locus 1; *4CL* locus 2). A dash (–) indicates locus not sequenced for that specimen. AA = Arnold Arboretum; cult. = in cultivation; PDT = collected by Peter Del Tredici. AA 1251–83A, cult., CG #4, wild origin: Namseo, between Taeha-dong and Manyang-dong, voucher: *Holman 09–16* (AAH) (KX256185; KX354396; KX354397); AA 1251–83B, cult., CG #3, wild origin: Namseo, between Taeha-dong and Manyang-dong, voucher: *Holman 08–13* (AAH); Jeodong, Jeodong, 35 m, 10 May 2008, *PDT 3*; Jeodong, Sol-song-na-mu, 22 Feb 2005, *Lee, Y.N.* (AAH); Namseo, Taeha Ryeong Protected Reserve, 440–480 m, 11 May 2008, *PDT 4–1*, *8*, *13* (–; KX354394; KX354395), *15*, *20*, and *24* (AAH); Namseo, below the Taeha Ryeong Protected Reserve, 310 m, 11 May 2008, *PDT 5–2* (AAH); Namseo, end of paved road #5, below the Taeha Ryeong Protected Reserve, 240 m, 11 May 2008, *PDT 7–2* and *3* (AAH); Nari, Nari Basin, 400 m, 11 May 2008, *PDT 8*; Ulleung, Naesujeon, 10 May 2008, *PDT 1* (AAH).

Distribution and Habitat—*Tsuga ulleungensis* is known only from Ulleungdo, where it grows on north facing slopes from 310 to 500 m above sea level (Fig. 10). Plants occur primarily on well-drained rocky ridges in a forest community type dominated by *Pinus parviflora* Siebold & Zuccarini (Kim 1988). Other woody plants commonly associated with *T. ulleungensis* include *Acer pseudosieboldianum* subsp. *takesimense* (Nakai) P.C. de Jong, *Camellia japonica* L., and *Fagus multinervis* Nakai.

Conservation—According to IUCN criteria, *T. ulleungensis* may be considered "critically endangered" due to its restricted geographic range (IUCN 2001). Pacific islands are a global hotspot for endemic conifers (Rumeu et al. 2014), and recognition of *T. ulleungensis* evidences uniqueness of the Ulleungdo flora.

Etymology—The epithet *"ulleungensis"* is derived from Ulleungdo, where the new species is found.

Key to Oceanic Hemlocks

1. Twigs hairy, stomatal bands snow-white, proximal (closest to the peduncle) 2–3 ovulate cone-scale bract margins entire to crenulate ..... *T. diversifolia*
1. Twigs glabrous, stomatal bands dull white to buff, proximal 2–3 ovulate cone-scale bract margins erose to crenulate ... 2
   2. Resin canals at midpoint of leaf length 0.8–1.5 mm in diam, proximal 2–3 ovulate cone-scale bract margins erose to denticulate (with many tiny sharply-pointed teeth distributed along the whole margin) ..... *T. sieboldii*
   2. Resin canals at midpoint of leaf length 0.40–0.7 mm in diam, proximal 2–3 ovulate cone-scale bract margins crenulate to entire (with small, rounded teeth on the sides and no teeth or wavy, large teeth towards central cone-scale bract notch) ..... *T. ulleungensis*

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Appendix 1. List of accessions examined for species other than *T. ulleungensis*. GenBank accession numbers for specimens used in molecular analyses are indicated as: (cpDNA; *4CL* locus 1; *4CL* locus 2), and new sequences are in bold. Dash (–) indicates locus not sequenced for that specimen. Specimen information is organized by species, locality, date, *collector, collection number* (herbarium). Specimens from botanical gardens also include the garden, specimen number in cultivation, and wild collection locality, if known. Plants included in the common garden phenology study are noted as CG#. AA = Arnold Arboretum; cult. = in cultivation; FHBG = Fay Hyland Botanical Garden, Orono, Maine; PDT = collected by Peter Del Tredici; USNA = United States National Arboretum.

Keteleeria davidiana (relevant publication: Wu et al. 2009), (NC011930; –;–).

Nothotsuga longibracteata (relevant publication: Wang et al. 2000), (–;AF144523;–); CHINA. USNA 70160, cult., wild origin: Fujian Prov., voucher: *Bentz SEB3* (NA)
(**KX249803**;–;–).

Tsuga canadensis UNITED STATES. FHBG, cult., wild origin: Maine, voucher: *Holman 08-02* (AAH) (**KX255640**; **KX354416**; –); (relevant publication: Wang et al. 2000), (–; AF144525; AF144526).

Tsuga caroliniana UNITED STATES. FHBG, cult., wild origin: North Carolina, voucher: *Holman 08-03* (AAH) (**KX256180**; **KX354414**; **KX354415**). AA705-93, cult., wild origin: North Carolina, voucher: *Holman 09-50* (AAH) (–; **KX354412**; **KX354413**).

Tsuga chinensis CHINA. Anhui Prov.: Huangshan, 23 Oct 1933, *W.C. Cheng 4594* (NA); Anhui Prov.: Huangshan, 12 Aug 1924, *A.N. Steward 7194* (NA); Fujian Prov.: Wuyishan, 1,800 m, 1 Jan 1981, *Y.-T. Zhang 2561* (NA); Guizhou Prov.: Yinjiang, Tongren, 840 m, 1931, *W.C. Cheng 7972* (AAH); Hubei Prov.: Fangxian, Shiyan, 8,000–9,000 ft, 1 May 1907, *E.H. Wilson 5096* (AAH); AA 17569, cult., wild origin: Hubei Prov., voucher: *Holman 09-72* (AAH) (–; **KX354410**; **KX354411**). Sichuan Prov.: Ziyang, 3 Aug 1989, *Qing-Sheng 207* (AAH); Sichuan Prov.: Lucheng, Kangding, N.E. of Tachienlu, 7,000–9,000 ft, 2 Jul 1908, *E.H. Wilson 2100* (AAH); Sichuan Prov.: Lixian, Ching-Chi Hsien, 8,500 ft, 1 Aug 1908, *E.H. Wilson 5097* (AAH); AA 233-2003, cult., wild origin: Sichuan Prov., voucher: *Holman 10-02* (AAH) (**KX256181**; **KX354408**; **KX354409**).

Tsuga chinensis TAIWAN. Chiayi County: Mt. Morrison, Kagi Prov., 2,500–3,833 m, 25 Oct 1918, *E.H. Wilson 10904* (AAH).

Tsuga chinensis VIETNAM. Ha Giang Prov.: Ngan Chai, Ha Giang Prov., Yen Minh dist., 1,400m, 26 Nov 2004, *S.K. Wu 705* (AAH).

Tsuga diversifolia JAPAN. Nagasaki Pref.: cult., wild origin unknown, 1863, *Maximowicz* (AAH); AA 17571A, cult., CG #1, origin: Tokyo Pref.: Shinjuku, Imperial Botanical Garden, wild origin unknown, 1897, voucher: *Holman 08-15* (AAH); AA 39-82A, cult., CG #7, origin: 1982 cutting of AA 17571, Tokyo Pref.: Shinjuku, Imperial Botanical Garden, voucher: *Holman 08-11* (AAH) (–; **KX354406**; **KX354407**); AA 1837-77A, cult., CG #6, wild origin: Iwate Pref.: Iwanebashi, Mount Hayachine, subalpine zone, 16 Sep 1977, *S.A. Spongberg 220* (AAH), voucher: *Holman 08-11* (AAH) (–; **KX374104**; **KX374105**); Aomori Pref.: Mt. Hakkoda, 1,000m, 03 Oct 1892, *C.S. Sargent* (AAH); Fukushima Pref.: Takayu, 24 Jun 1904, *Baurie 5747* (AAH); Fukushima Pref.: east slope of Mt. Higashiazuma, 1,700–1,975 m, 06 Jul 1984, *H. Ohashi 11831* (AAH); Fukushima Pref.: Hinoemata, Ayamedaira, Oze, 20 Jun 1951, *M. Mizushima 1195* (AAH); Fukushima Pref.: Tsuchiyuonsenmachi, Mt. Azuma, 05 Aug 1952, *K. Uno 2612* (AAH); Gifu Pref.: Mino, Mt. Yenasam, 16 Aug 1910, *Anonymous* (NA); Gifu Pref.: Takayama, Norikura, 27 Jun 1955, *H. Muroi 3852* (AAH); Gunma Pref.: Katashina, Konsei Pass, 2,000–2,020 m, 23 Aug 1982, *T. Yahara 6350* (AAH); Iwate Pref.: Iwanebashi, Mt. Hayachine, Aionzawa, 1,000m, 08 Jul 1987, *Tohda 2058* (AAH); Iwate Pref.: Rikuchukawai, Rikuchu, Mt. Hayachine, 1,000–2,000 m, 27 Sep 1915, *E.H. Wilson 7557* (AAH); Nagano Pref.: Chino, Mt. Yatsugadake, 27 Sep 1986, *R. Nicholson 1085-86* (AAH); Nagano Pref.: Matsumoto, Nakanoyu, Nagano, 26 Jul 1955, *H. Muroi 3436* (AAH); Nagano Pref.: Sancho, Kurayu-yama, 1,300–1,900 m, 25 May 1977, *E.W. Wood 3696* (AAH); Nara Pref.: Yamato, Shores of Lake Yamato, 05 Sep 1892, *C.S. Sargent* (AAH); Oita Pref.: Bungotaketa, Bungo: Mt. Sobo, 29 Aug 1911, *E.H. Wilson 'Drugoi'* (AAH); Tochigi Pref.: Shimotsukeosawa, Nikko region, 1,000–2,500 m, 16 Oct 1914, *E.H. Wilson 7646* (AAH); Tokushima Pref.: Shikoku, Mt. Tsurugi, Gyoba, 1,700 m, 06 Jun 1984, *T. Yamazaki 5271* (AAH); Yamagata Pref.: Akumi, Mt. Chokai, 14 Oct 1914, *E.H. Wilson 7187* (AAH); Yamanashi Pref.: Minamitsuru, Minamitsuru-gun, Mt. Fuji, 11 Jun 1978, *H. Ohba 78601* (AAH); Yamanashi Pref.: Oniwa, N.W. side of Mt. Fuji, 2,300 m, 15 Sep 1978, *Y. Tateishi 15712* (AAH); AA 1146-86, cult., wild origin: Yamanashi Pref., voucher: *Holman 09-71* (AAH) (**KX256182**; **KX354404**; **KX354405**).

Tsuga dumosa CHINA. Sichuan Prov.: Liangshan, Mountains of Kopati, Djago, and Muli, 2,900–3,535 m, 1 Jun 1928, *J.F. Rock 16163* (NA); Yunnan Prov.: Yangbi, w. side of Diancang Shan mountain range, 3,000 m, 19 Jun 1984, *B. Bartholomew 293* (AAH).

Tsuga dumosa MYANMAR. Kachin State: Jiangao Shan on trail E of Baduolin Yakou, 3,020m, 23 May 2006, *L. Heng 30356* (GH).

Tsuga heterophylla UNITED STATES. Washington State: Clearwater National Forest, Palisades-White Pass. *Peery 7-1* (**KX256183**;–;–).

Tsuga mertensiana (relevant publication: Wang et al. 2000), (–;AF144524;–).

Tsuga sieboldii JAPAN. USNA 55482-H, cult., wild origin: Mie Pref.: Ise City, Forest of Gegu Shrine, 1 Jan 1956, *Creech*, voucher: *Bentz 13* (AAH); AA 1007-80A, cult., CG #8, wild origin: Tochigi, Nikko, Japan, Tochigi Prefecture, Hosco, Nikko and Vicinity, 21 Aug 1980, voucher: *Holman 09-09* (AAH) (**KX256184**; **KX354398**; **KX354399**); AA 1007-80B, cult., CG #5, wild origin: Tochigi, Nikko, Japan, Tochigi Prefecture, Hosco, Nikko and Vicinity, 21 Aug 1980, voucher: *Holman 08-17*; AA 17577A, cult., CG #2, wild origin unknown, Kyoto, Japan, Imperial Forest School, Japan, 20 Mar 1908, voucher: *Holman 08-16* (AAH) (–; **KX354400**; **KX354401**); Aichi Pref.: Nagasuda, 1892, *C.S. Sargent* (AAH); Chiba Pref.: Amatsu, Tokyo Imperial University, Forest back of Amatsu, 7 Sep 1928, *Beattie 10352* (NA); Chiba

Pref.: Futtsu, Mt. Nokogiri, Nishitama-gun, 19 Mar 1953, *M. Miyushima 11411* (AAH); Chiba Pref.: Kamogawa, Awa-gun, Mts. Kiyosumi, Yomogi, 200 m, 9 May 1976, *Y. Tateishi 2317* (AAH); Gifu Pref.: Hida, 7 Aug 1933, *K. Shiota 6685* (AAH); Gifu Pref.: Hida, 7 Aug 1933, *K. Shiota 6746* (AAH); Gifu Pref.: Mino, 9 Aug 1933, *K. Shiota 6790* (AAH); Hiroshima Pref.: Miyajima, 17 Apr 1903, *Baurie 5349* (AAH); Hyogo Pref.: Fukuzaki, Hyogo Pref.: Mt. Nanakusa-yama, Kanzaki-gun, 200–300 m, 26 May 1979, *S. Tsugaru 5375* (AAH); Kagoshima Pref.: Yakushima, Kumage-gun, Yaku-cho, Yugo forestry road to Mt. Shichigo, 880–1,488 m, 30 Jul 1992, *S. Kobayashi 1731* (AAH); Kagoshima Pref.: Yakushima, 500–1,150 m, 21 Feb 1914, *E.H. Wilson 6037* (AAH); Kanagawa Pref.: Nakahara, Japonia. Yokohama, 1862, *Maximowicz* (NA); Kishi Pref.: Koya, 1,000m, 2 Dec 1914, *E.H. Wilson 7845* (AAH); Kyoto Pref.: Hiyoshi, Kbidani, Yotsuya, Funai-gun, 650m, 5 Jul 1995, *S. Tsugaru 22360* (AAH); Kyoto Pref.: Kameoka, Fudo-Saga-Kameoka-sen, Hodzu-cho, Kameoka-shi, 24 Jun 2001, *S. Tsugaru 30684* (AAH); Kyoto Pref.: Keihoku, near Koshiki-toge, Kitakuwada-gun, 780m, 6 Jul 1995, *T. Takahashi 2861* (AAH); Kyoto Pref.: Keihoku, Hattyo-rindo, Kitakuwada-gun, 490 m, 17 Sep 1994, *S. Tsugaru 20940* (AAH); Kyoto Pref.: Nantan, Hiyashi, Kyushu, 3 Aug 1914, *E.H. Wilson 6221* (AAH); Nagano Pref.: Nagawa, Minami-azumi-gun, Nyuyamaguchi, 900 m, 6 Jun 1983, *T. Yahara 6946* (AAH); Nagano Pref.: Shimoina, Urugi, 13 Jun 1957, *Yamazaki 3794* (AAH); Nara Pref.: Wakakusayama, 310 m, 18 May 2008, *PDT 15-11* (AAH); Nara Pref.: Wakakusayama, 310m, 18 May 2008, *PDT 15-12* (AAH); Osaka Pref.: Kawachinagano, Iwawaki-san, 570 m, 8 Jun 2004, *N. Havill 04-30* (GH); Osaka Pref.: Takatsuki, Myo-on-ji Temple, 375–400 m, 17 May 2008, *PDT 13-8* (AAH); Osaka Pref.: Takatsuki, Myo-on-ji Temple, 375–400 m, 17 May 2008, *PDT 13-9* (AAH); Osaka Pref.: Takatsuki, Nakahata, 370m, 9 Jun 2004, *N. Havill 04-34* (GH); Osaka Pref.: Toyono, Yoshikawa, Toyono-cho, 180 m, 9 Jun 2004, *N. Havill 04-33* (GH); Tosa Pref.: Motoyama, Shikoku, Shiraga, 1,000–1,600 m, 22 Nov 1914, *E.H. Wilson 7791A* (AAH); Tosa Pref.: Shikoku, 500–1,000 m, 19 Nov 1914, *E.H. Wilson 7791* (AAH); Wakayama Pref.: Kogawa, Tomisato, Oto-mura, Nishimuro-gun., 500 m, 11 Aug 1965, *G. Murata* (AAH); Wakayama Pref.: Koya, Kongo-buji Temple, Koya-cho, 820 m, 8 Jun 2004, *N. Havill 04-31* (GH); Wakayama Pref.: Koya, Mt. Benten, Koya-san, 920 m, 8 Jun 2004, *N. Havill 04-32* (GH).

Appendix 2. Third-generation DNA sequencing metrics.

Species	Barcode	Contigs	Read Length	Reads	Coverage
Nothotsuga longibracteata	CGTT	27	33	2,411,734	676
Tsuga canadensis	GATT	93	33	350,266	98
Tsuga caroliniana	CACTCA	23	100	2,651,733	2,253
Tsuga chinensis	ACGT	38	80	952,434	647
Tsuga diversifolia	CTGT	31	80	1,734,013	1,178
Tsuga heterophylla	AACT	61	50	1,855,346	788
Tsuga sieboldii	GATT	44	80	1,808,736	1,229
Tsuga ulleungensis	TCAT	30	80	1,726,604	1,173