Inheritance of nrDNA in artificial hybrids of *Cryptomeria japonica* cv. *Haara* and *C. japonica* cv. *Kumotooshi*

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ABSTRACT

Sequencing nrDNA of parents (*Cryptomeria japonica* cv. *Haara* and *C. japonica* cv. *Kumotooshi*) and seven artificial hybrids, revealed three of the seven (3/7) hybrids had nrDNA that was heterozygous, just as found in Kumo, whereas four of the seven (4/7) hybrids had nrDNA that was exactly like that of one parent, Haara. Sequencing petN-psbM, showed that cv. *Kumotooshi* was the male (pollen source) for all of the hybrids. The inheritance of nrDNA was not correlated with pollen source. If these results can replicated, this appears to raise a cautionary flag on the use of nrDNA in detecting hybridization. Published on-line **www.phytologia.org** *Phytologia 98(1): 37-41 (Jan. 5, 2016)*. ISSN 030319430.

KEY WORDS: *Cryptomeria japonica* cv. *Haara, C. japonica* ***cv.*** *Kumotooshi,* hybrids, inheritance, nrDNA, petN-psbM.

Sequencing of nrDNA spacer regions has been an important source of phylogenetic information in plant systematics for several years. The conserved nature of the multi-copy nrDNA (thousands of copies per cell) might be due to concerted evolution (Liao, 1999). Liao (1999) argues that because rRNAs are structural molecules, multiple gene copies are necessary to supply the demand for ribosomal subunits in the cell. Because these sub-units function only when assembled into a large complex, homogeneity of rRNAs is critical for regular, functional ribosome assembly and translation to function normally. Liao (1999) concludes that "a possible biological function of concerted evolution is to maintain homogeneous gene copies in a family so that homogeneous transcripts can be produced." However, concerted evolution is thought to be a slow process over numerous generations. Hybrids would seem likely to be heterozygous for both parents nrDNA. Thus, nrDNA (ITS) is often used for the analysis of hybridization. Recently, Adams (2015a,b) found that nrDNA detected 15 hybrids, whereas, maldehy, a single copy nuclear gene (SCN) detected 25 hybrids. nrDNA appeared more often to be the same as one of the parents, whereas the SCN gene (maldehy) was heterozygous indicating the plant(s) were of hybrid origin.

Chaing et al. (2001) found that in the artificial hybrids between *Begonia aptera* (pollen) and *B. formosana* (maternal), nrDNA was predominantly that of the maternal parent, *B. formosana* (diamonds, Fig. 1). Volkov, et al. (1999) reported that one of the parental nrDNAs was eliminated in the allopolyploid genome of cultivated tobacco. Fukuoka et al. (1994) found that the nrDNA in γ-ray irradiated tetraploid rice was homogenized in a short time.

Aguilar et al. (1999) made artificial hybrids between *Armeria villosa* ssp. *longiaristata* and *A. colorata*, then examined the inheritance of nrDNA in F_1 and F_2 generations. They found the expected additive pattern in polymorphisms for five of the six variable sites in F_1 plants. However, in the F_2

generation, there was a bias towards one parent (*A. colorata*). Backcrosses showed homogenization of five of the polymorphic sites to the recurrent parent.

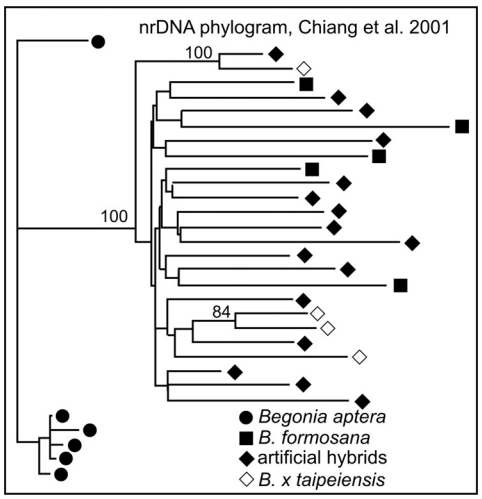

Figure 1. Phylogram based on nrDNA for *Begonia* and hybrids (adapted from Chiang, et al. 2001). Notice the grouping of the hybrids (triangles, nrDNA) with the maternal parent, *B. formosana* (shaded squares), rather than with the pollen (paternal) parent *(B. aptera*, shaded circles).

Okuyama et al. (2005) examined introgression in *Mitella* using nrDNA ITS and ETS, and cpDNA and found that cpDNA revealed the most introgression, ITS regions showed a moderate amount of introgression and the ETS region gave no evidence of introgression. They concluded that non-uniform concerted evolution between the ETS region and ITS regions may explain these different patterns of introgression.

Cryptomeria japonica D. Don (Sugi) is a monotypic genus (Farjon, 2005; Tsumura, 2011), endemic to Japan. Farjon (2005) argues that *C. fortunei* Hooibr. is conspecific, and a study (Kusumi et al. 2000) based on DNA sequencing, found no support for the recognition of *C. fortunei* separate from *C. japonica*. *Cryptomeria japonica* appears to have been introduced into China many years ago (Farjon, 2005) and is now widely cultivated in Japan, Taiwan, Korea, China and the Azores Islands (Tsumura, 2011). It is a very important commercial forest tree in Japan and the object of many detailed studies (see review, Tsumura, 2011) at the Forestry and Forest Products Research Institute and other institutes in Japan. Recently, Adams and Tsumura (2012) reported on the inheritance of leaf terpenoids from artificial hybrids of *C. japonica* cv. *Haara* X *C. japonica* cv. *Kumotooshi*. These hybrids were developed as part of a forestry improvement program at the Forestry and Forest Products Research Institute and other institutes in Japan.

In the Cupressaceae, breeding programs are rare, so the existence of parents and artificial (verified) hybrids is an important resource for studies on inheritance. This program afforded an unusual opportunity to examine the inheritance of nrDNA in hybrids in the Cupressaceae. As far as known to the authors, there are no reports on the inheritance of nrDNA in the Cupressaceae (or in conifers). The purpose of this paper is to report on the inheritance of nrDNA in artificial hybrids of *C. japonica* cv. *Haara* X *C. japonica* cv. *Kumotooshi*.

MATERIALS AND METHODS

Plant material: Crosses were made at the Forestry and Forest Products Research Institute and other institutes in Japan. Local cultivars of sugi (*Cryptomeria japonica*): 'Haara 4' (female parent) and 'Kumotooski' (male parent) were crossed and produced one hundred (100) progeny. Leaves were collected from individual hybrids 23,48,56, 65, 70, 81 and 83, growing at an outdoor nursery at the Institute. (Note: there are several clones of Haara at the Institute, so clone 4 is 'Haara 4'). Recently, the parent trees used in this cross died, but DNA was preserved. Leaf and DNA materials:

Parents: 14517 *Cryptomeria japonica* cv. *Haara*, 'Haara clone 4', DNA
14518 *C. japonica* cv. *Kumotooshi*, DNA

Seven (7) Hybrids (leaves in silica gel) (lab accession # - hybrid #):
14519 - 23, 14520 - 48, 14521 - 56, 14522 - 65, 14523 - 70, 14524 - 81, 14525 - 83.

Voucher specimens are deposited at the Dept. of Forestry and Forest Products Research Institute, Tsukuba, Ibaraki, 305-8687, Japan

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM), D (maldehy) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.).

The stability of sequence mixed base heights was investigated by analyzing 10 separate PCR - ITS amplification and subsequent sequencing using genomic DNA of Kumo (14518) and 10 using genomic DNA of hybrid 14520. These analyses were conducted as per the other genomic DNAs above.

RESULTS AND DISCUSSION

Sequencing of nrDNA of *C. japonica* cv. *Haara* resulted in 1179 bp that agreed well with AB23983 in GenBank. However, sequencing of cv. *Kumotooshi* (Kumo) proved difficult and only 802 bp was cleanly sequenced. Nevertheless, the Kumo sequence was identical to that of Haara, except in the positions 154, 468, and 505. In Kumo, each of these three positions was heterozygous, which seems to imply that Kumo, itself, may be of hybrid origin. Of course, these could be relictual from incomplete lineage sorting. In any case, we do know that parent Kumo did contain (from some event) heterozygous bases at three positions in its nrDNA. Thus, although not ideal, it is still instructive to follow the fate of these three positions in the artificial hybrids. Three of the hybrids had nrDNA very similar to Kumo, with some variation in the ratio of bases. Unfortunately, repeated sequencing from both forward and reverse was unable to obtain clean sequences in the area around site 505 for six of the hybrids (NA in Table 1). The nrDNAs of four hybrids (Table 1) were the same as Haara at positions 154 and 468.

Table 1. Variable sites in the nrDNA sequence for *Cryptomeria japonica* cv. *Haara, C. japonica* cv. *Kumotooshi* and their hybrids. The ratios of bases in parenthesis ( ) were obtained by measurements of the peak sizes on the chromatogram. NA = not available.

					petN-psbM (from pollen)		
	site 154	site 468	site 505	nrDNA type	site 145	site 146	
Haara	C	T	G	Haara	A	T	Haara
Kumo	S(C/G 1:0.71)	Y(C/T 1:0.64	R(A/G 1:0.76)	Kumo	C	G	Kumo
Hybrids:							Pollen parent
14519	S(C/G 1:0.7)	Y(C/T 1:1)	R(A/G 1:0.7)	Kumo	C	G	Kumo
14520	S(C/G 1:0.71)	Y(C/T 1:0.46)	R(A/G 1:0.58	Kumo	C	G	Kumo
14521	S(C/G 1:0.2)	Y(C/T 0.5:1)	NA	Kumo	C	G	Kumo
14522	C	T	NA	Haara	C	G	Kumo
14523	C	T	NA	Haara	C	G	Kumo
14524	C	T	NA	Haara	C	G	Kumo
14525	C	T	NA	Haara	C	G	Kumo

Sequencing of petN-psbM yielded two SNPs at sites 145 and 146 (Table 1), revealing that all the hybrids had Kumo as the male, pollen parent and Haara as the maternal (seed) parent. The inheritance of nrDNA patterns appears un-correlated with the pollen source (Table 1).

The stability of sequence base heights was investigated by analyses based on 10 separate PCR - ITS amplification and subsequent sequencing for Kumo (14518) and 10 for hybrid 14520. The results are very uniform (Table 2). Although, the first PCR cycle might preferentially amplify a given DNA strand, there seems no support from these data that this is happening for these two genomic DNAs.

Table 2. Variation in base height on chromatograms at positions 154, 469 and 505 for ITS sequences.

rep.	154, C/G	469, C/T	505, A/G
14518-1	1:0.7	1:0.55	1:0.7
14518-2	1:0.7	1:0.6	1:0.7
14518-3	1:0.7	1:0.6	1:0.9
14518-4	1:0.7	1:0.7	1:0.8
14518-5	1:0.7	1:0.6	1:0.8
14518-6	1:0.7	1:0.7	1:0.8
14518-7	1:0.7	1:0.6	1:0.8
14518-8	1:0.8	1:0.7	1:0.7
14518-9	1:0.7	1:0.7	1:0.7
14518-10	1:0.7	1:0.6	1:0.7
avg +/- 2 SD	1:0.71+/-0.062	1:0.635+/-0.116	1:0.76+/-0.14
14520-1	1:0.8	1:0.7	NA
14520-2	1:1.0	1:0.4	1:0.5
14520-3	1:1.0	1:0.5	1:0.7
14520-4	1:1.0	1:0.4	1:0.6
14520-5	1:1.0	1:0.4	1:0.6
14520-6	1:1.0	1:0.5	1:0.5
14520-7	1:1.0	1:0.4	1:0.6
14520-8	1:0.9	1:0.4	1:0.6
14520-9	1:0.8	1:0.4	1:0.5
14520-10	1:1.0	1:0.5	1:0.6
avg +/- 2 SD	1:0.95+/-0.17	1:0.46+/-0.194	1:0.58+/-0.134

CONCLUSION

Although it was unfortunate that one of the parents (Kumo) had heterozygous nrDNA, the results showing that three of the seven (3/7) hybrids had nrDNA that was heterozygous, just as found in Kumo, whereas four of the seven (4/7) hybrids had nrDNA that was exactly like that of one parent, Haara. If these results can replicated, this appears to raise a cautionary flag on the use of nrDNA in detecting hybridization and favoring a more wide-spread use of single copy nuclear (SCN) genes for the analysis of putative hybridization.

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