

CROSSABILITY RELATIONSHIPS BETWEEN NOBLE, MANCHURIAN AND CAUCASIAN FIRS

ANDREJ KORMUŤÁK^{1,3,4*}, BOŽENA VOOKOVÁ¹, TERÉZIA SALAJ¹, VLADIMÍR ČAMEK¹,
MARTIN GALGÓCI², PETER MAŇKA², PETER BOLEČEK³, ROMAN KUNA³,
AND JAROSLAV KOBLIHA⁴

¹Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences,
Akademická 2, P. O. Box 39A, 950 07 Nitra, Slovak Republic

²Mlyňany Arboretum, Slovak Academy of Sciences, 951 52 Slepčany, Slovak Republic

³Constantine Philosopher University, Hlinku 1, 949 74 Nitra, Slovak Republic

⁴Czech University of Agriculture in Prague, 165 21 Prague 6 – Suchdol, Czech Republic

Received July 31, 2012; revision accepted October 25, 2012

We used artificial hybridization to study the crossability of the noble fir (*Abies procera*) with Manchurian fir (*A. holophylla*) and Caucasian fir (*A. nordmanniana*), and found compatibility between *A. procera* of North American origin and the Asian species *A. holophylla* as evidenced by the 14% fraction of filled seeds obtained in *A. procera* × *A. holophylla* crossing. Crossing of *A. procera* with the Mediterranean species *A. nordmanniana* failed completely, producing only empty seeds.

Key words: *Abies procera*, *A. holophylla*, *A. nordmanniana*, interspecific hybridization, cpDNA markers.

INTRODUCTION

Recent taxonomic works on the true firs indicate that the genus *Abies* contains 39 species classified in 10 or 15 sections (Liu, 1971; Farjon and Rushforth, 1989). Controversy about sections is due in part to past neglect of the genetics of the genus, and in particular the crossability relationships between species, which have been found to reflect the genetic status of individual species or whole groups of species.

In their work on *Abies* interspecific hybrids, Klaehn and Winieski (1962) listed 8 spontaneous hybrids in nature, 11 natural arboretum or plantation hybrids, 39 confirmed artificial hybrids and 15 putative artificial hybrids produced by controlled pollination. At that time even preliminary data on crossability relationships within the genus *Abies* had not been available. Experiments by those authors, which they discussed in that work, employed controlled pollination of 12 female and 13 male parent species of Mediterranean, Asian and North American firs. They showed that species of a given geographical region intercross more readily than species from different regions. Mediterranean species were shown to be especially prone to

hybridize. Then Mergen et al. (1964) made 71 artificial pollination attempts using 10 species of Mediterranean, North American and Asian origin and found that it was easier to hybridize fir species from the same geographic region than from different regions. Artificial pollination experiments by Hawley and DeHayes (1985) supported that finding and showed genetic differentiation between species of the same geographic region belonging to different taxonomic sections. The North American species *A. balsamea* and *A. lasiocarpa* of sect. Balsameae intercrossed with each other but showed strong incompatibility with *A. concolor* of sect. Grandes. Crossing experiments by Critchfield (1988) using nine species of California firs of the Balsameae, Nobiles and Grandes sections, the Asian species *A. firma* and *A. veitchii* and/or the Mediterranean species *A. pinsapo* led the author to state that "...although crosses between North American species are sometimes successful, firs of the Eastern and Western hemispheres may be isolated from each other by genetic barriers that are almost or completely insurmountable."

Our crossing experiments carried out from 1976 to 2011 substantiate those conclusions. High hybridological affinity between the Mediterranean

*e-mail: nrgrkorm@savba.sk

species has been demonstrated (Kormuták, 1985). Obviously they are under strong reproductive isolation from the North American and Asian firs (Kormuták, 2004), but there are interhemispheric combinations of species exhibiting relatively high hybridological affinity. For example, as a pollen donor the Asian species *A. veitchii* has been reported to intercross with the Mediterranean species *A. alba*, *A. cephalonica*, *A. cilicica*, *A. nordmanniana* and *A. numidica* (Greguss, 1988). Besides these crosses, the author has suggested compatibility for the interhemispheric combinations *A. homolepis* × *A. cilicica* and *A. nordmanniana* × *A. veitchii*. There has been no attempt to validate these hybrids genetically or biochemically. The same applies to hybrids of *A. procera* with the North American species *A. lasiocarpa* and with the Asian fir *A. sibirica* (Mergen et al., 1964). The only validated hybrids of *A. procera* are *A. procera* × *A. magnifica* and its reciprocal; owing to the big difference in number of cotyledons between the parental species, early verification of the hybrids was possible based on this trait of their seedlings (Silen et al., 1965). Here we refer to the interspecific hybrid *A. procera* × *A. holophylla* as an additional interhemispheric cross whose validity was shown by cpDNA markers.

MATERIALS AND METHODS

ARTIFICIAL POLLINATION

Our controlled pollination experiment was performed in the Mlyňany Arboretum using two noble fir (*Abies procera* Rehd.) trees as mother plants and one Manchurian fir (*A. holophylla* Maxim.) and Caucasian fir [*A. nordmanniana* (Stev.) Spach] as males. The crossing variants attempted are listed in Table 1. Pollen of *A. holophylla* was collected in a natural stand of the species in Korea and delivered to Slovakia on ice and kept in a deep-freeze (-82°C) for one year. Fresh pollen of *A. nordmanniana* and *A. procera* was used in *A. procera* × *A. nordmanniana* crossing and in the control (*A. procera* self-pollination) soon after its collection. Female strobili were isolated a few days before reaching receptivity at the beginning of May. Table 1 gives the number of isolated and artificially pollinated female strobili in the two mother *A. procera* trees together with the number of harvested mature cones. The isolators were removed after complete closure of the ovuliferous scales and bracts of pollinated megastrobili. Mature cones were harvested at the beginning of September followed by hand-extraction of seeds. The cones of a given crossing variant collected from both mother trees were pooled and processed separately according to the given variants. The quality of seed progeny, expressed separately for each crossing

TABLE 1. Crossings performed in this experiment

Crossing variants	Number		Number of filled seeds per 400 seeds	Percentage of filled seeds
	Pollinated female strobili	Collected mature cones		
<i>A. procera</i> self-pollination	22	12	38	9.5
<i>A. procera</i> × <i>A. holophylla</i>	18	13	56	14
<i>A. procera</i> × <i>A. nordmanniana</i>	19	10	0	0

variant as the percentage of filled seeds, was judged according to the ČSN 48 1211 Czechoslovak standard (number of filled seeds per 400 analyzed seeds). The seeds of all three attempted crossing variants were sown in the nursery in autumn of the same year as the cone harvest. A small portion of the hybrid seedlings raised the following year were used in DNA analysis.

HYBRID VERIFICATION

The hybrid nature of *A. procera* × *A. holophylla* was verified by PCR-RFLP analysis of chloroplast DNA (cpDNA) in the parental trees and the hybrid seedlings. Owing to the paternal mode of cpDNA inheritance in conifers, identical restriction profiles of pollinating tree and hybrid seedlings were taken as unequivocal evidence supporting the hybridity of the latter.

Total DNA was extracted from young needles of the parental *A. procera* trees growing in the Mlyňany Arboretum and from needles of the paternal *A. holophylla* tree growing in Korea using the CTAB method (Murray and Thompson, 1980). A 10-seedling sample of *A. procera* × *A. holophylla* was used for DNA extraction soon after the seedlings protruded from the soil. The *rbcL*₁-*rbcL*₂ region of cpDNA was PCR amplified using the primer pair 5'-TGTCAC-CAAAACAGAGACT-3' and 5'-TTCCATACTTCA-CAAGCAGC-3' (Tsumura et al., 1995). DNA amplification was performed at 94°C for 4 min followed by 35 cycles of 93°C for 1 min, 56°C for 1 min and 72°C for 2 min. The last strand elongation at 72°C was allowed an additional 10 min. The obtained PCR products were digested with the restriction enzyme *Vsp* I, which has been found to discriminate cpDNA of *A. procera* from that of *A. holophylla* (Kormuták et al., 2004). The generated fragments were separated electrophoretically using 2% agarose gels with EtBr and 1x TBE buffer, pH 8.0.

HISTOLOGICAL ANALYSIS

Ovules of individual crossings were analyzed in permanent cytological preparations through the course of their development. The ovules were collected at

6–10-day intervals starting two weeks after pollination. Following fixation in Navashin fixative (Němec, 1962) and subsequent processing, longitudinal sections of the ovules were embedded in Canada balsam and examined microscopically.

RESULTS AND DISCUSSION

Of the two interspecific crossings attempted, only *A. procera* × *A. holophylla* proved compatible. The crossing yielded 14% filled seeds as compared with 9.5% filled seeds obtained from the self-pollinated control. *A. procera* × *A. nordmanniana* failed completely, producing only hollow seeds (Tab. 1). It is not certain whether the lower share of filled seeds in the control was due to the inbred nature of seed progeny or rather reflects reduced fecundity of *A. procera*. Mergen et al. (1964) reported 7% germinable seeds from controlled outcrossing of it. Crossability of *A. procera* with other species has been shown. In the intrasectinal cross *A. procera* × *A. magnifica* and its reciprocal, Silen et al. (1965) demonstrated high hybridological affinity between these species of North America. Using four maternal trees they obtained 39% germinable seeds per cone in *A. procera* × *A. magnifica*, and 45% in the reciprocal cross with the same parental trees. Another hybrid of *A. procera* with a North American species, *A. procera* × *A. lasiocarpa*, was intersectional (sect. Nobiles, sect. Balsameae); the cross yielded only 0.2% filled seeds (Mergen et al., 1964).

In light of these results, the *A. procera* × *A. holophylla* cross can be considered a success. The relatively low amount of sound seeds, only 14%, probably is due to the intersectional nature of the cross involving the North American species *A. procera* of sect. Nobiles and the Asian species *A. holophylla* of sect. Homolepides (Liu, 1971). According to Hawley and DeHayes (1985) and Critchfield (1988), intersectional hybrids of North American firs yielded a lower percentage of viable seeds than crosses of species belonging to the same section.

Silen et al. (1965) successfully used cotyledon number for early identification of *A. procera* × *A. magnifica* seedlings. We used the species-specific *rbcL₁-rbcL₂/Vsp I* restriction profiles of cpDNA instead to validate the hybridity of *A. procera* × *A. holophylla* cross. The absence of a restriction site in the *A. procera* PCR product contrasted with the presence of such a site in the PCR product of the male *A. holophylla*. An intact 1400 bp PCR product was detected electrophoretically in *A. procera* after digestion, as were two cpDNA restriction fragments (750 bp, 650 bp) in *A. holophylla*. Two restriction fragments of the same size as in *A. holophylla* were shared by the *A. procera* × *A. holophylla* seedlings; we take this as reliable proof of their hybridity (Fig. 1).

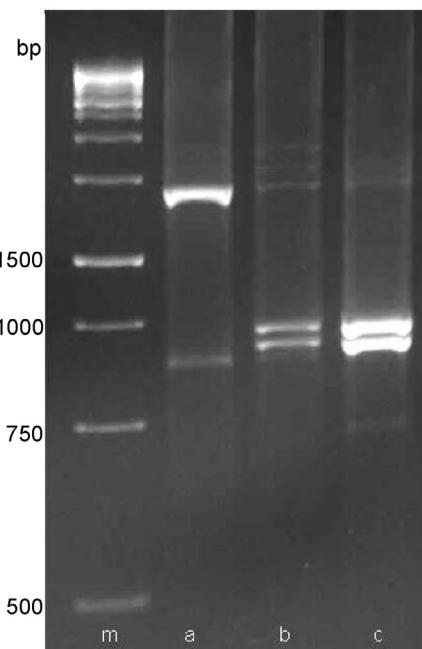


Fig. 1. *rbcL₁-rbcL₂/Vsp I* restriction profiles of *A. procera* (a), *A. holophylla* (c) and their hybrid (b). m – molecular size marker.

In contrast to the DNA hybridity proof given above, cytological evidence supports our earlier conclusions that a prezygotic hybridological barrier exists between the Mediterranean and North American firs (Kormut'ák, 1985, 2004). In the self-pollinated *A. procera* control we recorded young and more advanced embryos at the end of June and beginning of July; in *A. procera* × *A. nordmanniana* we detected aborted archegonia instead (Fig. 2). During the normal course of embryo development the archegonia are commonly observed to disintegrate; the presence of a darkly stained dead archegonia jacket surrounding the archegonia indicates their abortion due to the failure of egg cell fertilization (Owens and Molder, 1977).

The compatibility between the North American species *A. procera* and Asian *A. holophylla*, together with the reproductive isolation of *A. procera* from the Mediterranean *A. nordmanniana*, support Mergen et al.'s (1964) conclusion that North American species are more compatible with Asian than with European firs. *Abies* species originated from ancient Katazia, a region corresponding to modern-day central China and northern India (Krylov et al., 1986). Apparently the spread of firs via the Bering Strait along the Pacific coast of North America during their evolution was not accompanied by such profound differentiation as in the Mediterranean species, which deviated from the Asian firs during their migration from the site of origin.

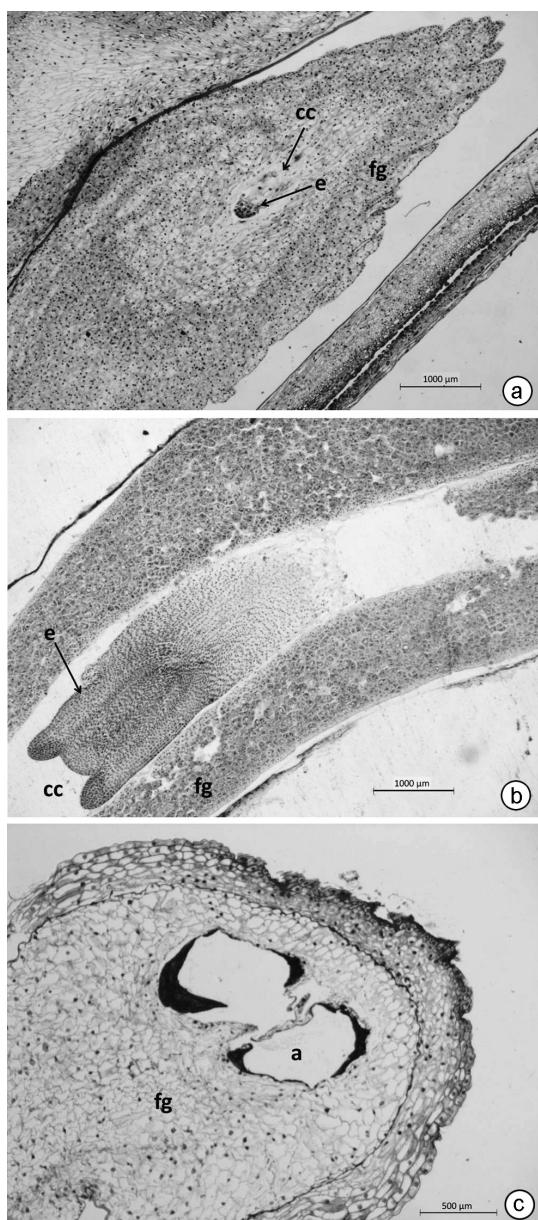


Fig. 2. Early embryogenesis in compatible *A. procera* × *A. holophylla* (a–b) and archegonia abortion in incompatible *A. procera* × *A. nordmanniana* (c). fg – female gametophyte; cc – corrosion cavity; e – embryo; a – archegonia. Bars = 2000 µm (a), 1000 µm (b), 500 µm (c).

gin. The European firs were under much stronger selective pressure than those in North America, as evidenced by the deep differentiation of *A. alba* during its migration from refugia in southern Italy and Greece and/or from a refugial area in the Pyrenees (Terhürn-Berson et al., 2004). Migrating a longer distance from the origin, *A. nordmanniana* evidently diverged more from the North American firs than *A. holophylla* did. The exceptions to the established hybridological pattern within the genus *Abies* may be deemed to reflect

the evolutionary history of individual species. We should expect more exceptions of this kind to be found when other combinations are attempted.

ACKNOWLEDGEMENTS

This study was supported by grants from the VEGA Grant Agency (project no. 2/0076/09) and the KON-TAKT Grant Agency (project no. ME914).

REFERENCES

- CRITCHFIELD WB. 1988. Hybridization of the California firs. *Forest Science* 34: 139–151.
- FARJON A, and RUSHFORTH KD. 1989. A classification of *Abies* Miller (Pinaceae). *Notes RBG Edinburg* 46: 59–79.
- GREGUSS L. 1988. Medzidruhová hybridizácia – náhrada za ustupujúcu jedľu bielu. *Lesnictví* 34: 797–808. (with English summary).
- HAWLEY GJ, and DEHAYES DH. 1985. Hybridization among several North American firs. I. Crossability. *Canadian Journal of Forest Research* 15: 42–49.
- KLAEHN FU, and WINIESKI JA. 1962. Interspecific hybridization in the genus *Abies*. *Silvae Genetica* 11: 130–142.
- KORMUTÁK A. 1985. Study on species hybridization within the genus *Abies*. *Acta Dendrobiologica*, VEDA, Bratislava.
- KORMUTÁK A. 2004. *Crossability relationships between some representatives of the Mediterranean, North American and Asian firs (Abies sp.)*. VEDA, Bratislava.
- KORMUTÁK A, VOOKOVÁ B, ZIEGENHAGEN B, KNOW HY, and HONG YP. 2004. Chloroplast DNA variation in some representatives of the Asian, North American and Mediterranean firs (*Abies* spp.). *Silvae Genetica* 53: 99–104.
- KRYLOV GV, MARADUDIN II, MICHEEV NI, and KOZAKOVA NF. 1986. *Firs*. Agropromizdat. Moscow. [in Russian]
- LIU TS. 1971. *A Monograph of the Genus Abies*. National Taiwan University, Taipei.
- MERGEN F, BURLEY J, and SIMPSON BA. 1964. Artificial hybridization in *Abies*. *Der Züchter* 34: 242–251.
- MURRAY MG, and THOMPSON WF. 1980. Rapid isolation of high molecular weight DNA. *Nucleic Acid Research* 8: 4231–4235.
- NĚMEC B. 1962. *Botanical Microtechnique*. Nakladatelství ČSAV, Prague. [in Czech]
- OWENS JN, and MOLDER M. 1977. Sexual reproduction of *Abies amabilis*. *Canadian Journal of Botany* 55: 2653–2667.
- SILEN RR, CRITCHFIELD WB, and FRANKLIN JF. 1965. Early verification of a hybrid between noble and California red firs. *Forest Science* 11: 460–462.
- TERHÜRNE-BERSON R, LITT T, and CHEDDADI R. 2004. The spread of *Abies* throughout Europe since the last glacial period: combined macrofossil and pollen data. *Vegetation History and Archaeobotany* 13: 257–268.
- TSUMURA Y, YOSHIMURA K, TOMARU N, and OHBA K. 1995. Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. *Theoretical and Applied Genetics* 91: 1222–1236.