Tree Genetics & Genomes (2006) 2: 53–60
DOI 10.1007/s11295-005-0033-x

ORIGINAL PAPER

**Nicholas Wheeler · Peggy Payne · Valerie Hipkins · Robert Saich · Stephen Kenny · Gerald Tuskan**

# Polymix breeding with paternity analysis in *Populus*: a test for differential reproductive success (DRS) among pollen donors

Received: 24 October 2005 / Accepted: 15 December 2005 / Published online: 2 February 2006
© Springer-Verlag 2006

**Abstract** Polymix breeding with paternity analysis (PMX/WPA) has been proposed as an alternative to traditional full-sib breeding and testing schemes. To fully capture the benefits of PMX/WPA, differential reproductive success (DRS) of pollen parents used in the polymix must be modest. DRS was evaluated in an operational test of PMX/WPA for a hybrid poplar breeding program. A 16-parent pollen polymix (*Populus nigra* L.) was used to pollinate seven clones of *Populus deltoides* (Bartr. ex. Marshall) under greenhouse breeding conditions. Progeny were grown out briefly and randomly sampled (357) prior to out-planting in field trials. Twenty-eight simple sequence repeat (SSR) loci were evaluated and 15 were selected for genetic characterization in small populations of three *Populus* spp (*P. nigra*, *P. deltoides*, and *P. balsamifera* spp *trichocarpa* Torr. & Gray). Seven loci were ultimately selected for paternity analysis of progeny. The average exclusion probability of the seven loci in *P. nigra* was 0.604; combined, the theoretical exclusion probability was 0.9999. However, only 95% of sampled progeny were unambiguously assigned a single paternal parent. Missing data likely accounted for most of the ambiguity. DRS was statistically significant though not prohibitive for practical utility of PMX/WPA as a breeding system. Of the 112 potential crosses in this study, 92 were represented. Eight of the 16 pollen parents contributed 83% of the progeny. Good pollen vigor, as measured by germination percent, did not ensure paternal success, but poor vigor was associated with lack of paternal success. PMX/WPA appears to be logistically and economically attractive for hybrid poplar breeding and testing.

N. Wheeler (✉)
Molecular Tree Breeding Services, LLC,
21040 Flumerfelt Rd SE,
Centralia, WA 98531, USA
e-mail: nickwheeler@scattercreek.com
Tel.: +1-360-2783535
Fax: +1-360-2783535

P. Payne
Boise Cascade LLC, Cottonwood Fiber Farm,
P.O. Box 500, 316 Attalia E. Road,
Wallula, WA 99363, USA

V. Hipkins · R. Saich
USDA Forest Service,
National Forest Genetic Electrophoresis Lab,
2480 Carson Road,
Placerville, CA 95667, USA

S. Kenny
Irrigated Agriculture Research and Extension Center,
Washington State University, Prosser,
24106 N Bunn Road,
Prosser, WA 99350-8694, USA

G. Tuskan
Environmental Sciences Division,
Oak Ridge National Laboratories,
P.O. Box 2008, Oak Ridge, TN 37830, USA

## Introduction

Polymix breeding with paternity analysis (PMX/WPA) of progeny has been proposed as an alternative to traditional full-sib breeding and testing schemes [4]. In short, PMX/WPA requires the application of pollen mixes of many male parents in controlled cross events, followed by paternity analysis of progeny using molecular markers. The approach offers good estimates of breeding values of parents and produces progeny populations with superior gain potential for forward selection. Other advantages of this approach include simpler and easier (thus, less expensive) breeding and testing programs, greater flexibility for inbreeding control, and convenient connections with other molecular genetic applications such as clonal fingerprinting and marker-aided selection [4]. These features are particularly relevant for trees, organisms which offer a number of obstacles to breeders (i.e., large, long-lived, and late-maturing habits requiring costly breeding and testing programs and facilities).

To fully capture the benefits of PMX/WPA, differential reproductive success (DRS) of parents used in pollen polymixes must not be high (most seed sired by only a few males). DRS in conifers (gymnosperms), though observed, is typically rather small (reviewed in Lambeth et al. 2001)

and insignificant relative to virtually all of the other factors controlling paternal success, such as amount and timing of pollen shed, location and distance of pollen donor relative to seed trees, and weather effects. Nakamura and Wheeler [8] concluded modest DRS in Douglas fir (*Pseudotsuga menziesii* Franco.) was likely due to genetic incompatibilities between male and female gametophytes (post-zygotic factors) but Nikkanen et al. [9] have demonstrated significant differences among spruce clones for pollen viability traits such as germination percentage and pollen tube growth rate. Such differences could arise from poor pollen vigor or genetic affects and could contribute to pre-zygotic competitive effects. Competition among male gametophytes has been well-studied in annual and non-woody angiosperms [7, 10, 11], but controlled studies with flowering forest trees are wanting.

The study described here results from the application of PMX/WPA for the creation of select hybrid poplar clones. Specifically, this study addresses the null hypothesis that all pollen parents in a polymix of pollens are represented equally in the progeny pool resulting from controlled inter-specific crosses with an array of seed parents (poplars are dioecious). Additionally, this study reports on the selection, optimization, and characterization of an array of micro-satellite simple sequence repeat (SSR) markers in three species of poplar.

## Materials and methods

### Plants, polymix construction, and controlled crosses

In the fall of 2003, multiple scions from each of seven select maternal *P. deltoides* Bartr. ex. Marshall (eastern cottonwood) clones originating from the upper mid-western United States, were grafted onto potted rootstock (*P. deltoides* var *siouxland*). In January of 2004, the grafts were moved into a greenhouse to encourage pistillate inflorescence emergence and receptivity. Similarly, in mid-January, branches bearing staminate inflorescences were collected from 16 select paternal *P. nigra* L. (black poplar) clones and placed in tap water baths in a greenhouse (15–18°C) to force pollen shed. All *P. nigra* clones originated from northern Italy; however, staminate cuttings were collected from an established clonal orchard located in eastern Washington. Seven of the 16 males had half-sib relatedness to one another (two pairs and a trio of half-sibs). Pollen was collected in glass beakers, funneled through 100 mesh screens to remove pollen catkins and poured into 20 ml glass vials (1/3 full) which were stoppered with cotton plugs. Vials were placed in desiccators containing silica gel and maintained at 4°C for the duration of the study (~1 week).

Upon completion of pollen collection, pollen germination trials were conducted by first rehydrating pollen aliquots for 2 h on dishes floating on a water bath in a hydration vessel and then, placing them on 1% agar containing 10% sucrose and minerals [1]. Pollen germination was scored after 18 h and reported as the proportion of pollen grains exhibiting pollen tubes equal to or greater than the diameter of the pollen grain. Individual clonal values were placed in categories (<10, 10–15, 15–20, and 20–25% germination). Beginning February 23, pollen mixes were created daily by combining equal weights of desiccated pollen from all 16 male clones. Prior to brush-applied pollination, the polymix was rehydrated for 2 h as described above. Individual inflorescences were pollinated once per day for 3 days and retained within protective paper bags. A single controlled-cross polymix family was created for each of the seven maternal *P. deltoides* clones. Seed capsules set and began to burst within ca. 30 days after pollination. Seeds were collected by family, weighed, and sown in flat trays. The rising crop (ca. 30 days after sowing) was transplanted into Ropac #3 trays (96 cells) in April, 2004, grown for ~30 additional days, and subsequently out-planted to a field test site near Pasco, WA. The hybrid poplar cross created here (*P. deltoides* × *P. nigra*) is one of the most common and valuable inter-specific hybrid taxa in the world and is recognized by the designation *Populus x canadensis* or *P. euromericana*. The hybrid is made with ease when *P. deltoides* serves as the female parent, though the reciprocal cross (*P. nigra* as female parent) virtually always fails [6].

### Molecular marker development

SSR markers were selected for this study because they are plentiful, co-dominant, and highly polymorphic in poplar. Though literally hundreds of SSR markers have been identified in poplar ([17] http://www.ornl.gov/sci/ipgc/ssr_resource.htm), few have been fully characterized and optimized for high throughput detection. One of the goals of this study was to identify and genetically characterize an array of SSR markers that were useful in three poplar species (*P. deltoides*, *P. nigra*, and *P. balsamifera* Torr. & Gray), the latter being a species native to the Pacific Northwest (black cottonwood) and common to hybrid poplar breeding programs.

*DNA extraction* DNA extraction was carried out on leaf tissue (progeny seedlings) using the DNeasy-96 Frozen Leaf Tissue Protocol following manufacturers instructions with tissue homogenization achieved via the Mixer Mill 300 (Qiagen, Valencia, CA) and from bud tissue (parents and population samples) using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following manufacturers' instructions. DNA quality and approximate quantity were assessed by comparing all samples against 50 ng of Lambda DNA standard on 0.8% agarose gels stained with ethidium bromide (EtBr) under UV light.

*SSR amplification and electrophoresis* A total of 28 candidate SSR primer pairs were evaluated (Table 1). SSR screening was achieved by amplifying approximately 1.5–2.5 ng of template DNA from *P. nigra* clones in a 10-μl final volume including 1× polymerase chain reaction (PCR) buffer, 2.0 mM $MgCl_2$, 0.4 μM of each dNTP, 0.4 μM of the forward and reverse primers, and 1 U of HotStarTaq DNA Polymerase (Qiagen, Valencia, CA). Amplifications were performed using a MJ Research PT-100

thermal controller with the following touchdown conditions: 15 min at 95°C, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (3×), 94°C for 30 s, 52°C for 30 s, 72°C for 1 min (3×), 94°C for 30 s, 50°C for 30 s, 72°C for 1 min (29×) followed by a final extension of 72°C for 7 min. The amplification product was then diluted to a ratio of 1:50 (amplification:dd$H_2O$) and 1 μl of dilute amplification product was added to 10 μl of Hi-Di Formamide containing 1.2% GeneScan-500 [ROX] size standard. Samples were subsequently denatured at 95°C for 2 min and placed immediately on ice for 3 min before the sample plate was loaded on an ABI Prism 3,100 Genetic Analyzer for detection of SSR product. ABI software packages, GeneScan Analysis Software and Genotyper Software v 3.7, were used to visualize and evaluate alleles at each locus.

### Molecular marker characterization

Each of the 28 candidate SSR primer pairs were evaluated for use in determining paternity in each of the three poplar species using the criteria of genetic interpretability (i.e., does the primer pair produce a single locus that segregates in Mendelian fashion, and are the resulting alleles interpretable), heterozygosity level, exclusion probability, and expected null allele frequency. Primer pairs were initially screened using full-sib, controlled-cross family progeny of each species (five progeny each in *P. deltoides* and *P. nigra* and six progeny in *P. trichocarpa*). Based on inability to amplify and/or the presence of uninterpretable peaks, 13 of the 28 candidate primer pairs were discarded from further study. The remaining 15 primer pairs produced 15 loci that were determined to be potentially useful for paternity analysis. These primer pairs/loci were selected based on:

1. 16 progeny from each of the three *Populus* species (48 individuals; unrelated to individuals in the polymix study),
2. Eight progeny from each of six controlled-cross families, i.e., two families from each of the three species (48 individuals), and
3. 12 parent trees of the six families noted above.

Parental genotypes were confirmed by simple exclusion analysis of the paternal contribution to each progeny in each species family thus, providing a basis for the evaluation of each locus in the areas of interpretability and presence of null alleles. Data from each species were scored using Genotyper Software v 3.7. CERVUS 2.0 [5] was used to calculate heterozygosity, parental exclusion probabilities, and estimated null allele frequencies. Also, the number, size, and frequency of alleles were calculated for each locus.

### Paternity assignment and reproductive success

In August 2004, one leaf was sampled from each of ~50 randomly selected progeny from each of seven polymix derived families and from those pollen parents not previously genotyped in the marker characterization exercise. DNA extraction, SSR amplification, and electrophoresis followed protocols noted previously. Paternity assignment for each progeny was determined using programming and formal language (PFL), a computer program for estimating pollen flow using paternity exclusion and SSR markers (http://www.fsl.orst.edu/pnwtirc/publications/pnwtirc_pubs_date.htm). PFL allows for exclusion calculated on the basis of one or more locus genotype mismatches. All crosses were evaluated at both one and two genotype mismatch levels. Missing data were accommodated in the program. Where two or more paternal parents could have potentially sired a progeny, partial paternity was assigned (e.g., two potential pollen parents=0.5 each). A test for deviation from expected reproductive success across all females was performed using chi-square analysis.

## Results and discussion

### Pollen germination and seed set

Date of pollen shed varied from February 3 to 17, with individual clones shedding pollen for up to 7 days. In general, pollen germination rates in this study were low, ranging from less than 10% to around 25%. Pollen germination rates of 10–55% are considered sufficient for adequate seed set in most controlled crosses [13]; it is unknown whether variation in germination percent might contribute to pollen competitiveness in polycrosses of poplar. In the few controlled crosses performed with single-clone pollen lots exhibiting low pollen germination percent (<10%) in this study, seed set was nil, and inflorescence development was abnormal. Four of the 16 pollen lots tested (25%) had germination scores of <10%. There was no relationship between date of pollen shed and germination percent ($r$=0.04).

Seed set was generally very good. Estimated seed set for polymix crosses exceeded 3,000 for six of the seven female *P. deltoides* clones (680–4,860 seeds), while 20 seed weights ranged from 0.0083 to 0.0150 g. The maternal clone with a few seeds also had the lowest number of inflorescences to pollinate.

### Molecular marker characterization

Thirteen of the selected SSR primer pairs produced ambiguous peaks or failed to yield an amplified product (Table 1). Fifteen SSR loci from the remaining 15 primer pairs were characterized for the three poplar species studied. Most loci were highly polymorphic in all three species: the average number of alleles per locus was 8.6, 10.7, and 11.1 for *P. nigra*, *P. deltoides*, and *P. balsamifera*, respectively. Across all species, 368 alleles were detected at the 15 select loci (mean=24.5 alleles per locus; range=11–41; Table 1). In general, relatively few alleles were shared among species, particularly between either

**Table 1** Microsatellite (*SSR*) primer pairs evaluated for use in poplar paternity analyses are listed along with ABI dye used for detection, forward and reverse primer sequence, *SSR* motif, primer pair utility (*U* Used in study, *I* discarded due to uninterpretable band patterns (i.e., non-specific amplification, allelic dropout), *P* discarded due to poor amplification), number of alleles detected, and number of alleles shared among species, disregarding nulls (*DP. deltoides*, *NP. nigra*, *TP. balsamifera*)

| Primer Name[a] | Dye | Forward primer | Reverse primer | Motif | Utility | Number of Alleles | | | | Number of Alleles Shared | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *D* | *N* | *T* | Total | DN | DT | NT | DNT |
| ORNL 029 | FAM | TGGTGATCCAGTTTTGGTGA | GTCCTTGCAAGCCATGAA | AC | U | 4 | 11 | 5 | 20 | 0 | 0 | 0 | 0 |
| ORNL 127 | FAM | TCAATGAGGGGTGCCATAAT | CTTTCCACTTTTGGCCCTTT | TG | U | 10 | 3 | 4 | 17 | 0 | 0 | 0 | 0 |
| PMGC 14 | FAM | TTCAGAATGTGCATGATGG | GTGATGATCTCACCGTTTG | CTT | U | 2 | 7 | 7 | 14 | 0 | 0 | 2 | 0 |
| PMGC 420 | HEX | ATGGATGAGAAATGCTTGTG | ACTGGCACACTCTTTAACTGG | GA | U | 4 | 3 | 10 | 16 | 0 | 1 | 0 | 0 |
| PMGC 433 | TAMRA | GCAGCATTGTAGAATAATAAAAG | AAGGGGTCTATTATCCACG | GA | U | 15 | 8 | 14 | 23 | 5 | 8 | 5 | 4 |
| PMGC 576 | HEX | GCTGTCTAACATGCCATTGC | AATTTACATTTCTTTATCATCACC | GA | U | 13 | 16 | 17 | 41 | 2 | 0 | 3 | 0 |
| PMGC 649 | FAM | CATCCATGATATCAAACCAAATTAG | TGTAATCCAAACATAAAATCCCAAG | GA | U | 17 | 7 | 19 | 32 | 4 | 7 | 1 | 1 |
| PMGC 2011 | HEX | TCTACGAGGAAAGGGAAGGG | CTTTATAATGCATCATAAAGTTC | GA | U | 13 | 9 | 8 | 21 | 2 | 6 | 2 | 1 |
| PMGC 2221 | HEX | GCTTTGGGAACATGCTTAT | CCAAATCTCCAATTTTATGG | ATT | U | 19 | 15 | 21 | 40 | 5 | 6 | 7 | 3 |
| PMGC 2235 | FAM | GCCAAAATAGTAAGTGTGATGG | CACACATTCTCTCATTCAAAGC | GA | U | 15 | 9 | 16 | 31 | 0 | 8 | 1 | 0 |
| PMGC 2571 | FAM | TCTCGCAGATTCATGTAACCC | GACTGTATGTTGACCATGCCC | GA | U | 1 | 1 | 9 | 11 | 0 | 0 | 0 | 0 |
| PMGC 2675 | FAM | CACACCGACAAATTATGAGTG | TTTTAGAGTGAATTTTCCTGCG | GA | U | 18 | 10 | 14 | 32 | 1 | 7 | 2 | 1 |
| PMGC 2804 | FAM | AAAGTTTTTCATTTTCAATCCTTG | TAATCGCCTATACACAGGCG | GA | U | 8 | 7 | 12 | 24 | 0 | 2 | 1 | 0 |
| PMGC 2885 | FAM | CATGATCAAATTGGATTTGAATG | AAAGATGAACATGGCTAGCTC | GA | U | 10 | 8 | 7 | 21 | 0 | 4 | 0 | 0 |
| WPMS03 | HEX | TTTACATAGCATTTAGCCTTTAGA | CCAAATCTCCAATTTTATGG | ATT | U | 10 | 14 | 3 | 25 | 0 | 1 | 1 | 0 |
| AG1 | HEX | CTTGTAATTAAGAGCAAGCCA | ATGTTAAACTACCTCAAACATATCC | AT | P | – | – | – | – | – | – | – | – |
| ORNL 015 | HEX | CGTGAGTTTTGAGGCCATTT | CATGGAAAGGATCACCCACT | [AT]14 | I | – | – | – | – | – | – | – | – |
| ORNL 203 | FAM | CCACCAGGCATGAGATATGA | TCAAACCGAAAGGTCAACAA | [TA]4 | I | – | – | – | – | – | – | – | – |
| ORNL 206 | HEX | CCGTGGCCATTGACTCTTTA | GAACCCATTTGGTGCAAGAT | [GCT]7 | I | – | – | – | – | – | – | – | – |
| ORNL 286 | HEX | TCAGGCAGAAGGGTAGAGGA | CCTGACCCTGCTTGCTTATC | [GT]4 | | | | | | | | | |
| [GA]8 | I | – | – | – | – | – | – | – | – | | | | |
| PMGC 603 | FAM | GTACCTATGAAAGTAGGCAACAC | TTTTTTATCACTATCTCAGATAC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 684 | FAM | GAAATTGAATATCTCTCACTTACC | TAATACGTGAAAAGTCAGGTTTTG | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2140 | HEX | GCTGTCAGAATCAAACACTTC | AAGCAGATAACTAAGACATGCC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2156 | FAM | GATCTCTCTTACATCACTCATC | GAATGTCTTTACTCCATTGTTGG | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2515 | FAM | GAAAAGGGATTGTTAATAAACCC | CCAAAATCATAAAAGACAGGGC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2585 | HEX | ACTGCTGTGTATTGCCCTAG | TAGTTGAAGTTGGAGCACAAC | GA | I | – | – | – | – | – | – | – | – |
| PMGC 2610 | HEX | AACACGCAAGAACATACATAAG | GATTAACATGTTTCGCTACGC | GA | I | – | – | — | – | – | – | – | – |
| PMGC 2862 | HEX | TTTGTAACTAATGAAGATTTGTAC | ATTTTTGTTCTTTTAACCAAAATTC | GA | P | – | – | – | – | – | – | – | – |

[a]Primers used in this study were provided by Dr. Steven Strauss, Oregon State University and Dr. Jerry Tuskan, ORNL. Primer development was as follows: ORNL markers: [15] PMGC markers: http://www.ornl.gov/sci/ipgc/ssr_resource.htm; WPMS markers: [12, 16]; AG1 marker: [2]

North American species (*P. deltoides* and *P. balsamifera*) and the European native *P. nigra* (5.2 and 6.8% alleles in common, respectively). *P. deltoides* and *P. trichocarpa* shared 13.6% of their alleles. Only 10 alleles (2.7%) were shared by all three species. Null alleles were detected in controlled crosses for six loci but were not considered shared between species because they likely do not represent the same mutational event. The mean allele frequency of the most common allele, across all 15 loci, was 0.308, 0.342, and 0.444 for *P. trichocarpa*, *P. deltoides* and *P. nigra*, respectively. Allele sizes and frequencies of all 15 loci evaluated are provided elsewhere (Appendix 1: Gene frequencies: http://www.ornl.gov/sci/ipgc/poplar polymix genefreg Appendix1.xls) Seven of the 15 loci characterized were ultimately deemed to be "informative" for all three species (Table 2) and were used for paternity testing in this study. For the seven selected loci, average observed heterozygosity was 0.681, 0.700, and 0.623 for *P. trichocarpa*, *P. deltoides*, and *P. nigra*, respectively. Similarly, the average exclusion probability for any one locus, given that the maternal genotype was known, was 0.754, 0.704, and 0.604. Although the Italian sample of *P. nigra* was the least genetically diverse of the three species evaluated, it theoretically possessed sufficient diversity to distinguish among all paternal parents. In the final analysis of all progeny and parents, the paternal exclusion probability for all seven loci combined was 0.9999. Measures of diversity (number of alleles, heterozygosity) observed for this breeding population of *P. nigra* were slightly lower than those detected in a previous study characterizing a range-wide collection of 23 individuals using nine SSR loci [16].

## Genotyping

Multi-locus genotypes were obtained for 357 hybrid poplar progeny. Over half of the progeny (58.3%) failed to amplify a paternal allele at one of seven loci; however, fewer than 5% were missing paternal genotypes at two or more loci. Null alleles accounted for a very small percentage of the missing data, while simple failure to amplify some primers in specific crosses appeared to be the cause in most cases. The failure to amplify a product was most acute on the maternal side (*P. deltoides*). For two markers (PMGC 2235 and PMGC 2675), five of the seven maternal parents displayed distorted allelic segregation due to a failure to amplify a product. For the remaining five

**Table 2** Properties of seven SSR loci selected for paternity studies in *P. deltoides* (*D*) by *P. nigra* (*N*) crosses (*P. nigra* is paternal parent)

| Locus | Species | *A* | *k* | *N* | $H_{(O)}$ | $H_{(E)}$ | Excl (1) | Excl (2) | Null freq | Use |
|---|---|---|---|---|---|---|---|---|---|---|
| PMGC 649 | T | 94–158 | 19 | 19 | 0.895 | 0.957 | 0.759 | 0.863 | 0.019 | 1 |
| | D | 100–152 | 17 | 17 | 0.765 | 0.959 | 0.753 | 0.859 | 0.098 | 1* |
| | N | 92–108 | 7 | 18 | 0.722 | 0.754 | 0.336 | 0.513 | 0.013 | 1 |
| PMGC 14 | T | 186–210 | 7 | 19 | 0.632 | 0.804 | 0.406 | 0.586 | 0.118 | 1 |
| | D | 188–191 | 2 | 19 | 0.579 | 0.508 | 0.122 | 0.186 | 0.079 | 2 |
| | N | 197–220 | 7 | 20 | 0.600 | 0.771 | 0.350 | 0.526 | 0.116 | 1 |
| PMGC 433 | T | 198–230 | 14 | 18 | 0.722 | 0.927 | 0.667 | 0.801 | 0.110 | 1 |
| | D | 193–221 | 15 | 19 | 0.684 | 0.909 | 0.630 | 0.774 | 0.118 | 1 |
| | N | 196–228 | 8 | 18 | 0.556 | 0.514 | 0.147 | 0.321 | 0.069 | 1 |
| PMGC 2221 | T | 67–138 | 21 | 18 | 0.667 | 0.959 | 0.759 | 0.863 | 0.167 | 1 |
| | D | 73–287 | 19 | 19 | 0.842 | 0.950 | 0.739 | 0.849 | 0.048 | 1 |
| | N | 81–136 | 15 | 19 | 0.526 | 0.937 | 0.700 | 0.824 | 0.265 | 1 |
| PMGC 2235 | T | 111–152 | 16 | 18 | 0.833 | 0.941 | 0.708 | 0.829 | 0.046 | 1 |
| | D | 114–161 | 15 | 18 | 0.611 | 0.927 | 0.670 | 0.803 | 0.190 | 1 |
| | N | 111–132 | 9 | 19 | 0.895 | 0.898 | 0.591 | 0.745 | 0.011 | 1 |
| PMGC 2675 | T | 165–232 | 14 | 17 | 0.353 | 0.922 | 0.656 | 0.793 | 0.441 | 2* |
| | D | 161–220 | 18 | 19 | 0.632 | 0.923 | 0.669 | 0.801 | 0.180 | 1 |
| | N | 152–208 | 10 | 17 | 0.412 | 0.863 | 0.521 | 0.688 | 0.347 | 1 |
| PMGC 2885 | T | 291–316 | 7 | 18 | 0.667 | 0.778 | 0.364 | 0.543 | 0.067 | 1 |
| | D | 297–316 | 10 | 19 | 0.789 | 0.839 | 0.483 | 0.657 | 0.013 | 1 |
| | N | 288–322 | 8 | 20 | 0.650 | 0.817 | 0.430 | 0.608 | 0.100 | 1 |
| AVERAGE OVER ALL SEVEN LOCI | T | | 14.0 | 18.1 | 0.681 | 0.898 | 0.617 | 0.754 | 0.138 | |
| | D | | 13.8 | 18.6 | 0.700 | 0.859 | 0.613 | 0.704 | 0.104 | |
| | N | | 9.3 | 18.7 | 0.623 | 0.793 | 0.439 | 0.604 | 0.132 | |

Values are given for *P. trichocarpa* as well. *A* Range of allele size in bp, *k* number of alleles, *N* number of individuals typed, $H_{(O)}$ observed heterozygosity, $H_{(E)}$ expected heterozygosity, *Excl(1)* average exclusion probability without information on one parent, *Excl(2)* average exclusion probability given information on known (female) parent, *Null freq* estimated null allele frequency, Use: 1 indicates likely useful locus, 2 indicates possibly useful locus

*Indicates issues with genetic interpretability

loci, only three of 35 cases displayed segregation distortion of maternal alleles ($p$=0.05, $\chi^2$≥3.84, 1 *df*). Marker PMGC 2235 is known to occur on chromosome IV which is comprised almost entirely of maternally biased segregation ratios [17]. Interestingly, two of the 16 paternal parents (numbers 12 and 16) possessed triploid genotypes and a third parent (number 9), a tetraploid genotype, for marker PMGC 2675. Whether this was causal in the aforementioned amplification problem is unknown. To properly account for paternal parents with three or four alleles in the paternal assignment program, additional dummy genotypes were created for these three parents. It should be noted that polyploidy in poplars is not uncommon [3].

## Paternal reproductive success

The PFL program assigned unambiguous paternity to 86% (309) of the progeny based on a single mismatch (one mismatch excludes a parent) and 94.7% based on two mismatches. Of the remaining 19 progeny, 15 were assigned two potential paternal parents, three were assigned three paternal parents, and a single individual could not be matched. For numerical summary purposes, multiple paternal assignments were treated proportionately. That is, each of two potential parents received credit for 0.5 progeny, and each of three potential parents received credit for 0.33 progeny. The theoretical expectation of exclusion probability exceeding 99% was above the observed value of 94.7%. Several factors likely contributed to this shortfall: genotyping error, including missing data, progeny that were undifferentiated genetically (half-sibs), and/or pollen contamination. Indeed, five of the 15 cases sporting two potential pollen parents assigned the half-sib paternal parents number 7 and number 8. Contamination, though unlikely, in the controlled-cross conditions of this experiment, was still possible and may have accounted for the lone progeny that had no parental assignment.

All 16 paternal parents in the polymix were represented in the progeny pool, although representation was highly variable (Table 3). Clearly, the null hypothesis that all parents would be represented equally was rejected ($p$<0.001, $\chi^2$=219.1, 15 *df*). Over all seven females, the expectation is that each paternal parent would be represented by 22.3 progeny (3.2 per individual female). Actual paternal contribution varied from 2.5 to 62.0 (1.0 to 17.0%) across all female parents and ranged from 0–12% for individual female parents. The top eight paternal parents sired 83.3% of the progeny. In general, the top eight parents were well represented in each cross, though there were instances in which these males failed to sire a progeny for a given female (i.e., Male number 1, Cross H4). The reverse was also true, i.e., a number of parents that faired poorly overall did quite well with individual females. The expected sample size for each male by female combination was too low to consider performing interaction chi-square tests.

It is noteworthy that paternal success appeared to be unrelated to pollen vigor, as measured by proportion of pollen grains germinating in a short term test ($r^2$=0.057). Indeed, the least represented pollen parent (number 8) had the highest pollen germination score (>25%). Still, reproductive success was clearly low for all parents with germination scores of one. Given the observation that a few single parent controlled crosses with pollen parents that had germination scores of one resulted in no filled seed, it is surprising to see any representation in the polymix progeny for these parents. One explanation could be the mentor pollen effect [14]. The mentor pollen technique has

**Table 3** Number of progeny assigned to each of 16 paternal parents in a polymix for each of seven crosses (H2–H9), the total number and proportion of progeny assigned each parent, and a measure of pollen viability based on germination percentage (<10%=1, 10–15%=2, 15–20%=3, and 20–25%=4)

| | Female (*P. deltoides*) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Male (*P. nigra*) | H2 | H3 | H4 | H5 | H6 | H8 | H9 | Total | Proportion of total | Pollen viability |
| 1 | 2.0 | 5.5 | 0.0 | 8.0 | 8.0 | 4.5 | 4.5 | 32.5 | 0.09 | 2 |
| 2 | 1.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 2.0 | 3.3 | 0.01 | 1 |
| 3 | 9.0 | 7.5 | 6.0 | 5.0 | 4.0 | 5.0 | 4.0 | 40.5 | 0.11 | 3 |
| 4 | 2.3 | 4.5 | 4.0 | 4.0 | 7.8 | 10.0 | 3.5 | 36.1 | 0.10 | 4 |
| 5 | 4.0 | 3.0 | 8.0 | 4.0 | 1.3 | 6.0 | 10.0 | 36.3 | 0.10 | 4 |
| 6 | 12.0 | 7.0 | 11.0 | 7.0 | 8.5 | 11.0 | 5.5 | 62.0 | 0.17 | 2 |
| 7 | 0.8 | 1.0 | 1.5 | 1.5 | 0.0 | 0.0 | 3.0 | 7.8 | 0.02 | 1 |
| 8 | 0.5 | 0.0 | 0.5 | 0.5 | 0.0 | 0.0 | 1.0 | 2.5 | 0.01 | 4 |
| 9 | 5.3 | 1.0 | 2.0 | 6.5 | 1.0 | 0.5 | 2.5 | 18.8 | 0.05 | 4 |
| 10 | 7.0 | 11.0 | 5.0 | 5.0 | 10.0 | 3.0 | 6.0 | 47.0 | 0.13 | 2 |
| 11 | 0.0 | 2.5 | 0.0 | 0.5 | 1.5 | 2.0 | 4.0 | 10.5 | 0.03 | 1 |
| 12 | 2.0 | 2.0 | 0.0 | 5.0 | 2.0 | 1.0 | 2.0 | 14.0 | 0.04 | 3 |
| 13 | 1.0 | 2.0 | 2.0 | 1.0 | 1.3 | 1.0 | 0.0 | 8.3 | 0.02 | 4 |
| 14 | 2.0 | 2.0 | 7.0 | 0.0 | 3.3 | 7.0 | 3.0 | 24.3 | 0.07 | 2 |
| 15 | 1.0 | 0.0 | 1.0 | 1.0 | 2.8 | 0.0 | 1.0 | 6.8 | 0.02 | 1 |
| 16 | 0.0 | 0.0 | 4.0 | 0.0 | 0.0 | 1.3 | 1.0 | 6.3 | 0.02 | 4 |
| Totals | 50.0 | 49.0 | 52.0 | 49.0 | 52.0 | 52.0 | 53.0 | 357.0 | 1.00 | |

been used to make inter-specific crosses that otherwise cannot be made. The desired pollen is mixed with irradiated (killed) compatible pollen before pollination and fertilization is facilitated. The combination of pollens in this case may have actually aided pollen lots of low viability.

Finally, all three paternal parents that possessed polyploid genotypes for marker PMGC 2675 were under-represented in the progeny pool (mean of 13 progeny per parent, overall) even though they all possessed high pollen germination scores.

Though the experimental design and statistical rigor of the experiment are insufficient to differentiate among all the possible reasons for unequal paternal success, the results do guide speculation. The lack of association between vigor and paternal success implies pre-zygotic competition based on number of pollen grains competing is not a critical factor although issues such as pollen tube growth rate may still be relevant (assuming rate of growth is unrelated to germination success). Post-zygotic competition in the form of genetic complementation between male and female parents could explain much of the pattern of paternal success observed. This may be particularly relevant in this case where inter-specific crosses were made. The correspondence between DRS and paternal half-sibs among the pollen parents appears to support this hypothesis. For instance paternal half-sib pairs number 7 and number 8, and number 2 and number 15 each exhibited very poor reproductive success for both individuals (mean of 1.5% in each case), while each of the three pollen parents in the half-sib that included number 5, number 12, and number 14 contributed significantly (mean of 7.0% per parent) to the progeny pool.

### Applicability of PMX/WPA for hybrid poplar breeding

PMX/WPA was conceived for recurrent selection breeding programs in which pedigree control is vital to reduce or eliminate inbreeding in advanced generations while simultaneously improving gain and decreasing breeding costs. For inter-specific $F_1$ hybrid breeding programs, such as the one described here, pedigree control for inbreeding is not directly relevant, though knowing the parentage of individual progeny is still valuable. Managing a pedigree against inbreeding would be relevant if reciprocal recurrent selection programs exist for each parental species to identify the best parents to enter the hybrid program. More importantly, PMX/WPA offers a method for increasing the number of parental combinations created, ostensibly improving the chances of finding the best combination at a reasonable cost. This is particularly true if the chosen alternative approach is a disconnected factorial design. A potential tradeoff, relative to traditional factorial designs, is that fewer progeny per cross are created with PMX/WPA, assuming that progeny test size remains the same for the two designs. It is necessary to understand the genetic variance structure of "among parents" vs "within parents" to determine if this trade off is significant. While it is too early to determine if gain was improved in this study, it is possible to evaluate the costs of deploying PMX/WPA relative to the traditional factorial mating design used in the hybrid poplar breeding in this program. For the combination of activities that included grafting of breeding materials, pollen handling, making controlled crosses, and seed sowing, the polymix approach cost was ~30% of the traditional factorial approach ($9,716.00 vs $31,556.00). The added costs of genotyping all parents and a few selected progeny would be nominal (<$500.00), making this approach economically quite attractive in an operational setting.

## Summary

Significant logistical and economic incentives exist for adopting PMX/WPA for the creation of hybrid poplars in the program described here. Though not particularly surprising, the skewed paternal contribution to the progeny pool is noteworthy and suggests follow-up studies to determine if pollen competition induced by polymix breeding prevents otherwise good crosses from occurring while simultaneously encouraging fertilization from pollen sources with low vigor. Of the 112 potential crosses in this polymix study, 92 were represented in the progeny pool. This contrasts with the 49 crosses that would have occurred under the factorial study. Even the least successful pollen parent could be represented by ~70 progeny in the operational field tests (0.7% success rate in sample × 9,800 progeny in test). Still, it would be desirable to have the remaining 20 potential crosses represented if the highest possible predicted gains are to be achieved.

**Acknowledgements** We wish to thank Dr. Steve DiFazio from the Environmental Sciences Division, Oak Ridge National Laboratory, and Dr. Steven Strauss, Department of Forest Science, Oregon State University for providing aliquots of primers needed to amplify 28 SSR loci evaluated in this study. We are grateful to Chuck Wierman, Brian Stanton and Clem Lambeth for reviewing earlier versions of this manuscript, and to anonymous reviewers at the journal.

## References

1. Brewbaker JL, Kwack BH (1963) The essential role of calcium ion in pollen germination and pollen tube growth. Am J Bot 50:859–865
2. Brunner AM, Rottmann WH, Sheppard LA, Krutovskii K, DiFazio SP, Leonardi S, Strauss SH (2000) Structure and expression of duplicate AGAMOUS orthologues in poplar. Plant Mol Biol 44:619–634
3. Dickmann DI, Stuart KW (1983) The culture of poplars in Eastern North America. Michigan State University, East Lansing, MI
4. Lambeth C, Lee B-C, O'Malley D, Wheeler NC (2001) Polymix breeding with parental analysis of progeny: an alternative to full-sib breeding and testing. Theor Appl Genet 103:930–943

5. Marshall TC, Slate J, Kruuk L, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7(5):639–655
6. Melchior GH, Seitz FW (1968) Interspezifische kreuzungssterilitat innerhalb der pappelsektion *Aigeriros*. [Interspecific cross sterility within the poplar section *Aigerios*], translated from German. Silvae Genet 17:88–93
7. Mulcahy D (1983) Models of pollen-tube competition in *Geranium maculatum*. In: Real L (ed) Pollination Biology, Academic, New York, pp 151–161
8. Nakamura RR, Wheeler NC (1992) Pollen competition and paternal success in Douglas-fir. Evolution 46:846–851
9. Nikkanen T, Aronen T, Haggman H, Venäläinen M (2000) Variation in pollen viability among *Picea abies* genotypes—potential for unequal paternal success. Theor Appl Genet 101:511–518
10. Quesada M, Winsor JA, Sephenson AG (1993) Effects of pollen competition on progeny performance in a heterozygous cucurbit. Am Nat 142:694–706
11. Smouse PE, Meagher TR (1994) Genetic analysis of male reproductive contributions in *Chamaelirium luteum* (L) Gray (*Liliacease*). Genetics 136:313–332
12. Smulders MJM, Van Der Schoot J, Arens P, Vosman B (2001) Trinucleotide repeat microsatellite markers for black poplar (*Populus nigra* L.). Molec Ecol Notes 1(3):188–190
13. Stanton BJ, Villar M (1996) Controlled reproduction of *Populus*. In: Stettler RF, Bradshaw HD Jr, Heilman PE, Hinckley TM (eds) Biology of *Populus* and its implication for management and conservation. Ch 5. NRC Research, Ottawa, Ontario, Canada, pp 113–138
14. Stettler RF (1968) Irradiated mentor pollen: its use in remote hybridization of black cottonwood. Nature 219:746–747
15. Tuskan GA, Gunter LE, Yang ZK, Yin TM, Sewell MM, DiFazio SP (2004) Characterization of microsatellites revealed by genomic sequencing of *Populus trichocarpa*. Can J For Res 34(1):85–93
16. van der Schoot J, Pospiskova M, Vosman B, Smulders MJM (2000) Development and characterization of microsatellite markers in black poplar (*Populus nigra* L.). Theor Appl Genet 101(1–2):317–322
17. Yin TM, DiFazio SP, Gunter LE, Riemenschneider D, Tuskan GA (2004) Large-scale heterospecific segregation distortion in *Populus* revealed by a dense genetic map. Theor Appl Genet 109:451–463