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Low Level of Pollen-Mediated Gene Flow from Cultivated to Wild Grapevine: Consequences for the Evolution of the Endangered Subspecies *Vitis vinifera* L. subsp. *silvestris*

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Abstract

A parentage and a paternity-based approach were tested for estimation of pollen-mediated gene flow in wild grapevine (*Vitis vinifera* L. subsp. *silvestris*), a wind-pollinated species occurring in Mediterranean Europe and southwestern Asia. For this purpose, 305 seedlings collected in 2 years at 2 locations in France from 4 wild female individuals and 417 wild individuals prospected from France and Italy were analyzed using 20 highly polymorphic microsatellite loci. Their profiles were compared with a database consisting of 3203 accessions from the Institut National de la Recherche Agronomique Vassal collection including cultivars, rootstocks, interspecific hybrids, and other wild individuals. Paternity was assigned for 202 (66.2%) of the 305 seedlings, confirming the feasibility of the method. Most of the fertilizing pollen could be assigned to wild males growing nearby. Estimates of pollen immigration from the cultivated compartment (i.e., the totality of cultivars) ranged from 4.2% to 26% from nearby vineyards and from hidden pollinators such as cultivars and rootstocks that had escaped from farms. In an open landscape, the pollen flow was correlated to the distance between individuals, the main pollinator being the closest wild male (accounting for 51.4–86.2% of the pollen flow). In a closed landscape, more complex pollination occurred. Analysis of the parentage of the 417 wild individuals also revealed relationships between nearby wild individuals, but in the case of 12 individuals (3%), analysis revealed pollen immigration from vineyards, confirming the fitness of the hybrid seedlings. These pollen fluxes may have a significant effect on the evolution of wild populations: on the one hand, the low level of pollen-mediated gene flow from cultivated to wild grapevine could contribute to a risk of extinction of the wild compartment (i.e., the totality of the wild individuals). On the other hand, pollen dispersal within the wild populations may induce inbreeding depression of wild grapevines.

Key words: *paternity analysis, pollen dispersal, rootstock, seedling, transgenic plant, true-to-type*

Gene flow between crops and their wild relatives is an important process that has major implications both for in situ conservation of genetic diversity and for plant breeding. Most domesticated plants are able to spontaneously hybridize with wild relatives present in their distribution area (Ellstrand 2003). Because diversity is often more important for wild populations than for cultivated, overwhelming gene flow from crops may deplete genetic diversity in wild populations (Ellstrand 2003), in some cases leading to the genetic extinction of wild populations (Levin et al. 1996; Wolf et al. 2001; Ellstrand 2003). Conversely, extensive flow from wild populations to the cultivated

compartment (i.e., the totality of cultivars) may increase the genetic diversity of the latter, leading to a reduction in divergence between compartments. Thus, for strategies for the conservation of wild plants, it is important to acquire knowledge about the genetic structure of the populations and the extent of gene flow (Ellstrand 2003).

Gene flow of important crops in sympatry with wild populations has recently received much attention because of the potential risk of transfer of transgenes (e.g., Dale 1994; Darmency 1994; Gliddon 1994; Giddings 2000; Saeglitz et al. 2000; Gueritaine et al. 2002; Lee and Natesan 2006) or of exotic genes (Davison 2005; Warren and James 2006) into natural populations. The risks are particularly evident for endangered species, as their small population size makes them vulnerable to pollen invasion (Ellstrand 2003). On the other hand, moderate gene flow between wild populations may help maintain genetic variation and reduce the risk of inbreeding depression (Storfer 1999; Keller and Waller 2002; Bailey and McCauley 2006).

Natural hybridization and introgression between wild and domesticated populations has been shown to be a widespread phenomenon in most annual species (Arnold et al. 1998; Darmency et al. 1998; Bartsch et al. 1999; Desplanque et al. 1999; Murray et al. 2002; Papa and Gepts 2003; Song et al. 2003; Andersen et al. 2005), whereas studies on contemporary gene flow in perennial species are still scarce or based on the genetic structure of populations (Luby and McNicol 1995; Coart et al. 2003; Breton et al. 2006; Coart et al. 2006).

Nuclear microsatellite markers [nuclear simple sequence repeat (nSSR)] have reached a level of parental resolution that enables analysis of genetic paternity (e.g., Streiff et al. 1999) and have been successfully used in numerous species (Bartsch et al. 1999; Streiff et al. 1999; González-Martínez et al. 2002; Viard et al. 2002; Burczyk et al. 2004; Alibert et al. 2005; Dunphy and Hamrick 2005; Yamamoto et al. 2005). Direct estimation of gene flow was made possible by comparing genotypes of potential parents with genotypes of seeds collected from maternal individuals or of naturally established seedlings or juveniles (Kameyama et al. 2001; Robledo-Arnuncio and Gil 2004). However, the exhaustive sampling of candidate parents required for paternity assignment often limits the spatial extent of analysis, leading to a very substantial number of offspring sired by unknown parents from outside the study area.

Because of its clonal nature due to vegetative propagation, grapevine (*Vitis vinifera* L.) is a good model for testing paternity-based approaches in perennial cultivated plants. *Vitis vinifera* is principally wind pollinated (Huglin 1998), and insects do not affect pollenization. Flower types are quite distinct between wild and cultivated vines: cultivars are mostly hermaphroditic while wild are dioecious; the female flowers, however, show anthers, but usually with sterile pollen (Caporali et al. 2003). Introgression from grape hermaphrodite cultivars (subspecies *sativa*) into wild dioecious populations (subspecies *silvestris*) has been suspected (Levadoux 1956), but quantitative data are lacking so far. Wild populations subsist in Europe, but are extremely rare (Arnold et al. 1998), and existing only as very small populations of a few individuals (This et al. 2000; Lacombe et al. 2003), often in close proximity to vineyards. Owing to the globalization of wine companies and markets, the cultivated grapevine has undergone a drastic reduction in diversity, resulting in the emergence of a small number of worldwide grown cultivars such as "Chardonnay," "Cabernet Sauvignon," and "Syrah," as well as the disappearance of local cultivars (Bouquet and Boursiquot 1999). Microsatellite markers have been developed for the characterization of grapevine (Thomas and Scott 1993; Bowers et al. 1996, 1999; Sefc et al. 1999; Pellerone et al. 2001; This et al. 2004) and can easily differentiate *Vitis* species as well as *V. vinifera* cultivars (This et al. 2006). They have also been useful for the characterization of wild populations (This et al. 2000; Aradhya et al. 2003; Grassi, Imazio, et al. 2003; Grassi, Labra, et al. 2003; Snoussi et al. 2004; Di Vecchi-Staraz et al. 2006).

In the present work, using 20 nSSR well distributed across the 19 grape chromosomes, we tested a direct paternity-based approach (Robledo-Arnuncio and Gil 2004) for the characterization of pollen-mediated gene flow between wild and cultivated populations of grapevine in the 2 locations in France displaying populations more than 15 individuals (Lacombe et al. 2003) suitable for such analysis. Gene flow was also evaluated using an indirect parentage analysis of 417 wild individuals collected in France. The consequences for grapevine conservation and evolution are discussed as well as the potential interest of perennial cultivated species.

Materials and Methods

Site Description

"Pic Saint-Loup" Site

The population (first described by This et al. 2000) is located 30 km northwest of the city of Montpellier (Herault, France) on the hillside of Pic Saint-Loup (Saint-Loup Peak). Twenty individuals (PSL) located at an altitude of between 250 and 600 m a.s.l. were observed (see Supplementary data, Figure S1) and 18 were genotyped. The site has a Mediterranean climate and is a typical "Garrigue" landscape (with scrub). Wind and lifting currents are frequent. The nearest commercial vineyards (part of the wine-producing area "Coteaux du Languedoc—Pic Saint-Loup"; 1128 ha) are located at a lower altitude (350 m).

"Grésigne" Site

The population is located inside the Grésigne forest, 70 km north of the city of Toulouse in the Tarn region (France). The area has a continental climate characterized by higher rainfall than the Pic Saint-Loup site. It consists of 11 distinct individuals (Gre), located at an altitude of between 230 and 300 m a.s.l., spread over an area of 3500 m along the D87 road (see Supplementary data, Figure S2). Two more individuals were identified here, but they turned out to be identical ("Gre6" = "Gre7" and "Gre14" = "Gre15").

The vineyards (770 ha) are located at a distance of about 10 km from the site.

Sample Collection

Seeds were collected on 4 wild female grapevines from both parental populations ("PSL12" and "PSL13" from Pic Saint-Loup, and "Gre8" and "Gre10") in 2000 and on PSL13 in 2001. Empty seeds were first excluded (by floating in water). Well-formed seeds were cold treated for a period of 2 months and planted on vermiculite for germination on a heated bench (28 °C). After transplantation in the field, recovered individuals were planted in the field ungrafted. Young leaves were collected on the plants and freeze-dried for DNA extraction.

In addition to the 18 "PSL" and 11 "Gre" plants, 388 wild individuals collected in France (Lacombe et al. 2003) and in Italy (Di Vecchi-Staraz 2007) were analyzed: young leaves were collected in the wild and treated as previously reported (This et al. 2000).

DNA Extraction and Microsatellite Markers

The 722 individuals (417 wild grapevines and 305 seedlings) were analyzed using a set of 20 microsatellite loci (nSSR) well scattered on the genome: VVMD5, VVMD7, VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, and VVMD32 (Bowers et al. 1996, 1999); VVIn16, VVIv67, VVIv37, VVIq52, VVIp60, VVIh54, VVIb01, VVIn73, and VVIp31 (Merdinoglu et al. 2005); VVS2 (Thomas and Scott 1993); and VMC1b11 and VMC4f3 (Di Gaspero et al. 2000; Adam-Blondon et al. 2004), and already used for the analysis of grape diversity (Lacombe et al. 2003; Di Vecchi-Staraz et al. 2007; Laucou V., in preparation). Total DNA extraction (DNeasy Tissue Kit, Qiagen Hilden, Germany), polymerase chain reaction amplification, sequencing, and detection of polymorphism were performed according to Adam-Blondon et al. (2004), slightly modified by Di Vecchi-Staraz et al. (2007).

Genetic Diversity within Populations

Standard measurements of genetic variation, including the average number of alleles per locus and per overall loci (mean number of alleles for each locus), allele frequencies, Nei unbiased gene diversity (GD; Nei 1987), observed heterozygosity (Ho), and standard deviations were calculated for the 2 wild parental populations, the seedling populations, and the whole set of wild and cultivar populations. For both wild parental populations, Wright's F_{is} were estimated following Weir and Cockerham (1984) and its significance ($F_{is} \neq 0$) tested after 1000 permutations. All calculations and tests were performed using GENEPOP software v. 3.4 (Raymond and Rousset 1995). We finally calculated the pairwise relationship coefficient (PRC; Goodnight and Queller 1999) and computed regressions of pairwise statistics on spatial distance using SPAGEDI software v. 1.2 (Hardy and Vekemans 2002).

Parentage and Paternity Analyses

Paternity tests and parentage assignment were performed using a database of 3203 nSSR multilocus genotypes of 2646 cultivars, 140 rootstocks, and interspecific hybrids (Laucou V., in preparation) and 417 wild grapevines (18 from PSL, 11 from Gre, and 388 from other origins), using FAMOZ software (Gerber et al. 2003). This database was obtained by analyzing genotypes from the Institut National de la Recherche Agronomique Vassal germplasm collection (http://www.montpellier.inra.fr/vassal). The cumulated exclusion probabilities for single parents and for parent pairs were calculated as well as parentage or paternity assignment threshold values before any further analysis. Parentage assignments were performed as described by Di Vecchi-Staraz et al. (2007). Paternities were assigned by calculating a logarithm of the likelihood ratio (log of odds ratio, LOD score). The latter is the likelihood of an individual being the parent of a given offspring divided by the likelihood of the individuals being unrelated (Gerber et al. 2000; Jones and Ardren 2003). In order to take into account possible typing errors (Ewen et al. 2000) and null alleles (Dakin and Avise 2004), we allowed a maximum number of 3 loci mismatches (Dakin and Avise 2004) in parentage assignment.

Results

Seedling Recovery

A total of 2061 seeds were collected on the 4 individuals ("PSL12", "PSL13", "Gre8", and "Gre10") and stored. Of these, 461 well-formed seeds (251 from Pic Saint-Loup and 210 from Grésigne) were planted, and 461 seedlings were obtained. After transplantation in the field, 305 individuals (120 from Pic Saint-Loup and 159 from Grésigne from the 2000 collection and 26 from Pic Saint-Loup from the 2001 collection) were recovered and analyzed.

Microsatellite Polymorphism and Genetic Diversity

The genetic diversity revealed by the 20 SSR markers is summarized in Table 1. The genetic diversity and heterozygosity of the cultivars (2646 individuals) were very high with a total number of 402 alleles, GD = 0.730 and Ho = 0.755 (Table 1). For the wild populations (417 individuals), it was lower because only 238 alleles were identified and GD = 0.684. The genetic diversity was even lower in the seedling populations (GD = 0.597) and was different according to the populations (GD ranging from 0.387 to 0.577). Heterozygosity in the wild population was also lower (0.596) than that for the cultivars (Table 1).

Exclusion Probabilities and LOD Score Threshold

The paternity exclusion probability for a single locus ranged from 26.7% (VVIn73) to 78.5% (VVIp31; Table 2). A probability of cumulated exclusion of 100% was reached using only 8 markers for paternity and 4 markers for the

Table 1. Genetic diversity indices of the grapevine populations analyzed in this study (wild populations, seedling populations, and cultivars) based on 20 nSSR markers

Population (geographical origin)	Sample size	GD[a] (σ)	Ho (σ)	MNA (σ)	NTA
Wild (France)	241	0.638 (0.02)	0.596 (0.01)	9.4 (3.7)	241
Cultivars (France)	588	0.730 (0.03)	0.750 (0.01)	13.2 (5.2)	264
Wild (all)	417	0.684 (0.02)	0.619 (0.01)	11.9 (5.0)	238
Cultivars (whole grape collection)	2646	0.769 (0.03)	0.755 (0.01)	20.1 (8.1)	402
Total wild populations and cultivars	3063	0.779 (0.03)	0.738 (0.01)	—	—
Wild population (Grésigne)	11	0.613 (0.04)	0.627 (0.03)	4.5 (1.4)	—
Wild population (Pic Saint-Loup)	18	0.598 (0.04)	0.593 (0.03)	4.6 (1.1)	—
Total parental wild populations	29	0.621 (0.03)	0.606 (0.02)	5.9 (1.6)	—
Seedlings from Gre8 (Grésigne)	65	0.387 (0.05)	0.476 (0.02)	2.7 (0.9)	—
Seedlings from Gre10 (Grésigne)	94	0.577 (0.04)	0.665 (0.01)	5.3 (1.2)	—
Seedlings from PSL12 (Pic Saint-Loup)	48	0.481 (0.06)	0.576 (0.02)	3.9 (1.5)	—
Seedlings from PSL13 (Pic Saint-Loup)	98	0.508 (0.03)	0.595 (0.01)	5.2 (1.4)	—
Total seedling populations	305	0.597 (0.03)	0.588 (0.01)	7.1 (2.2)	—

σ, standard deviation; MNA, mean number of alleles for each locus; NTA, total number of alleles.

[a] Nei (1987).

parent pair. The simulation for parentage analysis performed by FAMOZ identified a LOD score threshold of 4.8 to assess a potential single parent and 7.1 to assess a parent pair with the 20 nSSRs. Consequently, only pairs with LOD scores >7.1 were considered as valid.

Paternity Assignment of the Seedlings

In total, we assigned full parentage to 202 (66.2%) out of the 305 seedlings, but the success of the identification differed in the 2 populations.

Pic Saint-Loup Populations

At the Pic Saint-Loup site, the fathers of 120 out of 146 seedlings were unambiguously identified: 38 (79.2%) seedlings from the mother "PSL12" and 82 (83.7%, 2000 and 2001 all together) seedlings from the mother "PSL13" (Table 3). In both cases, the main pollinator was "PSL11" (father of 29 and 54 seedlings), and 7 other wild individuals ("PSL9", "PSL13", "PSL14", "PSL18", "PSL21", "PSL25" and "PSL26") also contributed to pollination, but to a lesser extent (1–8 seedlings). A total of 103 (85%) descendants from the 2 mothers were assigned to a wild male. Interestingly, no descendant resulted from the second closest male "PSL10" (<100 m). In only one case, because of missing data (9 loci), 2 putative parents were identified (ex aequo). Two "PSL12" seedlings and 15 "PSL13" seedlings were also assigned to fathers issued from cultivars (Table 3). We thus observed a mean rate of 10.8% (4.2% and 17.4% in "PSL12" and "PSL13", respectively) of

Table 2. Estimated exclusion probabilities (single parent, paternity, and parent pair) using 20 nSSRs

	Single locus exclusion probabilities		Cumulated exclusion probabilities	
Locus	Paternity	Parent pair	Paternity	Parent pair
VVIp31	0.7851	0.9284	0.7851	0.9284
VVMD28	0.7830	0.9263	0.9534	0.9947
VVIv67	0.7502	0.9071	0.9884	0.9995
VMC4f3	0.7478	0.9095	0.9971	1
VVMD5	0.7343	0.8931	0.9992	1
VVS2	0.7082	0.8777	0.9998	1
VMC1b11	0.6964	0.8680	0.9999	1
VVMD32	0.6959	0.8669	1	1
VVMD27	0.6943	0.8634	1	1
VVMD7	0.6937	0.8681	1	1
VVIv37	0.6892	0.8675	1	1
VVIp60	0.6183	0.8091	1	1
VVMD25	0.6128	0.7937	1	1
VVIh54	0.5945	0.7891	1	1
VVMD24	0.5175	0.7151	1	1
VVMD21	0.5058	0.7105	1	1
VVIb01	0.4774	0.6613	1	1
VVIn16	0.4829	0.6743	1	1
VVIq52	0.4015	0.5642	1	1
VVIn73	0.2671	0.4463	1	1

Table 3. Paternity assignment of the 146 seedlings issued from the wild grapevines "PSL12" and "PSL13" at the Pic Saint-Loup study site

Mother	Location	Father (sex)	Seedlings (2000)	Seedlings (2001)	Total (2000/2001)
PSL12	Within the stand	PSL9 (ND)	2	—	2
		PSL11 (male)	29	—	29
		PSL13 (female)	2	—	2
		PSL21 (ND)	1	—	1
		PSL25 (ND)	1	—	1
		PSL26 (ND)	1	—	1
	Outside the stand	cv. Cabernet sauvignon (hermaphrodite)	1	—	1
		cv. Cinsaut (hermaphrodite)	1	—	1
	—	ND	10	—	10
		Total seedlings from PSL12	48	—	48
PSL13	Within the stand	PSL11 (male)	37	17	54
		PSL14 (male)	1	0	1
		PSL18 (ND)	1	0	1
		PSL21 (ND)	1	0	1
		PSL25 (ND)	1	0	1
		PSL26 (ND)	7	1	8
	Outside the stand	cv. Cinsaut (hermaphrodite)	4	6	10
		cv. Carignan (hermaphrodite)	0	1	1
		cv. Marsanne (hermaphrodite)	2	0	2
		cv. Syrah (hermaphrodite)	2	0	2
	—	Ex aequo	1	0	1
	—	ND	15	1	16
		Total seedlings from PSL13	72	26	98

cv., Grapevine cultivars; ND, not determined.

pollination from outside the wild compartment (immigration pollen flow). We confirmed the presence of the cv. "Cinsaut" with some vines of cv. "Cabernet Sauvignon" in the nearest vineyards (in the plain in front of the hill at a distance of 350 m). The "Marsanne" and "Syrah" cvs were located within a distance of 500 m to 3 km. Knowing these distances and the distances between PSL individuals (Supplementary data, Table S1), we analyzed the relationship between reproductive success and geographical distance. Figure 1 clearly shows a logarithmic relationship for this population ($r^2 = 0.72$).

Finally, paternity analysis revealed no differences in pollen donors of seedlings from "PSL13" between 2000 and 2001 (Table 3). "PSL11" was the main donor in both years and intra- and intercompartment pollination was also observed in both years. However, the immigration pollen flow was higher in 2001 (26.9%) than in 2000 (11.1%).

Grésigne Populations

We analyzed a total of 159 seedlings from the 2 mothers "Gre8" and "Gre10". Paternity analysis identified the fathers of 60 seedlings (92.3%) from "Gre8" and of 22 (24.4%) from "Gre10". In all, the fathers of 82 seedlings (51.6%) were identified at this site. Concerning "Gre8", the male "Gre9" was the father of 56 descendants (86.2%; Table 4), and 1 seedling was from "Gre6." We also identified 3 cases

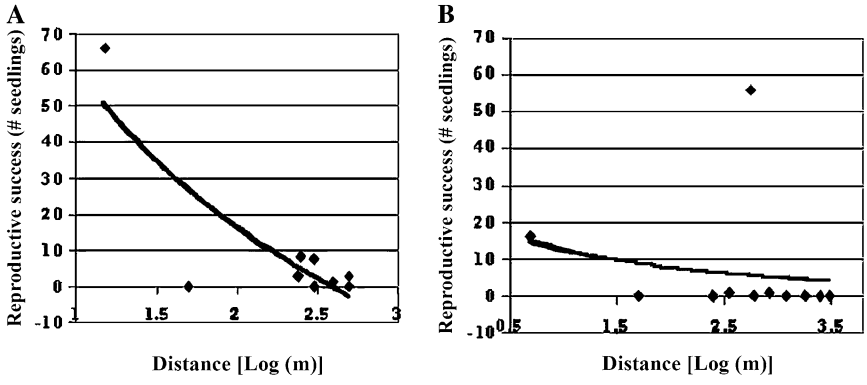

Figure 1. Estimated relationships between reproductive success of male parents and their geographical distance from females (log of distance in meters) within the Pic Saint-Loup (**A**) and Grésigne (**B**) wild grapevine populations in France.

Table 4. Paternity assignment of the 159 seedlings issued from the wild grapevines "Gre8" and "Gre9" at the Grésigne study site

Mother	Location	Father (sex)	Seedlings
Gre8	Within the stand	Self-fertilization	3
		Gre6 (female/hermaphrodite)	1
		Gre9 (male)	56
	—	Ex aequo	1
	—	ND	4
		Total seedlings from Gre8	65
	Inside the stand	Gre12 (female/hermaphrodite)	16
		Gre1 (female)	1
		Gre4 (ND)	1
Gre10	ND	cv. Gros Cabernet (hermaphrodite)	1
		cv. Petit verdot (hermaphrodite)	2
		r. Aramon rupestris Ganzin n° 9 (male)	1
	—	ND	72
		Total seedlings from Gre10	94

ND, Not determined; cv., grapevine cultivars; r., rootstocks (*Vitis* ssp.).

of self-fertilization. Concerning "Gre10", "Gre12" contributed to the pollination of 16 seedlings and "Gre1" and "Gre4" both contributed to the pollination of 1 seedling. We were unable to assign paternity to 72 seedlings (77%; Table 4) from "Gre10". Nevertheless, among these 72 half sibs, the maximum number of alleles per locus was 7 (VVS2), so they were issued from a minimum of 3 fathers. Furthermore, these potential fathers often shared alleles with the mother, indicating a possible wild origin of this gene flow. Interestingly, we did not identify pollination from the nearest male "Gre11" (<10 m). In all, 78 seedlings at this site (93% of the assigned seedlings) were assigned to wild parents, mainly "Gre12" (17%). Only 3 seedlings (3.2%) were assigned to cultivars (cv. "Petit verdot" and cv. "Gros Cabernet"), and 1 was issued from pollen of the rootstock "Aramon rupestris Ganzin n° 9." These cultivars and this rootstock were not recorded in the official list of cultivars of this wine-producing area. Because the geographical position of these 3 potential fathers was not identified, we only considered the wild fathers for the analysis of reproductive success (Supplementary data, Table S2). For this population, there was no relationship between reproductive success and geographical distance ($r^2 = 0.25$; Figure 1). However, these results should be interpreted with caution because only 52% of the seedlings were characterized.

Parentage in the Wild Parental Populations

In order to check for evidence of pollen flux in natural conditions, we searched for parentage relationships between wild individuals and with any of the cultivars in the database. In a very few cases, we found a complete relationship (i.e., both parents compatible with the offspring), but most of the time, only incomplete parentage was identified (revealed as 2 individuals sharing at least one allele at each locus). In total, direct relationships were revealed for 41 individuals including individuals from Pic Saint-Loup and Grésigne populations with high LOD scores (Supplementary data, Table S1). The location of the individuals was often strongly in favor of these relationships. Twelve individuals collected in the wild (including "Gre12") were also revealed as possible offspring of cultivars (from Pyrenees and Corsica populations), a small percentage of the total (~3%) (Supplementary data, Table S2). In addition several indirect relationships were also identified (Supplementary data, Tables S3 and S4).

Discussion

In the present work, we analyzed wild individuals and seedlings obtained from open pollination on wild female plants with 20 well-distributed nSSR markers and compared their profiles with a large database consisting of 3203 unique multilocus SSR genotypes. Paternity of 202 seedlings (66.3%) was identified, demonstrating the feasibility of paternity-based methods for estimation of gene flow in perennial plants. The extent of cross-pollination from cultivated to wild grapevines must be evaluated in more than 2 different environmental conditions (distance from vineyards, size of wild population, and landscape) before a detailed assessment of its impact on the wild subspecies *Vitis vinifera silvestris* is possible. Nevertheless, the parentage analysis of 417 wild individuals collected in very different situations confirmed both the intra- and intercompartment flux.

Microsatellite Markers, Database, and Paternity Analysis

Microsatellite markers were confirmed as very powerful markers for paternity analysis as already demonstrated for olive tree (Mookerjee et al. 2005), particularly the set of 20 markers defined for this study that allowed a high value of cumulated exclusion probabilities. Paternity analysis assigned a lower number of ex aequo than expected (Mookerjee et al. 2005), but the number of nSSRs was much greater than usually needed (up to 9, Slavov et al. 2005). The method used for parentage analysis (also accounting for mistyping) was extremely accurate in the discovery of paternity. Applied to the analysis of gene flow,

this paternity-based method enabled direct estimation of pollen-mediated gene flow from cultivated to wild grapevine. Parentage analysis enabled the identification of both contemporary and past gene flow, analyzing the seedlings and the wild individuals, respectively, but with some underestimation due to the nonidentification of some pollen donors. We confirmed that covering a large distance for sampling is not a guarantee of the identification of pollinators (Ellstrand et al. 1999) as in Grésigne. However, our results demonstrated the power of a large database for paternity analysis and the feasibility of such analysis for grapevine, when the cultivated compartment is so overwhelmingly abundant compared with the few wild individuals that still exist. The size of clonal cultivated populations of this species explained the success in identifying pollen donors from vineyards.

The unsuccessful characterization of the pollen donors for about one-third of the seedlings (102) was most probably due to the absence from the database of some old and extinct cultivars or of wild parents either extinct or not yet discovered. Parentage or paternity analyses have often shown high proportions of offspring with no potential father within the population studied (Streiff et al. 1999; Lian et al. 2001). Even if long-distance pollen flow is a common phenomenon in wind-pollinated species (Sato et al. 2005), hidden pollinators living in the natural sites studied are much more probable, as demonstrated in the present work.

Gene Flow within the Wild Compartment

We analyzed gene flow in grapevine using 2 different methods and at 2 sites with very different environments. As expected for an outcrossing dioecious subspecies (*V. v. silvestris*), we identified gene flow within the wild populations. Despite the complexity of pedigrees in wild populations, the parentage analysis of the wild individuals also revealed possible kin relationships. These relationships involved individuals usually located close to one another—in the case of Pic Saint-Loup, the farthest were "PSL7" and "PSL22" (at a distance of 3000 m)—and in an environment with no serious barriers, such as on the same side of the peak. Even if wild grapevines are dioecious (Zohary 1995), we also identified 3 seedlings issued from self-fertilization of a supposedly female individual. In this case, pollen sterility may not be as complete as previously recorded (Caporali et al. 2003). Given the importance of these findings, additional analysis of flower type and pollen viability should be conducted.

The analysis of these 2 different sites revealed some differences. In an open Mediterranean landscape with frequent wind, the large majority of seedlings were issued from pollination by the nearest male located at a distance of few meters, and reproductive success decreased with distance. Conversely, in the natural closed landscape of forests, there seems to be less correlation between reproductive success and distance. The pollen flow was probably perturbed by the surrounding vegetation. Similarly, no pollen flow was revealed between individuals on opposite sides of the peak in the Pic Saint-Loup population. The 2 wild populations were located too far apart for us to discover any gene flow between them. Finally, the analysis of 2 consecutive years revealed a constancy of flux over time from the closest male and the involvement of other wild males in pollination. In conclusion, the pollen flow was correlated to the distance between individuals, as previously observed for other wood species (Garcia et al. 2005). The distances recorded here are lower than those reported for wind-pollinated species such as pine and oak (more than 1–2 km; Streiff et al. 1999; Schuster and Mitton 2000) and than the maximum distance covered by grapevine pollen (Turner and Brown 2004). The structure of the landscape had an effect on pollen flow rates. A landscape genetic approach, including landscape composition, configuration, and matrix quality could provide information about the interaction between landscape features and microevolutionary processes, such as gene flow (Manel et al. 2003).

Gene Flow between Wild and Cultivated Compartments

Both sites also differed in their proximity to commercial vineyards, with Pic Saint-Loup located close to commercial vineyards, while Grésigne is located farther away. In both cases and in both years, we observed cross-pollination from cultivars to wild individuals, but at much higher frequencies at the "Pic Saint-Loup" site, even if no wild individuals from this site were revealed as intercompartment crosses. Vestiges of past introgressions were also identified. Even if rare (~3%), they nevertheless demonstrate the fitness of the hybrid seedlings. In general, the male parent identified is consistent with its probable presence in the corresponding regions, for example, cv. "Cinsaut" near "Pic Saint-Loup" population, cv. "Calitor" near Grésigne population. In the case of cv. "Gros Cabernet", which has not been cultivated for a long time (more than 50 years), and cv. "Petit Verdot", which has never been much cultivated in the region of Grésigne forest, their presence is more surprising. Nevertheless, Odart (1843) described the widespread cultivation of cv. "Gros Cabernet" during the 19th century in the region of our site. Similarly, the rootstock "Aramon" rupestris Ganzin n° 9 of interspecific origin (Galet 1988) was planted in France after 1877, and its presence in the area of Grésigne is possible. As normal viticulture does not allow flowering of the rootstock, the pollen is likely to have come from an escaped individual living in wild conditions.

In conclusion, for the first time in this perennial species, we identified a low level of pollen-mediated gene flow (from 4.2% up to 26%) from the cultivated to the wild compartment, but with different intensity in different years. Gene flow was also high in the open areas close to vineyards (10 cases) and lower in the forest, where 4 cases of cross-pollination were identified.

An Endangered Subspecies?

Pollen-mediated gene flow immigration in the subspecies *V. v. silvestris* was revealed in this work, though at a relatively low rate compared with that reported for wind-pollinated

woody species (Burczyk et al. 1996, 2004; Dow and Ashley 1996; Harju and Nikkanen 1996; Streiff et al. 1999; Pakkanen et al. 2000; Buiteveld et al. 2001). Cross-pollination between wild and cultivated populations accounted for a fair percentage of this gene flow. The analysis of seedlings grown ex situ probably overestimated this rate. Further investigation is needed to understand the gene flow realized by naturally regenerated seedlings because factors such as seed dispersal, seed predation, competition, and environment may influence the success of naturally regenerated seedlings especially at high temperatures and in dry climates (González-Martínez et al. 2002). Nevertheless, parentage analysis of in situ wild individuals demonstrated that some descendants from past pollen flow between cultivated and wild species did survive in the wild and that gene flow does occur from the cultivated toward the wild compartment. This flow over many generations might have an effect on the evolution of such small wild populations discovered in grape (Arnold et al. 1998; Grassi, Imazio, et al. 2003; Lacombe et al. 2003; Grassi et al. 2006). In the future, intercompartment gene flow may lead to the extinction of the wild populations per se, causing the loss of some specific or rare alleles of the wild subspecies, reducing their diversity, and their adaptability (Wolf et al. 2001; Ellstrand 2003; Papa and Gepts 2003). It is also important to note that the pollen flow could arise from cultivars and rootstocks escaped from farming. Because transgenic grapevines may become a reality in the future (Iocco et al. 2001), the risk of introgression of exotic genes to the wild compartment exists and may have important consequences for the conservation of wild populations of grapevine in the future.

Wild populations display lower genetic diversity than cultivated populations. The reasons for the low heterozygosity of wild populations could be their small size as well as the intrapopulation pollen flow and the absence of inter–wild population flow. Furthermore, according to Ellstrand (2003), the crop-to-wild gene flow should decrease genetic variation. Such mating strongly affects both individual and population dynamics and survival, and there is a significant risk of extinction and/or potential inbreeding depression (Keller and Waller 2002). "Gre8" is the most exemplary case: its seedlings had the lowest genetic diversity and heterozygosity observed. It displayed a relatively high selfing rate and a direct parentage relationship with the most successful pollen donor ("Gre9").

Gene flow from cultivated to wild grapevines revealed in this study also raises the question of the substantial reality whether current wild populations (Slatkin 1987) truly represent ancestral forms of grapevine (Zohary 1995; Arroyo-Garcia et al. 2006; Imazio et al. 2006). Even so, conservation efforts should focus on the remnant populations of wild grapevine to maintain their genetic integrity as well as their survival.

In conclusion, we demonstrated the feasibility of a paternity-based approach to estimate contemporary and past gene flow in grape, a vegetatively propagated plant. The approach was mainly based on the size and representativeness of the database. It could thus be used for other perennial species, where similar germplasm collections and databases are available and wild genetic resources are endangered.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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