

# An Empirically Derived Model of Field-Scale Gene Flow in Winter Wheat

Todd A. Gaines, Patrick F. Byrne,\* Philip Westra, Scott J. Nissen,  
W. Brien Henry, Dale L. Shaner, and Phillip L. Chapman

## ABSTRACT

The potential introduction of wheat (*Triticum aestivum* L.) cultivars with transgenic traits has generated increased interest in pollen-mediated gene flow (PMGF). The objectives of this study were to estimate wheat PMGF between commercial fields across multiple years and locations, and to compare estimates from large fields to those from smaller experimental plots. The study was conducted in a total of 56 commercial field locations in eastern Colorado in 2003, 2004, and 2005. We measured PMGF by tracking the movement of an imidazolinone herbicide resistance gene from resistant to susceptible cultivars, sampled at distances of 0.23 to 61 m. At least one sample from all 56 fields and from all 18 evaluated cultivars had detectable PMGF. The highest observed PMGF was 5.3% at 0.23 m. The farthest distance at which PMGF was detected was 61 m and the highest PMGF at that distance was 0.25%. Higher levels and greater distances of PMGF were detected in commercial fields than in experimental plots. Based on estimates from a generalized linear mixed model with a random location effect, the distance required to ensure 95% confidence that 95% of locations have PMGF less than 0.9% is 41.1 m for cultivars heading earlier than the pollen source and 0.7 m for cultivars heading later than the pollen source. These confidence limits should represent the highest levels of PMGF expected to occur in winter wheat in the west-central Great Plains and will be useful for wheat biotechnology regulation.

T.A. Gaines, P.F. Byrne, Colorado State Univ., Dep. of Soil and Crop Sciences, 1170 Campus Delivery, Fort Collins, CO 80523; P. Westra, S.J. Nissen, Dep. of Bioagricultural Sciences and Pest Management, 1177 Campus Delivery, Fort Collins, CO 80523; W.B. Henry, USDA/ARS Central Great Plains Research Station, Akron, CO 80720, present address, Room 326 Dorman Hall, Stone Blvd., Mississippi State, MS 39762; D.L. Shaner, USDA/ARS Water Management Research, 2150 Centre Avenue, Fort Collins, CO 80526; P.L. Chapman, Dep. of Statistics, 1877 Campus Delivery, Fort Collins, CO 80523. Received 23 July 2007. \*Corresponding author e-mail: Patrick.Byrne@colostate.edu.

**Abbreviations:** ALS, acetolactate synthase; AIC, Akaike information criterion; GLMM, generalized linear mixed model; GWM, general wheat model; IR, imidazolinone-resistant; IS, imidazolinone-susceptible; PCR, polymerase chain reaction; PMGF, pollen-mediated gene flow; RHC, relative heading class.

GENE FLOW, defined as movement between populations that results in genetic exchange (Hedrick, 2005), can occur in plants through pollen dispersal. Gene flow in wheat has historically been a concern only for seed production, as wheat is considered a self-pollinating species with generally less than one to two percent outcrossing (Poehlman and Sleper, 1995). More recently, wheat cultivars and experimental lines with novel non-transgenic traits such as herbicide resistance (Newhouse et al., 1992) or transgenic traits such as herbicide and pathogen resistance (Zhou et al., 2003; Schlaich et al., 2006) have generated increased interest in gene flow between wheat fields. Understanding gene flow via pollen movement will be critical to ensure coexistence of transgenic and non-transgenic crops (Messean et al., 2006). Although no transgenic wheat cultivars have been commercialized to date,

Published in Crop Sci. 47:2308–2316 (2007).

doi: 10.2135/cropsci2007.05.0248

© Crop Science Society of America

677 S. Segoe Rd., Madison, WI 53711 USA

All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher.

biotechnology regulatory agencies require robust estimates of cross-pollination frequency between wheat fields to determine isolation distances for field evaluation of experimental transgenic lines.

Wheat seed production guidelines address gene flow between fields. In Colorado, isolation distances of 3 m are required for certified seed production (Anonymous, 2003). Several authors have suggested that to ensure adequate purity in wheat seed production, a larger isolation distance is needed, such as 30 m (Matus-Cadiz et al., 2004) or 45 m (Hanson et al., 2005). Gene flow in wheat has been detected at distances as great as 2.75 km, though at the very low level of 0.01% (Matus-Cadiz et al., 2007).

Pollen movement is the first step required for gene flow to occur. Synchrony in flowering between fields is necessary, because viable pollen must successfully enter florets, germinate on stigmas, and fertilize ovules (Waines and Hegde, 2003). Anthesis in wheat can last up to 10 d (De Vries, 1973) and stigmas can be receptive for a period of four to 13 days (De Vries, 1971). Cross-pollination in wheat can be affected by both genetic and environmental factors. Cultivars are known to vary in receptivity to gene flow under controlled conditions (Griffin, 1987; Hucl, 1996; Hucl and Matus-Cadiz, 2001; Lawrie et al., 2006; Martin, 1990). The optimal temperature for pollen production is 21°C (Porter and Gawith, 1999), but freezing or near-freezing temperatures at the boot, heading, and anthesis stages can kill anthers, resulting in male sterility (Shroyer et al., 1995) that leaves the surviving stigmas more receptive to foreign pollen. High temperatures or drought can increase cross-pollination frequency (Briggs et al., 1999; Waines and Hegde, 2003) by increasing glume opening, which is associated with higher outcrossing (De Vries, 1971; Hucl, 1996; Waines and Hegde, 2003).

Pollen source size is known to influence outcrossing in recipient wheat (De Vries, 1974). Several studies in wheat have used single row pollen sources to measure gene flow via pollen over close distance (Griffin, 1987; Hucl, 1996; Martin, 1990). Other studies have used pollen source blocks ranging in size from 5 m<sup>2</sup> (Hucl and Matus-Cadiz, 2001) to 50 m<sup>2</sup> (Matus-Cadiz et al., 2004) to 1640 m<sup>2</sup> (Hanson et al., 2005). Matus-Cadiz et al. (2004) suggested that further research at the commercial field scale was necessary, because small pollen sources may underestimate gene flow in wheat.

Realistic measurements of gene flow require adequate sampling over space and time to reveal the range of variation that may occur (Rieger et al., 2002). The release in 2001 of a non-transgenic herbicide-resistant winter wheat cultivar in Colorado presented a unique opportunity to monitor gene flow at the commercial field scale. This cultivar, Above (Haley et al., 2003), has single-gene imidazolinone herbicide resistance due to a mutant allele at the acetolactate synthase (ALS) locus on the D genome (Anderson et

al., 2004). Above and several subsequently released cultivars were developed through induced mutagenesis followed by conventional plant breeding (Newhouse et al., 1992; Tan et al., 2005). Above was first planted by commercial grain producers in fall of 2002 and the first summer during which PMGF could have occurred on a large scale was 2003. In this system, gene flow is identified by the presence of heterozygous imidazolinone-resistant (IR) plants in the progeny of imidazolinone-susceptible (IS) plants.

Ensuring coexistence of transgenic and non-transgenic wheat, including appropriate biotechnology regulations for transgenic wheat field trials and appropriate seed production standards, will require knowledge of commercial field-scale gene flow variation. A comparison between gene flow in commercial fields and in experiments with small pollen sources would also aid in evaluating previous wheat gene flow studies. This study utilized the novel IR trait to (i) estimate gene flow in wheat between commercial fields across years and locations and (ii) compare gene flow estimates from commercial field sampling to those obtained from small experimental plots.

## MATERIALS AND METHODS

### Commercial Fields

#### Sample Collection

Field sites were located in eastern Colorado where IR winter wheat cultivars Above or Bond CL (Haley et al., 2006) were grown bordering IS winter wheat during 2003, 2004, and 2005. Commercial field sites were either a single IS cultivar planted adjacent to an IR cultivar or cultivar strip trials, where multiple IS cultivars were planted in strips parallel to IR wheat. Dates of heading, when approximately 50% of heads were visible above the flag leaf, were recorded for both pollen source and recipient cultivars. Heading typically occurs two to three days before anthesis.

Commercial field samples were collected by hand harvesting single rows of the IS cultivar along a transect perpendicular to the field border at distances from 0.23 to 61 m from the IR border. Each sample was a composite of at least 20 subsamples of all wheat heads in a 1 m length of row, spaced at least 3 m apart, in a single row at each distance from the IR pollen source. For the strip trials, a single cultivar was represented by one to three composite samples at different distances from the IR cultivar. Samples were grouped by distance and threshed individually with a stationary thresher, starting with samples from the farthest distances and proceeding to the closest distances to minimize potential contamination between samples.

A total of 455 samples were collected from 56 locations in eastern Colorado (Table 1). The samples included 18 commercial wheat cultivars, representing six relative heading classes (RHC) for eastern Colorado (Table 2). Relative heading classes range from 1 to 8, with 1 being earliest and adjacent classes differing by approximately 1.5 d (S. Haley, personal communication, 2006). At all locations IS wheat was sampled next to Above, with the exception of one location where IS wheat was sampled next to Bond CL. Above is classified as an

**Table 1. Number of commercial wheat field sample locations and total plants screened.**

Year	Locations	Samples	Plants screened × 10 <sup>6</sup>
2003	17	123	1.06
2004	17	167	1.41
2005	22	165	1.54
Total	56	455	4.01

**Table 2. Wheat cultivars sampled in commercial fields.**

Relative heading class <sup>†</sup>	Cultivar	Number of locations	Number of samples
1	Prairie Red	11	72
1	TAM 107	1	7
2	Jagger	8	57
3	Halt	2	7
5	Akron	11	45
5	Alliance	3	16
5	Ankor	21	50
5	Avalanche	16	29
5	Enhancer	8	18
5	Hatcher	2	14
5	Jagalene	13	41
5	Yuma	2	9
5	Yumar	2	4
6	Ike	1	8
6	Millennium	1	1
6	Platte	1	5
6	Trego	19	52
8	Prowers 99	3	20

<sup>†</sup>Relative rating for eastern Colorado on a scale of 1 to 9 with 1 being earliest (S. Haley, personal communication, 2006).

RHC 3 cultivar, and Bond CL is considered to be in RHC 5. All locations had some degree of heading date overlap between IS and IR cultivars sufficient to provide flowering synchrony. Sampling sites included 25 cultivar strip trials (177 samples) and 36 large commercial fields (278 samples). Some locations included both commercial field transects and cultivar strip trials. Commercial field locations ranged in size from 32 to 130 ha for pollen source and recipient fields, and border length varied from 200 to 800 m. Cultivar trial pollen source and recipient plots ranged from 6- to 24-m wide with a 200- to 400-m long border.

### Field Screening Method

To detect cross-pollination, samples were screened for herbicide resistance using a field-based method. Field plots consisted of 17-m long plots with six rows (2003) or four rows (2004 and 2005). The design had four replicates and 15,000 total seeds planted per sample (2003) or three replicates and 11,250 total seeds planted per sample (2004 and 2005). Seed numbers were estimated based on 200-kernel mass for each sample. Known homozygous IR plants (Above) were included as checks in separate plots. Plots were planted in October and treated in April at the three to five leaf stage with imazamox (an imidazoli-

none herbicide) at 44 g ha<sup>-1</sup>, 0.25% (v/v) non-ionic surfactant (Activator 90, Loveland Industries Inc., Greeley, CO), and 2.5% (v/v) urea ammonium nitrate applied in a spray volume of 187 L ha<sup>-1</sup> at 206 kPa. A second imazamox application of 35 g ha<sup>-1</sup> was applied in early May to ensure adequate differentiation among homozygous resistant, homozygous susceptible, and heterozygous phenotypes. In this system, most plants derived from IS cultivar seeds are expected to be killed by the herbicide treatment, and only plants derived from cross-pollinated seeds are expected to survive.

The number of surviving plants was determined in late May. A heterozygous phenotype, indicative of gene flow, was identified by mild foliar chlorosis, stunting, increased tillering, and twisted spikes. This phenotype was consistent with greenhouse observations of imidazolinone-treated heterozygous plants (Pozniak and Hucl, 2004). We estimated the total number of emerged plants in each plot by counting subsamples. The subsample plant population was used to estimate the average number of plants per meter of row, and that number was multiplied by the number of meters of row in a plot to estimate the total number of emerged plants.

### Survivor Verification

Resistance to ALS-inhibiting herbicides in plants can occur via spontaneous mutation (Saari et al., 1994). To verify that plants scored as heterozygous carried one copy of the ALS mutant allele from Above and one copy of the wild-type ALS allele from the susceptible parent, we conducted polymerase chain reaction (PCR) analysis on a subsample of plants. Proprietary PCR-based protocols and primers specific to the D genome ALS mutant allele from Above and the wild-type D genome ALS allele were provided by the BASF Corp. (Research Triangle Park, NC). Reactions were performed on a PTC-100 thermal cycler (Bio-Rad Laboratories, Hercules, CA). Amplification products were separated on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

### Data Analysis

Percent PMGF was calculated as (number of verified survivors/total number of plants) × 100. Analysis of variance for PMGF was performed in SAS Proc GLM (SAS, 2004) on verified PMGF results from samples taken within 6 m of the pollen source. Means separation was conducted using Fisher's Least Significant Difference (LSD) at  $\alpha = 0.05$ .

### Experimental Plots

In a separate experiment, a Nelder wheel design (Nelder, 1962) was used to measure gene flow from smaller pollen sources. Plots were planted in fall 2003 and 2004 and sampled the following summer. Alternating strips of the IS cultivars Prairie Red and Halt were planted around a central 10-by-10 m block of Above. Dates of 50% heading for the three cultivars were recorded. Wind speed and direction data were obtained from a permanent USDA weather station 400 m from the site. Samples were collected by hand harvesting all wheat heads within a 1 m<sup>2</sup> area at 1, 3, 7, 11, 15, 23, 31, and 39 m from the edge of the pollen source along eight transects in north, northeast, east, southeast, south, southwest, west, and northwest directions. In 2004, two additional transects were

sampled on a north by northwest and a west by northwest orientation. Farther distances were sampled where the size of the recipient wheat plot permitted, up to 70 m from the pollen source. These samples were processed separately from commercial field samples and evaluated using a greenhouse screening method.

## Greenhouse Screening Method

Samples from the 2004 Nelder wheel were screened by planting 12 rows of 15 seeds each in rectangular flats with commercial potting media (Sunshine Mix #3, SunGro Horticulture, Bellevue, WA). Each sample was planted in two flats in each of two replications. Flats were placed in a greenhouse under natural light supplemented with 400 W sodium halide lamps to provide a 14 h daylength and watered as required. Plants were sprayed at the two- to three-leaf stage with imazamox at 35 g ha<sup>-1</sup>, 0.25% (v/v) non-ionic surfactant, and 1.0% (v/v) urea ammonium nitrate in a research track sprayer (DeVries Manufacturing Corp., Hollandale, MN) calibrated to apply 187 L ha<sup>-1</sup> at 206 kPa. Two days after treatment, plants were clipped to approximately 1 cm above the newest emerging leaf. Plants that re-grew and displayed an injured phenotype were identified as heterozygous survivors. The number of survivors divided by the number of emerged plants in each sample was used to calculate PMGF.

Samples from the 2005 Nelder wheel experiment were screened with a more efficient method of soaking seeds in imazamox, described by Gaines et al. (2007). A foliar application of imazamox as described for the 2004 samples was applied 10 to 14 d after emergence to eliminate any susceptible plants that escaped imazamox treatment during seed soaking. The number of survivors divided by the expected number of emerged plants in each sample was used to calculate PMGF. The methods used in 2004 and 2005 were compared by correlating PMGF estimates from a set of commercial field samples taken in 2003.

## Empirical Model

As a starting equation for this empirical modeling approach, we used the following "General Wheat Model" (GWM) proposed by Gustafson et al. (2005):

$$PMGF = 10^{-(0.2\sqrt{x})} \quad [1]$$

The GWM can be rewritten as

$$Y = a \times 10^{-bx^c} \quad [2]$$

where  $Y$  is PMGF,  $a$ ,  $b$ , and  $c$  are model parameters describing the height, steepness, and curvature of the line, respectively, and  $x$  is the distance in m between pollen source and recipient plants. Gustafson et al. (2005) fixed  $a$  at 1,  $b$  at 0.2, and  $c$  at 0.5 based on graphing data sets from published studies and adjusting model parameters to obtain a line that exceeded 95% of available data and had conservative (high-end) predictions of gene flow at all distances. Because heading date in the receiving field relative to the pollen source likely influences receptivity to gene flow (Waines and Hegde, 2003), an additional term was added to the model

$$Y = a \times 10^{-bx^c + dz^f} \quad [3]$$

where  $d$  is a model parameter,  $z$  is RHC, and  $f$  is the exponent in a power transformation of  $z$ . Based on preliminary fitting,

both  $c$  and  $f$  were fixed. By fixing these exponential values, the model becomes a linear combination of parameters, thereby substantially reducing the complexity of calculating standard errors for the value of interest (PMGF). A generalized linear mixed model (GLMM) was estimated using the maximum likelihood method in SAS Proc Nlmixed (SAS, 2004). The GLMM was fit separately to commercial field and experimental plot data. Model fit was judged using the Akaike information criterion (AIC) (Burnham and Anderson, 2002).

Equation [3] was modified for additional analysis in Proc Nlmixed. For a given location,  $H_i$  (number of heterozygous plants) is binomially distributed with  $n_i$  trials and a probability given by

$$a \times 10^{-bx^c + dz^f} + I_i \quad [4]$$

where  $I_i$  is a random, normal location effect with mean zero and standard deviation  $\sigma$ . Three lines were computed based on the model parameter estimates. The first line is a model of the median response, which represents a typical location where  $I = 0$ .

$$H = \hat{a} \times 10^{-\hat{b}x^{\hat{c}} + \hat{d}z^{\hat{f}}} \quad [5]$$

The second line represents an estimate of the response for a location at the upper 95th percentile of locations,  $10^3$ , where

$$\hat{\chi} = \log_{10}(\hat{a}) - \hat{b}x^{\hat{c}} + \hat{d}z^{\hat{f}} + 1.645\hat{\sigma} \quad [6]$$

and where 1.645 is the upper 95th percentile of a standard normal distribution.

The third line represents an upper 95% confidence limit on  $10^3$  and requires a standard error of  $\hat{\chi}$  calculated using established methods for variances of linear combinations of parameter estimates. An interpretation of this line is that we have 95% confidence that 95% of locations in a given RHC have gene flow less than this bound at a given distance.

## RESULTS

### Commercial Fields

Resistance to an imidazolinone herbicide proved to be a reliable and easily implemented trait for estimating gene flow. To verify our phenotypic scoring, a total of 92 suspected heterozygous plants were sampled for PCR analysis over the three years of the study and all were confirmed as having both mutant and wild-type alleles at the D genome ALS locus. In 2004 and 2005, we tested two single plants that were considered ambiguous as to whether they were homozygous or heterozygous for imidazolinone resistance. Both plants were scored homozygous mutant by PCR and were not included in the PMGF calculations.

The highest observed gene flow in a sample was 5.3% in 'Jagger' at 0.23 m, while no gene flow was detectable in 125 (27%) of the samples at distances ranging from 0.3 to 61 m from the pollen source. Gene flow was detected in at least one sample from every location and from each of 18 cultivars tested. Across all three years, 33% of samples were taken within 6 m of the pollen source and 67% of total observed gene flow occurred within that distance; 77% of samples were taken within 30 m and 92% of observed gene flow occurred within that distance. The farthest

**Table 3. Pollen-mediated gene flow in wheat samples collected at distances  $\leq 6$  m from the pollen source, by cultivar and year.**

Cultivar	Year					
	2003		2004		2005	
	Mean —%—	Number of samples	Mean —%—	Number of samples	Mean —%—	Number of samples
Akron	0.04 b <sup>f</sup>	8	0.12 bc	7	0.10 b	3
Alliance	0.14 b	1	0.24 abc	4		
Ankor	0.42 b	4	0.57 a	6	0.07 b	6
Avalanche	0.15 b	2				
Enhancer	0.12 b	5				
Halt					0.02 b	2
Hatcher					0.05 b	6
Ike	0.13 b	4				
Jagalene			0.03 c	4	0.30 b	8
Jagger	2.47 a	5	0.21 abc	8	0.28 b	9
Platte					0.40 b	1
Prairie Red	1.14 b	2	0.46 ab	9	0.56 b	12
Prowers 99					0.02 b	9
TAM 107					1.66 a	3
Trego	0.09 b	4	0.18 abc	3	0.07 b	6
Yuma	0.03 b	4				
Yumar	0.09 b	2	0.07 bc	2		
Mean	0.46		0.27		0.29	
LSD ( $\alpha = 0.05$ )	1.32		0.41		0.61	

<sup>f</sup>Means with the same letter within a column are not significantly different at  $\alpha = 0.05$ .

distance at which gene flow was detected was 61 m, our furthest sampling point, and the maximum outcrossing at that distance was 0.25% in a Prairie Red sample.

Analysis of variance for gene flow in samples taken within 6 m of the pollen source indicated that cultivar, year, and a cultivar-by-year interaction were all significant

( $P < 0.01$ , data not shown). Cultivars with mean PMGF  $> 1\%$  in at least one year were Jagger, Prairie Red, and TAM 107 (Table 3), all of which are early heading cultivars (RHC 1 or 2, Table 2). Ankor (RHC 5) also had relatively high cross-pollination rates in two of the three years.

## Experimental Plots

A total of 191 samples was analyzed from the Akron Nelder wheel sites in 2004 and 2005 (Table 4). The Pearson correlation coefficient between results of the 2004 and 2005 screening methods on a common set of commercial field samples from 2003 was 0.90 ( $n = 28$ ,  $P < 0.0001$ ), indicating that the two methods gave comparable results. In 2004 an average of 675 plants was screened per sample. Ninety-one of the 98 samples had no detectable gene flow. In 2005 an average of 5700 plants was screened per sample. Seventy-six of the 93 samples had no detectable gene flow. Maximum gene flow observations were greater in 2004 than in 2005, while mean and maximum gene flow observations were higher for Prairie Red than for Halt (Table 4). The farthest PMGF was observed in 2005 in a Prairie Red sample (0.02%) at 31 m from the pollen source (Table 4). A subsample of 13 putative heterozygous IR plants from 2004 and 2005 was verified heterozygous by PCR.

## Empirical Model

### Model Parameters

The Nlmixed procedure was used to fit values of  $c$  in Eq. [5]. The value of 0.53 was determined to provide the best fit based on AIC. We fixed  $c$  at 0.5, that is, a square root

transformation of distance, because it was very close to our fitted value and is supported by the literature (Gustafson et al., 2005). For relative maturity, the response of the parameter  $d$  fit for each value of  $z$  (RHC) was not linear, contrary to our initial expectations. Instead, fitted values of  $d$  appeared to be much higher for low values of  $z$  (RHC 1 and 2) and similar for values of  $z$  from 3 to 8. Testing various exponential transformations of  $z$  using Proc Nlmixed to fit values of  $f$  in Eq. [5] indicated that  $f = -1.4$  provided the best fit based on AIC. According to USDA weather station data, no single prevailing wind direction occurred during morning hours of the heading and anthesis periods from 2003,

**Table 4. Pollen-mediated gene flow (PMGF) results from Nelder wheel experimental plots (EP) and commercial fields (CF) for cultivars Halt, Prairie Red, and TAM 107.**

Cultivar	Site type	Year	Samples			PMGF			
			Year Analyzed	With PMGF	Distance range (m)	Mean <sup>†</sup>	Maximum <sup>†</sup>	Maximum distance <sup>‡</sup>	Mean 1–3 m <sup>§</sup>
Prairie Red	EP	2004	42	6	1–42	0.04	0.74	11 (0.15)	0.11 (9)
		2005	50	13	1–69	0.01	0.09	31 (0.02)	0.04 (10)
	CF	2003	10	7	0.15–37	0.26	1.15	37 (0.01)	1.14 (2)
		2004	26	24	0.3–46	0.24	1.06	46 (0.10)	0.55 (6)
TAM 107 <sup>¶</sup>	EP	2004	36	36	0.3–61	0.33	1.08	61 (0.25)	0.63 (7)
		2005	36	36	0.3–61	0.33	1.08	61 (0.25)	0.63 (7)
	CF	2005	7	7	0.3–61	0.85	3.71	61 (0.10)	2.15 (2)
		2005	7	7	0.3–61	0.85	3.71	61 (0.10)	2.15 (2)
Halt	EP	2004	56	1	1–55	0.003	0.15	15 (0.15)	0.00 (10)
		2005	43	4	1–70	0.004	0.06	7 (0.02)	0.01 (10)
	CF	2004	1	0	14	0.0	—	—	—
		2005	6	1	3–61	0.01	0.03	3 (0.03)	0.03 (1)

<sup>†</sup>Percent PMGF, defined as (number of resistant survivors/total plants screened)  $\times 100$ .

<sup>‡</sup>Maximum distance in m at which PMGF was observed, with percent PMGF from the sample in parentheses.

<sup>§</sup>Percent PMGF in samples collected 1 to 3 m from the pollen source, with the number of samples in parentheses.

<sup>¶</sup>Prairie Red was derived via backcrossing with TAM 107 as a recurrent parent and is expected to be  $> 95\%$  genetically similar to TAM 107.



2004, and 2005. A direction parameter was therefore not included in the GLMM because differences initially associated with direction were determined to be more likely due to random location variation than a consistent trend with wind direction across locations.

### Prediction Estimates

The final equation for the GLMM was:

$$H = a \times 10^{-bx^{0.5}} + dz^{-1.4} + I_i \quad [7]$$

Parameter estimates were produced in Proc Nlmixed and used to construct estimates of the lines for a median location (Eq. [5]), a location at the upper 95th percentile (Eq. [6]), and an upper 95% confidence limit for the upper 95th percentile of locations for each relative heading class  $z$  (Fig. 1A-1F). Model predictions were converted from probabilities to percentages for graphing purposes. Data from commercial fields and cultivar strip trials were included in the GLMM. Parameters and standard errors in parentheses estimated from the GLMM were  $a = -6.75$  (0.02),  $b = 0.41$  (0.01),  $d = 2.54$  (0.11), and  $\sigma = 1.14$  (0.12), all significant with  $P < 0.0001$ . Median location estimates (Eq. [5]) and upper 95% confidence limits on the upper 95th percentile of locations by RHC ( $z$ ) are shown in Fig. 2A and 2B.

### Commercial Field and Experimental Plot Comparison

Higher levels of PMGF were detected for Prairie Red in commercial fields than experimental plots (Table 4). We detected PMGF at farther distances and in a higher proportion of commercial field samples as well. Results for Halt were similar between commercial fields and experimental plots, although the number of Halt samples from commercial fields was small compared to Prairie Red (Table 4). Samples collected from 1 to 3 m from the pollen source also showed much higher PMGF in commercial fields than in experimental plots for Prairie Red (Table 4). Fitting the GLMM to commercial field data resulted in substantially higher predictions of PMGF. Based on commercial field data, the distance required to ensure 95% confidence that 95% of locations would have PMGF less than 0.9% was 41.1 m for RHC 1, 6.4 m for RHC 2, and 0.7 m for RHC 5. If a 0.5% threshold was selected, then the distances were 61.6 m for RHC 1, 15.7 m for RHC 2, and 5.2 m for RHC 5. Model predictions based on experimental plot data were substantially different, as the distance required to ensure 95% confidence that 95% of locations would have PMGF less than 0.1% was 4.4 m for RHC 1 and 0.3 m for RHC 3.

### DISCUSSION

The first widespread commercial release of IR wheat into the agroecosystem provided a unique opportunity to estimate gene flow in wheat at the commercial field scale. The

advantage of using a released cultivar for estimating gene flow was the ability to sample multiple cultivars in different locations across three years, at a scale and under conditions typical of commercial production. Results from our commercial field study are based on larger pollen sources and longer pollen source–recipient borders than typically reported in gene flow studies. Herbicide-resistant progeny were detected in each cultivar evaluated, indicating that all these cultivars are receptive to some level of PMGF.

At three of 56 locations (5%), all samples exceeded the estimated 95% confidence limit for the upper 95th percentile of locations, which is acceptable within the prediction limit. These included one location each of Jagger (Fig. 1B), 'Ankor' (Fig. 1D), and 'Platte' (Fig. 1E). In addition, one sample of Ankor out of 10 total samples at one cultivar strip trial location in 2003 exceeded the prediction limit (Fig. 1D). Weather stations near the three locations that exceeded model predictions recorded near-freezing or freezing temperatures at some point during boot and heading stages (Colorado Agricultural Meteorological Network, available at <http://ccc.atmos.colostate.edu/~coagmet>; accessed 15 June 2006; verified 12 Sept. 2007). This may have been cold enough to cause low levels of male sterility in the recipient fields by killing developing anthers (Shroyer et al., 1995), thus increasing the rate of outcrossing.

The RHC parameter explains significant variation in these data, indicating that cultivars that headed earlier than the pollen source (RHC 1 and 2) had higher levels of gene flow (Fig. 2A and 2B). The 95% confidence limit estimated for RHC 1 is high in part due to the influence of gene flow observations at greater distances (Fig. 1A). Whether the higher PMGF estimates in classes 1 and 2 are due to timing of heading relative to the pollen source or are confounded with other genetic attributes of Prairie Red and TAM 107 (both RHC 1) and Jagger (RHC 2) is difficult to determine because these cultivars are the only ones sampled in their respective classes. Based on the observation that Prairie Red and Jagger had wider floret opening at anthesis (Gaines, 2006), genetic variation in floret opening and stigma receptivity to foreign pollen may account for part of the cultivar variation.

Less variation in PMGF was observed in experimental plots than in commercial fields. Gene flow was not detected as far from the pollen source, lower average PMGF was found, and a higher percentage of samples had no detectable gene flow. This was probably due to the small area of the pollen source. The association of earlier relative heading with higher gene flow was consistent with the commercial field results. Prairie Red is earlier heading than Halt and had higher mean and maximum gene flow; however, this relationship would have been difficult to infer based solely on experimental plot data because only two cultivars were included in the design.

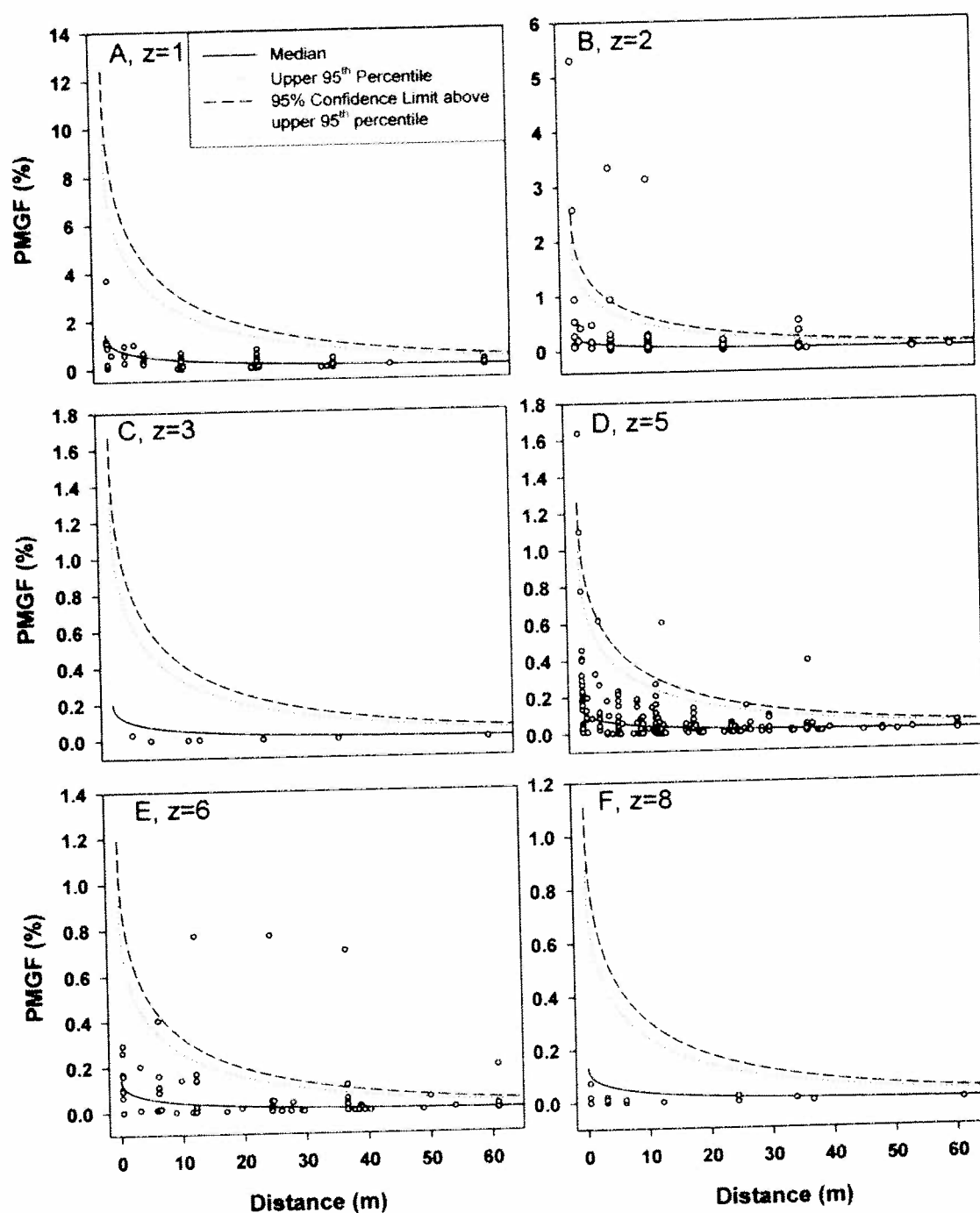


Figure 1. Generalized linear mixed model estimates for pollen-mediated gene flow (PMGF) by relative heading class (z) graphed with data points from each respective class (A-F).

Modeling wheat PMGF with a GLMM allows for two important improvements over the GWM proposed by Gustafson et al. (2005). First, it allows the inclusion of a random location effect which combines such site-specific variables as wind speed and direction, temperature, humidity, and topography. Second, a GLMM allows PMGF to be modeled explicitly as a multiple of a binomial random variable, for which values of zero are allowed.

This approach allows the binomial variance to be modeled separately from the location variance. Advantages of Proc Nlmixed over other considered alternatives include the ability to explicitly estimate  $\epsilon$  (the transformation used for distance), to compare models using AIC to evaluate fit, and to provide an approximate covariance matrix of all model parameters including  $\sigma$  (the standard deviation of the random location effect). Because many locations

were sampled, an estimate of location to location variation enables a prediction of gene flow at a typical location and an upper estimate of gene flow. Allowing for the random effect in predictions of upper bounds is very important, as the upper 95% confidence limit line for the upper 95th percentile line is approximately 10 times the median location estimate (Fig. 2A and 2B).

Median and upper 95% confidence limit gene flow estimates in this study are higher under early heading date classes than the GWM proposed by Gustafson et al. (2005). Part of this difference may be due to the sampling of many more cultivars and locations in this study than the studies used to develop the GWM (Griffin, 1987; Hucl, 1996; Hucl and Matus-Cadiz, 2001; Martin, 1990; Matus-Cadiz et al., 2004). Four of the five data sets used to develop the GWM measured gene flow in spring wheat cultivars, as opposed to winter wheat cultivars in our study. Winter wheat in Colorado is vulnerable to environmental stresses such as freezing temperatures during floral development and drought stress, two factors known to increase outcrossing. The pollen source in this study, Above, tends to head earlier than most varieties. Recipient cultivars that were earlier heading than Above tended to have higher gene flow, while later ones had less. This result is consistent with the report of Matus-Cadiz et al. (2004) that noted overall gene flow was higher if the pollen source headed later than the recipient plot. This trend may indicate that bordering fields are more receptive to gene flow if environmental conditions cause some male sterility in recipient plants. If under these conditions a recipient cultivar is earlier than the neighboring pollen source, pollen will be available for male sterile florets when the source cultivar sheds pollen.

In summary, we have shown that significant gene flow occurs in wheat at the commercial field-scale, with higher levels and greater distances of PMGF than in smaller experimental plots. Factors associated with elevated gene flow included a pollen source that sheds pollen somewhat later than the receiving fields and weather conditions that may have caused some level of male sterility in the recipient plants. Flowering traits specific to certain cultivars may also result in higher levels of PMGF. A GLMM was used to fit a median estimate of PMGF based on relative heading date and an upper estimate to account for unpredictable environmental variables, such as wind speed and direction. Based on variation due to these and other factors, our confidence limits should represent the highest levels of gene flow expected to occur in wheat in the west-central Great

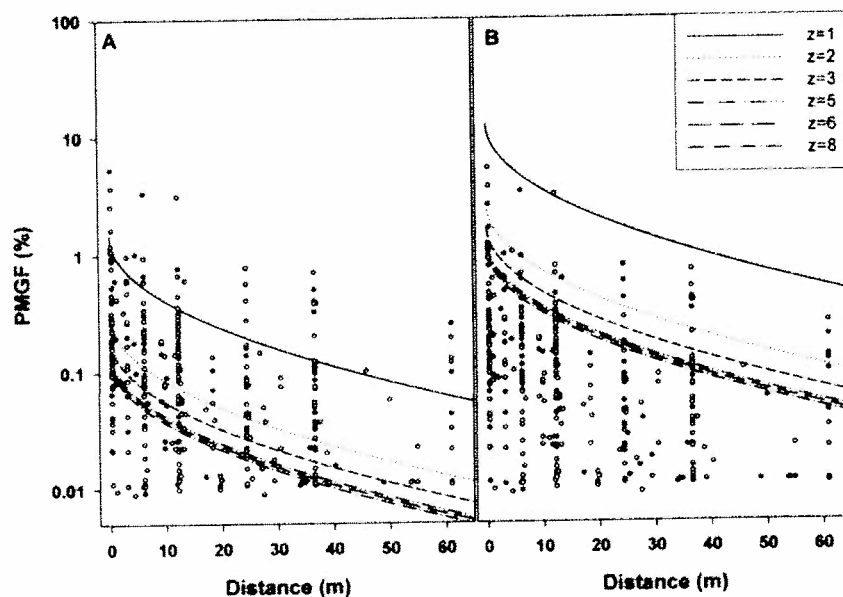


Figure 2. Generalized linear mixed model estimates for pollen-mediated gene flow (PMGF) by relative heading class ( $z$ ) with all data points. A, median location estimates. B, 95% confidence limits above the upper 95th percentile of locations. PMGF is on a logarithmic scale.

Plains. Our results will be useful to biotechnology regulatory agencies, seed production organizations, wheat growers, and others seeking to minimize gene flow in wheat.

### Acknowledgments

The authors would like to thank the wheat producers who provided access to their fields for this study, BASF Corporation for providing the PCR protocols, and Dr. Christopher Preston for development of the seed soaking technique. Funding was provided by the USDA Biotechnology Risk Assessment Program award number 2003-33120-13993. Assistance with statistical analysis was provided by Ambika Smith and Samuel Broderick. We are also grateful to Brad Erker and Linda Munk of the Colorado Seed Growers Association, Jerry Johnson, Alan Helm, Tim Macklin, and Bruce Bosley of Colorado State University Cooperative Extension, James Wittler, USDA-NRCS, and Paul Campbell, USDA-ARS, for their assistance.

### References

- Anderson, J.A., L. Matthiesen, and J. Hegstad. 2004. Resistance to an imidazolinone herbicide is conferred by a gene on chromosome 6DL in the wheat line cv. 9804. *Weed Sci.* 52:83-90.
- Anonymous. 2003. Colorado small grain standards. Colorado Seed Growers Association, Colorado State University, Fort Collins. Available at: [http://seeds.colostate.edu/CSGA/documents/standards/smallgrain\\_standards.pdf](http://seeds.colostate.edu/CSGA/documents/standards/smallgrain_standards.pdf) (verified 12 Sept. 2007).
- Briggs, K.G., O.K. Kiplagat, and A.M. Johnson-Flanagan. 1999. Floret sterility and outcrossing in two spring wheat cultivars. *Can. J. Plant Sci.* 79:321-328.
- Burnham, K.P., and D.R. Anderson. 2002. Model selection and inference: A practical information-theoretic approach. 2nd ed. Springer, New York.
- De Vries, A.P. 1971. Flowering biology of wheat, particularly in view of hybrid seed production- Review. *Euphytica* 20:152-170.



- De Vries, A.P. 1973. Some aspects of cross-pollination in wheat (*Triticum aestivum* L.). 2. Anther extrusion and ear and plant flowering pattern and duration. *Euphytica* 22:445–456.
- De Vries, A.P. 1974. Some aspects of cross-pollination in wheat (*Triticum aestivum* L.). 4. Set on male sterile plants as influenced by distance from pollen source, pollinator: Male-sterile ratio and width of male sterile strip. *Euphytica* 23:601–622.
- Gaines, T.A. 2006. Estimating landscape level gene flow in wheat and jointed goatgrass. M.S. Thesis, Colorado State University, Fort Collins, CO.
- Gaines, T.A., C. Preston, P.F. Byrne, W.B. Henry, and P. Westra. 2007. Adventitious presence of herbicide resistant wheat in certified and farm-saved seed lots. *Crop Sci.* 47:751–756.
- Griffin, W.B. 1987. Outcrossing in New Zealand wheats measured by occurrence of purple grain. *N.Z. J. Agric. Res.* 30:287–290.
- Gustafson, D.L., M.J. Horak, C.B. Rempel, S.G. Metz, D.R. Gigax, and P. Hucl. 2005. An empirical model for pollen-mediated gene flow in wheat. *Crop Sci.* 45:1286–1294.
- Haley, S.D., J.J. Johnson, F.B. Peairs, J.S. Quick, P. Westra, J.A. Stromberger, S.R. Clayshulte, B.L. Clifford, J.B. Rudolph, A. Giura, B. Seabourn, O.K. Chung, Y. Jin, and J. Kolmer. 2006. Registration of 'Bond CL' wheat. *Crop Sci.* 46:993–995.
- Haley, S.D., M.D. Lazar, J.S. Quick, J.J. Johnson, G.L. Peterson, J.A. Stromberger, S.R. Clayshulte, B.L. Clifford, T.A. Pester, S.J. Nissen, P.H. Westra, F.B. Peairs, and J.B. Rudolph. 2003. Above winter wheat. *Can. J. Plant Sci.* 83:107–108.
- Hanson, B.D., C.A. Mallory-Smith, B. Shafii, D.C. Thill, and R.S. Zemstra. 2005. Pollen-mediated gene flow from blue aleurone wheat to other wheat cultivars. *Crop Sci.* 45:1610–1617.
- Hedrick, P.W. 2005. Genetics of populations. 3rd ed. Jones and Bartlett, Sudbury, MA.
- Hucl, P. 1996. Out-crossing rates for 10 Canadian spring wheat cultivars. *Can. J. Plant Sci.* 76:423–427.
- Hucl, P., and M. Matus-Cadiz. 2001. Isolation distances for minimizing out-crossing in spring wheat. *Crop Sci.* 41:1348–1351.
- Lawrie, R.G., M.A. Matus-Cadiz, and P. Hucl. 2006. Estimating out-crossing rates in spring wheat cultivars using the contact method. *Crop Sci.* 46:247–249.
- Martin, T.J. 1990. Outcrossing in 12 hard red winter wheat cultivars. *Crop Sci.* 30:59–62.
- Matus-Cadiz, M.A., P. Hucl, and B. Dupuis. 2007. Pollen-mediated gene flow in wheat at a commercial scale. *Crop Sci.* 47:573–581.
- Matus-Cadiz, M.A., P. Hucl, M.J. Horak, and L.K. Blomquist. 2004. Gene flow in wheat at the field scale. *Crop Sci.* 44:718–727.
- Messean, A., F. Angevin, M. Gomez-Barbaro, K. Menrad, and E. Rodriguez-Cerezo. 2006. New case studies on the coexistence of GM and non-GM crops in European agriculture. [Online]. EUR 22102 EN. Institute for Prospective Technological Studies, European Commission. Available at: <http://ftp.jrc.es/eur22102en.pdf> (verified 12 Sept. 2007).
- Nelder, J.A. 1962. New kinds of systematic designs for spacing experiments. *Biometrics* 18:283–307.
- Newhouse, K.E., W.A. Smith, M.A. Starrett, T.J. Schaefer, and B.K. Singh. 1992. Tolerance to imidazolinone herbicides in wheat. *Plant Physiol.* 100:882–886.
- Poehlman, J.M., and D.A. Sleper. 1995. Breeding field crops. 4th ed. Iowa State Univ. Press, Ames, IA.
- Porter, J.R., and M. Gawith. 1999. Temperatures and the growth and development of wheat: A review. *Eur. J. Agron.* 10:23–36.
- Pozniak, C.J., and P.J. Hucl. 2004. Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Sci.* 44:23–30.
- Rieger, M.A., M. Lamond, C. Preston, S.B. Powles, and R.T. Roush. 2002. Pollen-mediated movement of herbicide resistance between commercial canola fields. *Science* 296:2386–2388.
- Saari, L.L., J.C. Cotterman, and D.C. Thill. 1994. Resistance to acetolactate inhibiting herbicides. p. 83–139. *In* S. B. Powles and J. A. M. Holtum (ed.) *Herbicide resistance in plants: Biology and biochemistry*. Lewis Publishers, Boca Raton, FL.
- SAS. 2004. SAS help and documentation, Vers. 9.1.3. SAS Institute Inc., Cary, NC.
- Schlaich, T., B.M. Urbaniak, N. Malgras, E. Ehler, C. Birrer, L. Meier, and C. Sautter. 2006. Increased field resistance to *Tilletia caries* provided by a specific antifungal virus gene in genetically engineered wheat. *Plant Biotech. J.* 4:63–75.
- Shroyer, J.P., M.E. Mikesell, and G.M. Paulsen. 1995. Spring freeze injury to Kansas wheat. Publication C-646. Kansas State University, Manhattan, KS.
- Tan, S.Y., R.R. Evans, M.L. Dahmer, B.K. Singh, and D.L. Shaner. 2005. Imidazolinone-tolerant crops: History, current status and future. *Pest Manage. Sci.* 61:246–257.
- Waines, J.G., and S.G. Hegde. 2003. Intraspecific gene flow in bread wheat as affected by reproductive biology and pollination ecology of wheat flowers. *Crop Sci.* 43:451–463.
- Zhou, H., J.D. Berg, S.E. Blank, C.A. Chay, G. Chen, S.R. Eskelsen, J.E. Fry, S. Hoi, T. Hu, P.J. Isakson, M.B. Lawton, S.G. Metz, C.B. Rempel, D.K. Ryerson, A.P. Sansone, A.L. Shook, R.J. Starke, J.M. Tichota, and S.A. Valenti. 2003. Field efficacy assessment of transgenic Roundup Ready wheat. *Crop Sci.* 43:1072–1075.