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# Nuclear and Cytoplasmic DNA Sequence Data Further Illuminate the Genetic Composition of Leyland Cypresses

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**Abstract. Leyland cypress [×*Hesperotropsis leylandii* (A.B. Jacks. & Dallim.) Garland & G. Moore, Cupressaceae] is a well-known horticultural evergreen conifer in the United Kingdom, United States, Australia, New Zealand, and other countries. As demonstrated by previous studies, this taxon is a hybrid between alaska (nootka) cypress [*Callitropsis nootkatensis* (D. Don) Oerst. ex D.P. Little] and monterey cypress [*Hesperocyparis macrocarpa* (Hartw. ex Gordon) Bartel]. However, the genetic background of leyland cypress cultivars is unclear. Are they $F_1$ or $F_2$ hybrids or backcrosses? In this study, six individuals that represent major leyland cypress cultivars and two individuals each of its two putative parental species were collected, and three nuclear DNA regions (internal transcribed spacer, leafy and needly), three mitochondrial (mt) DNA regions (*cox*I, *atp*A, and *rps*3), and two chloroplast (cp) DNA regions (*mat*K and *rbc*L) were sequenced and analyzed. Sequencing results of nuclear DNA regions revealed that leyland cypress cultivars consist of putative $F_1$ and $F_2$ hybrids as well as backcrosses. Analysis of the cp and mt DNA from six cultivars of leyland cypress revealed that their cytoplasmic (cp and mt) genomes came from alaska cypress. Our findings will provide important instructions and background knowledge on the management of these major leyland cypress cultivars as well as future studies. Meanwhile, alaska cypress and monterey cypress may have diverged with each other ≈46 million years ago. The fact that they can produce fertile hybrids indicates that hybridization events may have played an important role in the evolutionary history of the cypress family (Cupressaceae).**

Leyland cypress, a rapidly growing hybrid conifer that possesses important ornamental and economic values, is widely cultivated in the United Kingdom, United States, Australia, New Zealand, and many other countries (Hinesley et al., 2008; James, 2011; Lindstrom, 1992; Mitchell, 1996; Sturrock, 1989). Leyland cypress cultivars have been postulated to be spontaneous hybrids between monterey cypress and alaska (nootka) cypress (known by many common names including alaska cypress, nootka cypress, yellow cypress, alaska yellow cypress, alaska cedar, nootka cedar, yellow cedar, and alaska yellow cedar) (Farjon, 2005; Mitchell, 1996; Owens et al., 1964). Leyland cypresses were first raised through spontaneous hybridization in the United Kingdom when alaska cypress and monterey cypress were imported and planted together (see Adams et al., 2006, for a brief review); during the period from 1888 to ≈1911, many popular cultivars were generated there (Adams et al., 2006; Mitchell, 1996; Owens et al., 1964). Leyland cypresses have morphological traits such as the size of the cone, the number of scales, and the number of seeds that are intermediate between alaska cypress and monterey cypress (Yamaguchi et al., 2000). Leyland cypresses exhibit over-parent heterosis. They grow taller and faster than both parent species and

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tolerate a wide range of soil type (e.g., clay, sand, acid, alkaline, etc.), different light conditions (full sunlight or partial shade), and various sites from mesic to semiarid (Yamaguchi et al., 2000). Thus, many cultivars have been selected that differ in coloration and growth habit for use in shelterbelts, hedges, landscape plantings, wood production, and Christmas tree plantations in the United Kingdom, United States, Australia, New Zealand, and other countries (Mitchell, 1996; Yamaguchi et al., 2000). There are more than 40 cultivars of leyland cypresses. Six of the most popular cultivars are Castlewellan, Galway Gold, Green Spire, Haggerston Grey, Leighton Green, and Naylor's Blue (Adams et al., 2006; James, 2011; Mitchell, 1996).

Although leyland cypress has long been regarded as a hybrid between monterey cypress and alaska cypress, it was only at the end of the last century when this hypothesis was tested using DNA data. Generally, nuclear DNA regions are biparentally inherited, whereas cp and mt DNA regions are paternally inherited in the cypress family, especially in the subfamily (Cupressioideae) that monterey cypress and alaska cypress belongs to (Kondo et al., 1998; Mogensen, 1996; Neale et al., 1989, 1991; Sakaguchi et al., 2014; Whittle and Johnston, 2002). Yamaguchi et al. (2000) examined the 152-bp nuclear ribosomal DNA (nrDNA) sequences (*18S* rDNA) and 436-bp cp DNA (*rbc*L) sequences that were generated from a single leyland cypress tree, a single alaska cypress tree, and two monterey cypress trees. One base difference in the nrDNA sequences was found between alaska cypress (A) and monterey cypress (T). The leyland cypress tree had nucleotides of both species at this site (A and T). Two bases were found to differ between alaska cypress and monterey cypress in cp DNA (*rbc*L) sequences. The *rbc*L sequence of leyland cypress was identical to that of alaska cypress. They proposed a hybrid origin of this leyland cypress tree with monterey cypress and alaska cypress as parental species and alaska cypress as the likely cp genome donor to leyland cypress (Yamaguchi et al., 2000). Subsequently, Adams et al. (2006) investigated the hybrid origin of leyland cypresses by surveying 25 leyland cypress, three monterey cypress, and two alaska cypress plants using random amplified polymorphic DNA (RAPD). Principal coordinates analysis of a total of 77 RAPD bands revealed that leyland cypress individuals were ordinated in an intermediate position between monterey cypress and alaska cypress; and several complementary bands from either monterey cypress or alaska cypress were found in leyland cypress. Combining with preliminary intersimple sequence repeat results, which showed a similar pattern to RAPD results, both of these dominant markers suggested the hybrid origin of leyland cypress from parental species, monterey cypress and alaska cypress (Adams et al., 2006).

Art. H.6.2 of the International Code of Nomenclature for algae, fungi, and plants [Melbourne Code (McNeill et al., 2012)] requires that nothogeneric name of a bigeneric hybrids must be a combination of the names of the parental genera. However, the generic name for leyland cypress became unstable because the Latin names for the genera of their parent species, alaska cypress and monterey cypress, are controversial (for a detailed review on this issue, see Garland and Moore, 2012). In previous publications, alaska cypress has been assigned to four different genera (*Cupressus* L., *Chamaecyparis* Spach, *Callitropsis* Oerst., *Xanthocyparis* Farjon & T.H.Nguyên) as has monterey cypress (*Cupressus*, *Callitropsis, Hesperocyparis* Bartel & R.A.Price, and *Neocupressus* de Laub.) (De Laubenfels, 2009). According to recent molecular phylogenetic researches (Little, 2006; Little et al., 2004; Mao et al., 2010, 2012; Terry et al., 2012) and nomenclature publications (Adams et al., 2009; Garland and Moore, 2012; Little, 2006; Little et al., 2004; Mill and Farjon, 2006), the Old World cypresses (Fig. 1) clustered into a monophyletic clade, and the New World cypresses (Fig. 1), together with alaska cypress and vietnamese golden cypress (*Xanthocyparis vietnamensis* Farjon & Hiep), formed another monophyletic clade (Fig. 1); these two clades were supposed to be sisters with each other (Mao et al., 2010, 2012) or that either of them were sisters to junipers (*Juniperus* L.) (Little, 2006; Little et al., 2004). Several taxonomic treatments have been proposed for these taxa (Adams et al., 2009; Christenhusz et al., 2011; Farjon, 2005; Farjon et al., 2002; Little, 2006) [for details, see Fig. 1 (K. Rushforth prefers to treat both alaska cypress and monterey cypress as part of *Cupressus* but is of the opinion that the generic status of the parent species is not relevant to the science reported here)], but a recent taxonomic treatment (Adams et al., 2009; Fig. 1D) represented major contributions of previous works well and has been accepted by a broad botanical community (e.g., Baldwin et al., 2012; Garland and Moore, 2012; Mao et al., 2012; Yang et al., 2012). Therefore, the latest legitimate nomenclature of leyland cypress was proposed as ×*Hesperotropsis leylandii* (Garland and Moore, 2012).

There is still little known about the detailed genetic background of leyland cypress. It has not been well substantiated whether all forms of this taxon are $F_1$ hybrids as early cultivation history documented or if some of the cultivars are $F_2$ hybrids or backcrosses to the parental species. The origin of their cytoplasmic (cp and mt) genome is still not known with certainty. In this study, we analyzed six leyland cypress cultivars, two alaska cypress putative parents and two monterey cypress putative parents, by sequencing three nuclear DNA regions [internal transcribed spacer (ITS), leafy and needly], two cp DNA regions (*mat*K and *rbc*L) as well as three mt DNA regions (*atp*A, *cox*I, and *rps*3). Our purpose was to confirm the hybrid/backcross origin of leyland cypress cultivars using multiple DNA regions from all three plant genomes and further investigate the genetic background of leyland cypress.

## Materials and Methods

**Plant materials.** Leaf samples used in a previous study (Adams et al., 2006) were obtained for six leyland cypress cultivars. These cultivars were Green Spire and Haggerston Grey (whose maternal parent have been said to be alaska cypress), Leighton Green and Naylor's Blue (reported to have monterey cypress as the maternal parent), Castlewellan [reputedly from seed in a cone from monterey cypress (cv. Lutea) growing near an alaska cypress (cv. Aurea)], and 'Galway Gold' (of unknown origin but often thought to be a renaming of 'Castlewellan' or a sister seedling) (Adams et al., 2006). Leaf materials were obtained from the two putative parent species of leyland cypress, monterey cypress, and alaska cypress, which were used in Adams et al. (2006). These two species are both endemic to the west coast of North America, but because leyland cypress was originally raised in the United Kingdom, samples of two accessions for each of the two species were obtained from Botanic gardens in the United Kingdom (including from both putative parent trees of 'Castlewellan' and possibly 'Galway Gold'). Adams and Rushforth (Adams et al., 2006) collected fresh foliage from living trees and placed the

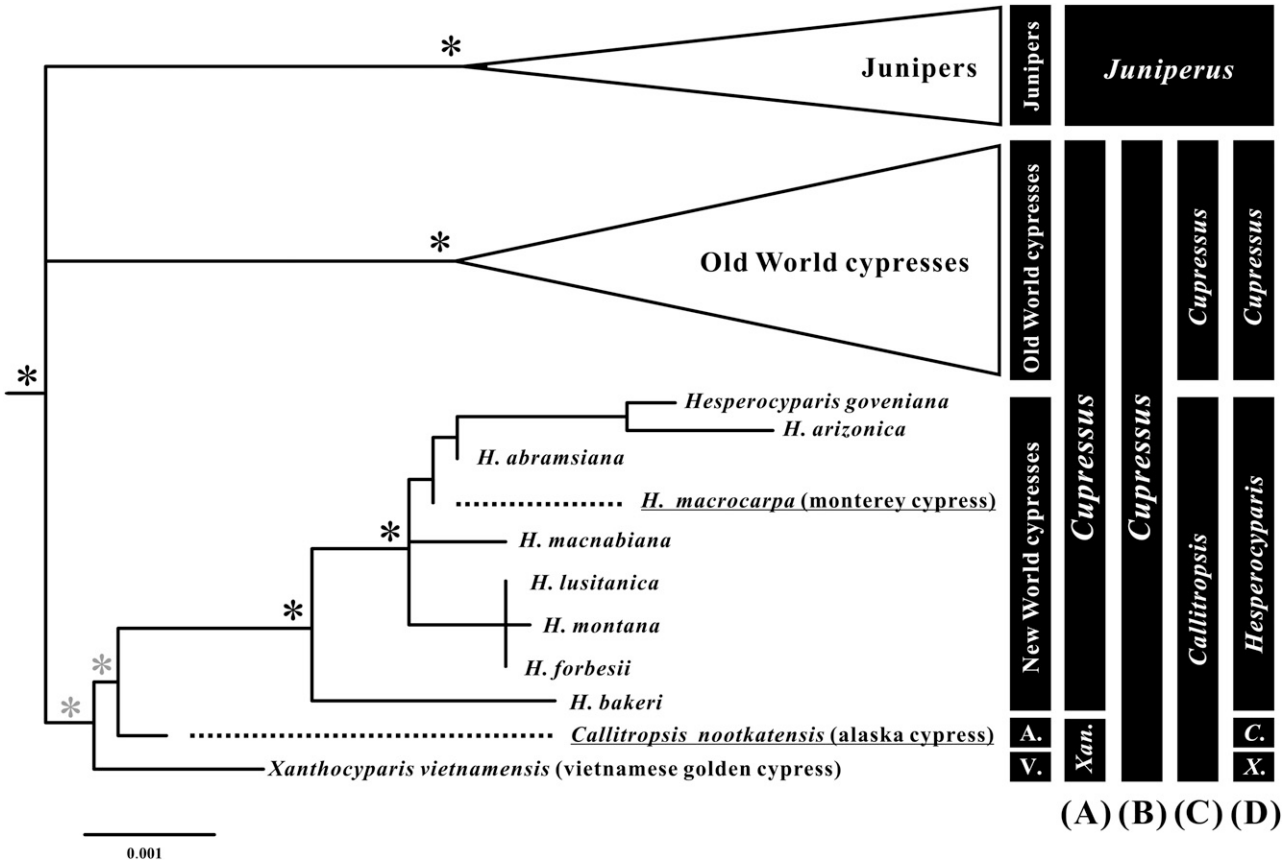


Fig. 1. A cladogram that shows phylogenetic relationships among the Old World cypresses, the New World cypresses, alaska cypress, vietnamese golden cypress, and junipers (courtesy of Mao et al., 2012: Fig. S1), and taxonomic treatments of different authors. Black bars to the right of the cladogram illustrate common names of all involved taxa, (**A**) the taxonomic treatment of Farjon (2005), (**B**) the taxonomic treatment of Christenhusz et al. (2011), (**C**) the taxonomic treatment of Little (2006), and (**D**) the taxonomic treatment of Adams et al. (2009). Brach lengths represent genetic distances as calculated using maximum likelihood approach, black and gray asterisks above branch indicate strong and moderate bootstrap support values, respectively: A. = alaska cypress; V. = vietnamese golden cypress; *C.* = *Callitropsis nootkatensis*; *X.* = *Xanthocyparis vietnamensis*; *Xan.* = *Xanthocyparis*.

leaves in silica gel, transported them back to the laboratory, and subsequently stored them at –20 °C. Detailed information of each accession is listed in Table 1.

**Data collection (molecular sequences).** Total genomic DNA was extracted from 20 mg dried leaf using a modified CTAB method (Doyle and Doyle, 1987). Using these primers from previous studies, three nuclear DNA regions [ITS, *leafy* and *needly* (Little et al., 2004; Yang et al., 2012)], three mt DNA regions [*cox*I, *atp*A, and *rps*3 (Mao et al., 2012; Ran et al., 2010)], and two cp DNA regions [*mat*K and *rbc*L (Mao et al., 2010)] were amplified and sequenced.

Polymerase chain reactions (PCRs) were performed in a 25-μL volume containing 20 μL of sterile water, 2.5 μL of 10 × PCR buffer, 0.25 μL of 10 mM dNTPs, 1 μL of 5 μM each primer, 0.25 μL of 5 U·μL$^{-1}$ r*Taq* DNA polymerase enzyme (TakaRa, Dalian, China), and 1 μL of extracted DNA (20 to 40 ng·μL$^{-1}$). The PCR protocols used to amplify were as follows: initial denaturation at 95 °C for 5 min, 37 cycles of 40 s denaturation at 95 °C, annealing at 55 to 60 °C for 1 min (55 °C for ITS, *leafy* and *rbc*L; 58 °C for *needly* and *mat*K; 60 °C for *cox*I, *atp*A, and *rps*3), and elongation at 72 °C for 1 min 10 s followed by a final elongation period at 72 °C for 7 min.

Following previous studies, PCR products of nuclear DNA regions were purified using an Agarose Gel DNA Purification kit, and then cloned using pMD19-T vector following the recommended protocol (TakaRa) and transformed into competent *Escherichia coli* (Migula) Castellani & Chalmers strain JM109 at 42 °C. The transformed bacteria were screened on solid Luria Broth media with 150 mg·mL$^{-1}$ ampicillin at 37 °C overnight, and five positive clones were amplified using universal primers (M13-47 and RV-M) for each nuclear DNA region of each individual. PCR product purifications, sequencing reactions, and successive purifications were performed and capillary analyses were run on a DNA sequencer (ABI 3130XL; Applied Biosystems, Foster City, CA) following the manufacturer's protocols. All sequences that were determined in this study were submitted to National Center for Biotechnology Information GenBank (KJ849621–KJ849731).

**Data analysis.** DNA sequence alignments were conducted using Clustal_X 1.83 (Thompson et al., 1997) and then manually modified in MEGA 5.0 (Tamura et al., 2011) according to the original chromatogram. Subsequently, haplotypes (genotypes) and variable sites of mt and cp DNA sequences as well as each of the three nuclear DNA regions were detected using DnaSP Version 5 (Librado and Rozas, 2009). To investigate the hybridization events in the cultivation history of leyland cypress, the Neighbor network method as implemented in Splitstree 4.11.3 (Huson and Bryant, 2006) was used to reconstruct reticulate networks based on sequences of each nuclear DNA region. For distance calculations, we excluded insertions/deletions (indels)

Table 1. Collecting sites, voucher, and documented history of alaska cypress, monterey cypress, and leyland cypress samples that were adopted in this study (see also Adams et al., 2006).

| Common names | Cultivar | Vouchers | Collecting sites and documented history |
|---|---|---|---|
| Alaska cypress | Aurea | Adams R.P. 9956 | Castlewellan, U.K. This tree was planted in 1892. Putative pollen parent of 'Castlewellan' and possibly 'Galway Gold'. |
| Alaska cypress | | Adams R.P. 10069 | Westonbirt, U.K. |
| Monterey cypress | Lutea | Adams R.P. 9953 | Castlewellan, U.K. Seed parent of 'Castlewellan' and possibly 'Galway Gold'. |
| Monterey cypress | | Adams R.P. 9954 | Castlewellan, U.K. |
| Leyland cypress | Green Spire | Adams R.P. 9463 | Haggerston Castle, U.K. In 1888, seeds were collected from alaska |
| Leyland cypress | Haggerston Grey | Adams R.P. 9464 | cypress at Leighton Hall and germinated. Six unusual seedlings were sent to Haggerston Castle in 1892. Adams R.P. 9463 and 9464 were collected from two of the six trees. |
| Leyland cypress | Naylor's Blue | Adams R.P. 9469 | Leighton Hall, U.K. In 1911, seeds from monterey cypress at Leighton |
| Leyland cypress | Leighton Green | Adams R.P. 9470 | Hall, U.K., were germinated and two unusual seedlings were obtained. Adams R.P. 9469 and 9470 were collected from these two trees. |
| Leyland cypress | Galway Gold | Adams R.P. 9482 | Castlewellan, U.K. Origin unknown but often thought to be a renaming of 'Castlewellan'. |
| Leyland cypress | Castlewellan | Adams R.P. 9957 | Castlewellan, U.K. This sample was taken from the original tree, which was reputedly generated from a seed of a monterey cypress 'Lutea' (Adams R.P. 9953) growing near an alaska cypress 'Aurea' (Adams R.P. 9956) in 1962. |

and used the K2P model (Kimura, 1980). The relative robustness of the clades was estimated by performing 1000 bootstrap replicates based on which a 95% confidence network was constructed for each data set (Huson and Bryant, 2006).

## Results

For nuclear DNA regions, alignments of molecular cloning DNA sequences (eight to 10 per individual) from the six leyland cypress cultivars as well as their putative parent species, alaska cypress and monterey cypress, generated data matrices of 1019, 818, and 882 bp for ITS, *leafy,* and *needly*, respectively. A total of 19 variable sites and four indels (insertions/deletions) were detected in the ITS data matrix, among which 18 sites and four indels are heterozygous in the six putative hybrids (Fig. 2A). Twenty-five variable sites and four indels were detected in the *leafy* data matrix, among which 23 sites and four indels are heterozygous in the six putative hybrids (Fig. 2B). Twenty-six variable sites and one indel were detected in the *needly* data matrix, among which 13 sites and one indel are heterozygous in the six putative hybrids (Fig. 2C). In these, there are 21 sites (indels), 13 sites (indels), and one site in ITS, *leafy*, and *needly,* respectively, in which the six leyland cypress cultivars showed heterozygous sites, which likely originated from homozygous sites in alaska cypress and monterey cypress.

Meanwhile, alignment of the two cp DNA regions of the six leyland cypress individuals, alaska cypress and monterey cypress, generated data matrices of 964 and 959 bp for *rbc*L and *mat*K, respectively. Eleven nucleotide differences were found between alaska cypress and monterey cypress (*rbc*L, nine; *mat*K, two).

According to the alignment of the three mt DNA regions, several differences were found between the species: *atp*A, 784 bp, two differences; *cox*I, 858 bp, one difference; and *rps*3, 937 bp, one difference. No intraspecific variation was detected in either alaska cypress or monterey cypress. Because common inheritance without recombination can be assumed for both cp and mt genomes in plants, the two cp DNA regions were concatenated into one unit for analysis; so did the three mt DNA regions.

## Discussion

**Nuclear DNA regions.** In contrast with the Yamaguchi et al. (2000) study that used only a single heterozygous site in the *18S* rDNA to examine the hybrid origin of leyland cypress, in the present study, three nuclear DNA regions and more abundant variable sites were used to analyze the hybrid/backcross origins of leyland cypress cultivars.

Combinations of variable sites (indels) in each nuclear DNA region lead to one to three genotypes in each individual. Neighbor network analyses suggested three points. First, most nuclear genotypes of leyland cypress were identical with (or very close to) nuclear genotypes of either alaska cypress or monterey cypress [ITS: 10 of 14, *leafy*: 11 of 13, *needly*: eight of 13 (Fig. 3)], whereas the other nuclear genotypes of leyland cypress were most likely recombinants between/among nuclear genotypes of putative parents as inferred from their intermediate phylogenetic positions (Fig. 3). Jackson and Dallimore (1926) reported that fertile offspring have been raised from seeds taken from leyland cypress cultivars. Alleles in $F_1$ hybrids from both parent species may have experienced recombination during meiosis when they generate pollens or ovules, so $F_1$ hybrids usually contribute recombinant alleles (genotypes) to their offspring (e.g., backcrosses and $F_2$ hybrids). Thus, leyland cypress cultivars that possess nuclear genotypes identical to those of either alaska cypress and monterey cypress are likely $F_1$ hybrids; meanwhile, leyland cypress cultivars that possess recombinant genotypes are most likely backcrosses or $F_2$ hybrids.

It would be interesting to compare classifications of the six leyland cypress putative hybrids (Table 2). The ITS sequence data classify 'Green Spire', 'Haggerston Grey', and 'Naylor's Blue' as hybrids between alaska cypress and monterey cypress. 'Castlewellan' and 'Galway Gold' are classified as backcrosses

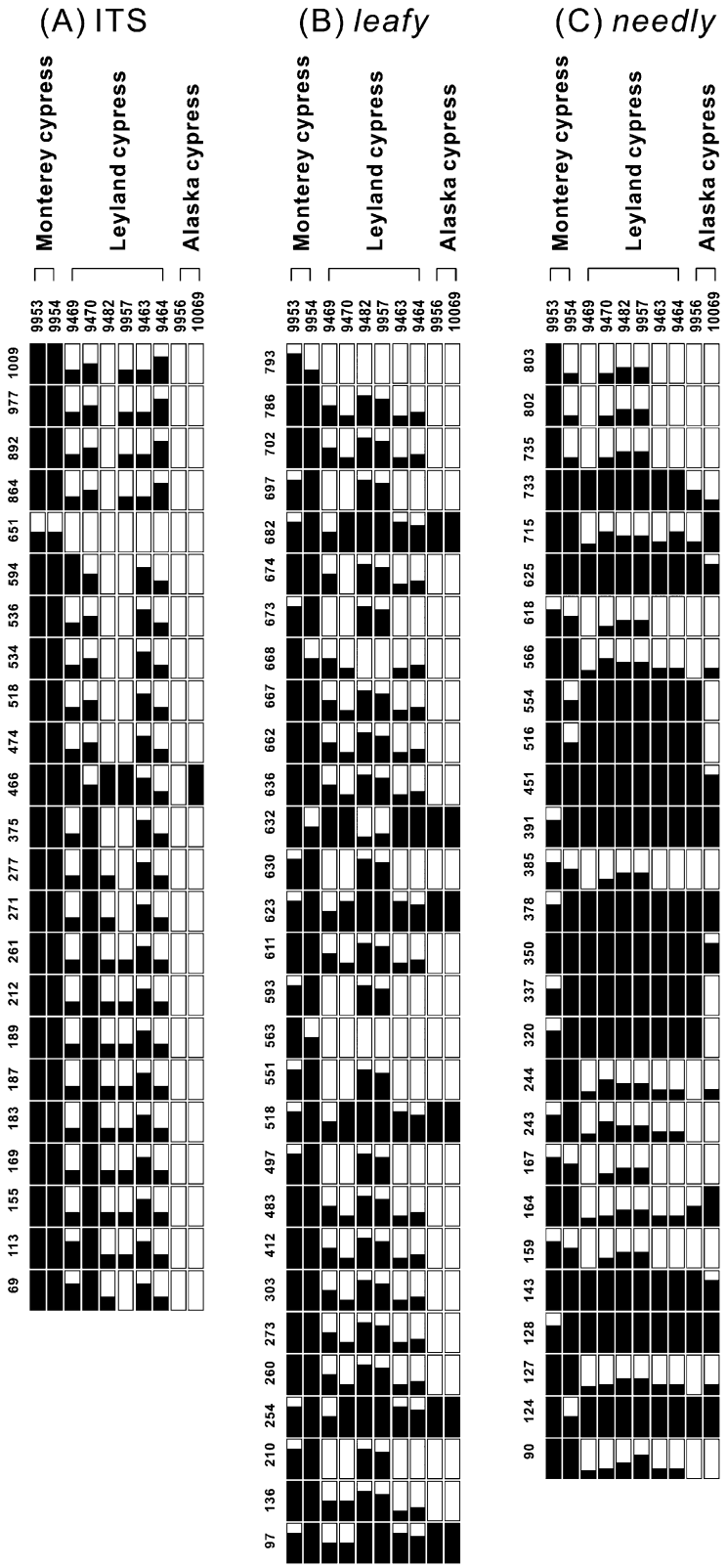


Fig. 2. A summary of variable nucleotide sites in DNA sequences of the three nuclear DNA regions: (**A**) internal transcribed spacer (ITS), (**B**) *leafy*, and (**C**) *needly* among sampled trees of alaska cypress, monterey cypress, and leyland cypress. Each cell represents a nucleotide site in a certain DNA sequence of an individual tree; the proportion of black and white colors in each cell represents the proportion of two different nucleotides at a certain site among all DNA sequences, which were generated from successful molecular cloning of each individual tree. Numbers of nucleotide sites are labeled on the left of each subfigure and abbreviated voucher numbers (collector's name was omitted) are marked at the top of each subfigure.

to alaska cypress and 'Leighton Green' is classified as a backcross to monterey cypress (Table 2).

In contrast, the *leafy* sequence data classify 'Green Spire', 'Haggerston Grey', 'Naylor's Blue', 'Castlewellan', and 'Galway Gold' as hybrids between alaska cypress and monterey cypress and 'Leighton Green' as a possible backcross to alaska cypress (Table 2).

A different classification was found by the *needly* sequence data. 'Castlewellan' and 'Galway Gold' were classified as putative hybrids and 'Green Spire', 'Haggerston Grey', and 'Naylor's Blue' as possible backcrosses to alaska cypress (Table 2). 'Leighton Green' appeared to be an $F_2$ generation hybrid (Table 2).

Neighbor network analyses (Fig. 3) suggested leyland cypress cultivars consist of both $F_1$ hybrids between alaska cypress and monterey cypress, and backcrosses as well as $F_2$ hybrids. Note that different nuclear DNA regions may have experienced different evolutionary histories; therefore, all available nuclear DNA regions should better be considered so as to precisely determine the property of hybrids.

Second, among the three nuclear DNA regions, ITS performed the best in identifying hybrids. Notice that phylogenetic relationships among genotypes using the ITS sequence data are simpler (Fig. 3A) than these using the other two nuclear DNA regions (Fig. 3B–C). In addition, the intraspecific genetic distances between ITS genotypes of either putative parental species are much shorter than intraspecific genetic distance between/among *leafy* and *needly* genotypes (Fig. 3). Usually, the nuclear ITS regions in plants are composed of multiple (reiterated) copies and are subject to concerted evolution (Alvarez and Wendel, 2003). During this process, different copies become homogenized to the same DNA sequence or at least become almost identical as a result of various mechanisms such as gene conversion or high-frequency unequal crossing over (Alvarez and Wendel, 2003). The nuclear ITS region, therefore, experiences a faster lineage sorting process than most nuclear DNA regions that are of single- or low-copy. As a result of these characteristics, the nuclear ITS is not only a highly efficient marker in delimitating closely related species (e.g., Wang et al., 2011), but also an effective marker in identifying recently originated hybrids (e.g., Feng et al., 2013).

Lastly, the six leyland cypress cultivars most likely originated from alaska cypress individuals that possess few intraspecific

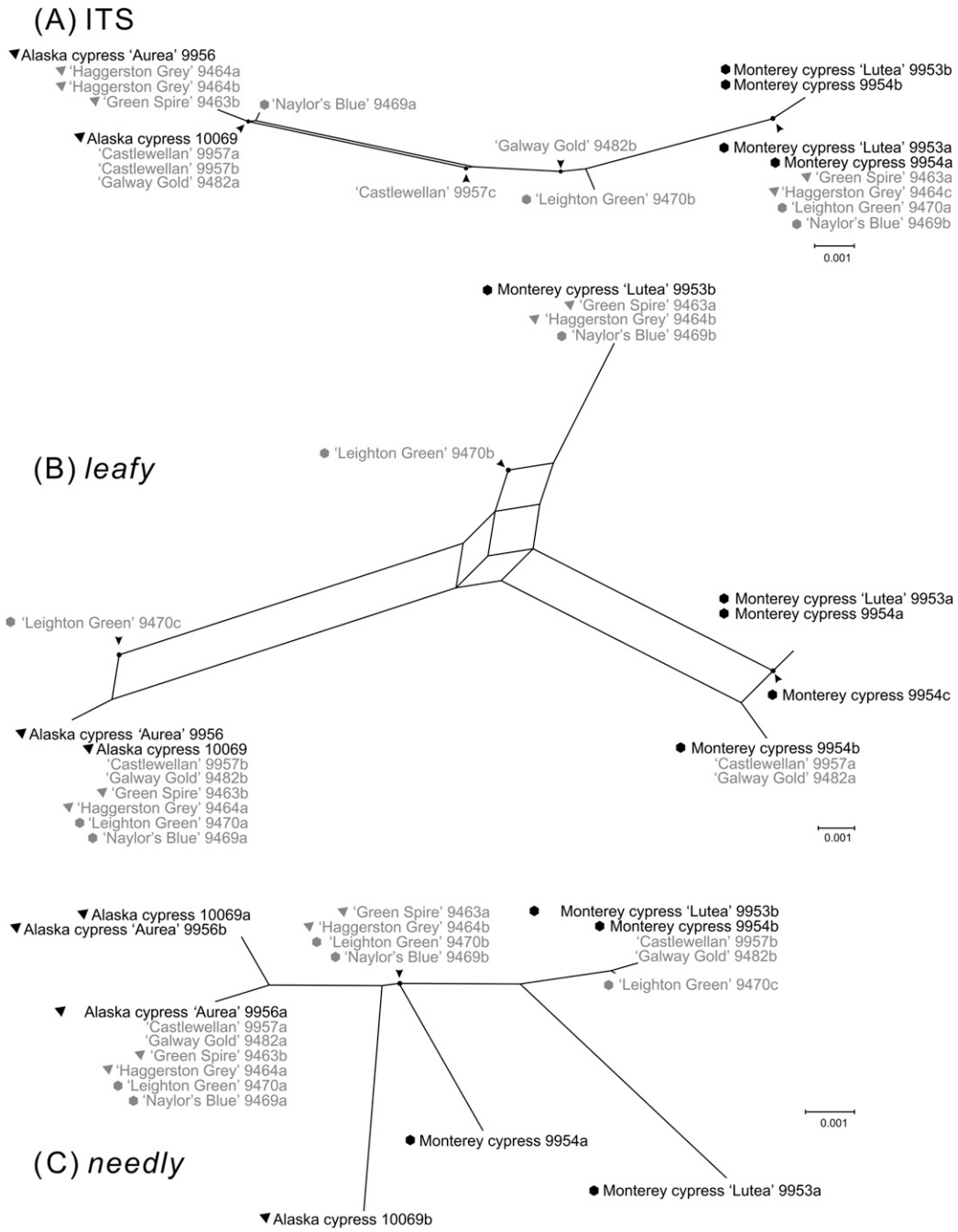


Fig. 3. Neighbor network among genotypes (alleles) of sampled trees of alaska cypress, monterey cypress, and leyland cypress according to DNA sequences of the three nuclear DNA regions: (**A**) internal transcribed spacer (ITS), (**B**) *leafy*, and (**C**) *needly*. Genotypes (alleles) that were detected from alaska cypress and monterey cypress are presented in black color, and alaska cypress and monterey cypress samples are marked with black triangular and black hexagon, respectively; genotypes (alleles) of leyland cypress cultivar samples were presented in gray color; those cultivars whose maternal parent were documented as alaska cypress and monterey cypress are marked with gray triangular and gray hexagon, respectively.

genetic variations. As shown in Figure 3, all genotypes (ITS and *leafy*) or most genotypes (*needly*) of alaska cypress are very closely related to each other. Both alaska cypress individuals were homozygous and they shared the same *leafy* genotype with each other as well as with all hybrid individuals. The ITS genotypes of both alaska cypress individuals are homozygous and they differ with each other by one mutation, and each of them shares the same ITS genotype with two hybrid individuals. One ITS genotype of another hybrid individual was closely related to these two ITS genotypes of alaska cypress. High intraspecific variation was found among the three *needly* genotypes of alaska cypress, but only one genotype was shared between this species and all hybrid individuals. In contrast, high-level genetic variation among genotypes of *leafy* and *needly* was detected in monterey cypress (Fig. 3).

**Cytoplasmic DNA regions.** Chloroplast and mitochondrial genomes of most Cupressaceae species have been shown to be paternally inherited (Kondo et al., 1998; Mogensen, 1996; Neale

Table 2. Classification of hybrid form for the six leyland cypresses as revealed by three different nuclear DNA markers, internal transcribed spacer (ITS), *leafy,* and *needly*.

| Leyland cypress cultivar | Classification | Parents or backcross parent |
|---|---|---|
| The ITS sequence data: | | |
| Green Spire, Haggerston Grey, Naylor's Blue | Hybrids | Alaska cypress × monterey cypress |
| Castlewellan, Galway Gold | Backcross to | Alaska cypress |
| Leighton Green | Backcross to | Monterey cypress |
| The *leafy* sequence data: | | |
| Green Spire, Haggerston Grey, Naylor's Blue | Hybrids | Alaska cypress × monterey cypress |
| Castlewellan, Galway Gold | Hybrids | Alaska cypress × monterey cypress |
| Leighton Green | Backcross to | Alaska cypress |
| The *needly* sequence data: | | |
| Green Spire, Haggerston Grey, Naylor's Blue | Backcross to | Alaska cypress |
| Castlewellan, Galway Gold | Hybrids | Alaska cypress × monterey cypress |
| Leighton Green | $F_2$ generation | Leighton Green |

Table 3. Putative origin of the chloroplast (cp) and mitochondrial (mt) genomes of leyland cypress cultivars that were inferred based on DNA sequences and documentary history.

| Leyland cypress cultivar | Literature report of maternal seed source | DNA basis for inheritance of cp and mt genomes |
|---|---|---|
| Castlewellan | Monterey cypress | Alaska cypress (paternal) |
| Galway Gold | Monterey cypress | Alaska cypress (paternal) |
| Green Spire | Alaska cypress | Alaska cypress (maternal) |
| Haggerston Grey | Alaska cypress | Alaska cypress (maternal) |
| Leighton Green | Monterey cypress | Alaska cypress (paternal) |
| Naylor's Blue | Monterey cypress | Alaska cypress (paternal) |

et al., 1989, 1991; Sakaguchi et al., 2014; Whittle and Johnston, 2002); this holds true for alaska cypress and monterey cypress.

In the present study, all six leyland cypress individuals shared the same cp and mt haplotype. These were identical to the cp and mt haplotype of alaska cypress. Therefore, it is reasonable to propose that the cp genome and mt genome were inherited through the same method (e.g., paternally or maternally) in leyland cypress hybrids.

A summary of the historical literature concerning reports of the maternal parentage of leyland cypress cultivars (see the review in Adams et al., 2006) is given in Table 3. The literature reports that seed cones were collected from monterey cypress for 'Castlewellan', 'Galway Gold', 'Leighton Green', and 'Naylor's Blue' (Adams et al., 2006). For 'Green Spire' and 'Haggerston Grey', Owens et al. (1964) reported that in 1888, some seeds were collected from alaska cypress growing at Leighton Hall and sown. Six of the resulting seedlings had different foliage from the other seedlings. C.J. Leyland took the six unusual plantlets (called clones 1 to 6; note that they were not clonally derived but siblings from the same maternal source) and planted them at his home, Haggerston Castle. Plant 2 (clone 2) was later named 'Haggerston Grey' and is extensively planted. Plant 1 (clone 1) was named 'Green Spire' and is also widely cultivated. From this account, it seems credible that the maternal parent of 'Green Spire' and 'Haggerston Grey' was alaska cypress, but of course there is always a possibility that these records have become confused. If 'Green Spire' and 'Haggerston Grey' were from alaska cypress seed (Table 3) and have cp and mt DNA of alaska cypress, then it appears that inheritance was maternal. This could be caused by maternal leakage. As Mogensen (1996) shows, both maternal and paternal cytoplasmic organelles may be present in the fertilized zygote; then either maternal or paternal organelles are eliminated. However, the process may not be 100% effective as Owens and Morris (1991) note that in the Cupressaceae, "presumably, most of the proembryo cytoplasm is of paternal origin, but some maternal organelles may be included during the migration." Wagner et al. (1991) found paternal leakage of mt DNA in 125 seedlings from a cross of jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*P. contorta* Douglas ex Loudon): 119 seedlings displayed maternal inheritance, but six seedlings displayed paternal inheritance. Cato and Richardson (1996) reported 99% paternal and 1% maternal inheritance of cp DNA in monterey pine (*Pinus radiata* D. Don). Grivet et al. (1999) reported 94 offspring with paternal mt DNA and two with maternal DNA in norway spruce [*Picea abies* (L.) H. Karst.]. Shiraishi et al. (2001) found the cp genome exhibited paternal inheritance in 97.5% (352) and maternal in 2.5% (nine) of the progeny of a cross within japanese cypress [*Chamaecyparis obtusa* (Siebold & Zucc.) Endl.]. It appears that maternal or paternal leakage may be small but not uncommon. In summary, the appearance of 'Green Spire' and 'Haggerston Grey' from the same maternal tree source [alaska cypress (Table 3)] may be the result of maternal leakage, but confusion in the literature records cannot be eliminated. Additional research is needed to clarify the matter.

**Evolutionary implications.** Molecular dating analyses revealed that alaska cypress and monterey cypress shared a common ancestor ≈46 million years ago (Mya) [credible interval: 72 to 21 Mya (Mao et al., 2012)]. In this study, our results clearly support leyland cypress as hybrids between alaska cypress and monterey cypress. Most importantly, some leyland cypress individuals were identified as putative backcrosses or $F_2$ hybrids, suggesting that hybrids between these two parent species are fertile. Under natural conditions, such hybridization events among conifer species will lead to the formation of hybrid zones (e.g., Little, 2004), interspecific gene flow or introgression (Li et al.,

2012), and even new hybrid species (e.g., Ren et al., 2012; Sun et al., 2014). Hybridizations usually lead to merging or combining of different evolutionary lineages. When reconstructing phylogenetic trees based on multiple loci that have undergone different inheritance pathways, species or lineages that experienced hybridization events usually exhibit incongruences of phylogenetic placements among different gene trees (e.g., Guggisberg et al., 2009).

Hybridization events may have played an important role in the evolutionary history of Cupressaceae (Wang and Ran, 2014; Yang et al., 2012). Molecular phylogenetic studies suggested that historical hybridization events may have occurred in the redwood subfamily (Sequoioideae) and the Southern Hemisphere cypress subfamily (Callitroideae) (Yang et al., 2012) and are very common in the cypress subfamily (Cupressoideae) (e.g., Peng and Wang, 2008; Yang et al., 2012). In the last subfamily, phylogenetic incongruences have been observed among genera: *Cupresssus* (the Old World cypresses), *Xanthocyparis–Callitropsis–Hesperocyparis* (vietnamese golden cypress, alaska cypress, the New World cypresses), and junipers (Little, 2006); among subgenera and clades in junipers (Mao et al., 2010); and among species in *Thuja* L. (arborvitaes) and *Thujopsis* Siebold & Zucc. ex Endl. (asunaro) (e.g., Peng and Wang, 2008), *Chamaecyparis* (false cypresses) and *Fokienia* A. Henry & H. H. Thomas (fujian cypress) (e.g., Liao et al., 2010). Given the fact that two Cupressaceae species that diverged with each other ≈46 Mya can still generate fertile hybrids, we expect that more hybridization events among genera, (intrageneric) clades, and taxa will be discovered in the Cupressaceae as well as other conifer families considering the fast development of sequencing technology (Twyford and Ennos, 2012).

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