Original article

New insights in the recognition of the European ash species *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl as useful tools for forest management

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Abstract – Common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl) are the most common ash species in Western Europe. The former is considered to be a highly valuable timber tree and contamination of its seed lots by the latter is strongly undesirable. We studied molecular, physiological and morphological characteristics that can help to detect the presence of *F. angustifolia* at the population level, either in seed and/or seedling samples, or in adult ash leave samples from natural or managed populations. First we developed two molecular markers, which correspond to a RAPD-SCAR marker and a nuclear microsatellite-derived marker. Results indicate that these markers are almost specific to one or the other species for a set of populations sampled across Europe. Second, first year dormancy was studied using germination tests without stratification. *F. excelsior* seeds did not germinate at all, while germinations were observed in samples of *F. angustifolia* and introgressed populations after 16 weeks. In addition, *F. angustifolia* embryo/seed length ratios at the dispersal stage were significantly higher than those from *F. excelsior* populations. This study provides straightforward and robust tools for avoiding commercial problems of impurity of seed lots and can help forest managers to certify common ash stands.

ashes / RAPD-SCAR / dormancy / discriminant markers / hybridization

Résumé – De nouveaux outils pour la reconnaissance des frênes européens *Fraxinus excelsior* L. et *F. angustifolia* Vahl : une aide au diagnostic. Le frêne commun (*Fraxinus excelsior* L.) et le frêne oxyphylle (*F. angustifolia* Vahl) sont les deux espèces de frêne les plus répandues en Europe occidentale. Le frêne commun étant le seul à posséder une importante valeur commerciale, la contamination de lots de graines par du frêne oxyphylle est fortement indésirable. Nous avons étudié des caractéristiques moléculaires, morphologiques et physiologiques qui pourront se révéler très utiles à la détection de la présence de *F. angustifolia* à l'échelle populationnelle, dans des lots de graines ou de semis, ainsi que dans des échantillons de feuilles d'arbres adultes issus de populations naturelles ou exploitées. Nous avons développé deux marqueurs moléculaires, un SCAR-RAPD et un marqueur issu d'un locus microsatellite nucléaire. Testés sur un ensemble de populations européennes, ces marqueurs se sont révélés quasi-spécifiques de l'une ou l'autre des deux espèces. D'autre part, nous avons étudié la dormance des graines en première année par des tests de germination sans stratification. Aucune germination n'a été observée sur un ensemble de descendances de frêne commun, alors que des germinations ont été observées sur toutes les descendances de frêne oxyphylle après seize semaines, ainsi que sur certaines descendances issues de populations introgressées. Enfin, nous avons montré que les embryons de *F. excelsior* à maturité dispersive occupent significativement moins de place dans la graine que ceux de *F. angustifolia*. Cette étude permet de fournir des outils rapides et efficaces qui pourront permettre d'éviter d'éventuels problèmes commerciaux liés à la pureté de lots de graines, et d'aider à la certification des peuplements de frêne.

frênes / SCAR-RAPD / dormance / marqueurs discriminants / hybridation

1. INTRODUCTION

The identification of closely-related species can require multiple lines of evidence, particularly if gene flow still occurs. A single type of trait, either morphological or genetic, can be insufficient for efficient separation of groups, as it is the case for the common ash (*Fraxinus excelsior* L.) and the narrow-leaved ash (*F. angustifolia* Vahl.) in Western Europe. The former is sought-after because of its tough and elastic wood and its rapid growth. The latter, with a more Mediterranean distribution [3, 15], has wood that is considered of

lower quality under oceanic climates [19], and thus its presence is undesirable in common ash stands (see [11] for OECD certification scheme of forest reproductive materials). Unfortunately, morphological characters frequently fail to distinguish individuals of the two species, particularly in zones of sympatry where interspecific hybridization has been suspected [18, 28]. Hence, different attempts have been made to distinguish the two ash species with molecular tools. Recently, a chloroplast microsatellite marker [10] was revealed to be of limited use because of the monomorphism observed in populations where both species and putative hybrids were present (Fernandez-Manjarrés and Gérard, unpublished results). This feature is common in forest tree species complexes like oaks, and can originate from a shared ancestral polymorphism or

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from recurrent gene flow and "pollen swamping" [14]. On the other hand, Jeandroz et al. [6] developed nine quasi species-specific RAPD markers, which were used to detect putative hybrid individuals in a population from a zone of sympatry. Of the above markers, only two RAPD loci were robust in a further study [20]. Overall such markers have poor repeatability, and a way around these problems is the development of Sequence Characterized Amplified Region (SCAR) markers [4]. Therefore, currently available molecular markers are not sufficient for ash species determination.

Even when molecular markers are efficient tools for discriminating closely-related species (e.g. [13]), useful information can also be obtained by examining physiological responses. Seeds of both ash species are known to exhibit dormancy, which is particularly long for *F. excelsior* (2 to 6 years) [23, 27, 28]. Following Nikolaeva [12], seeds of this species are characterized by an underdeveloped embryo and morphophysiological dormancy. The effect of storage conditions, temperature and stratification on the germination of *F. excelsior* seeds (e.g. [25, 26]), *F. angustifolia* seeds (e.g. [17, 24]) or both (e.g. [16, 19]) is well documented, and some studies had pointed out differences in the strength of dormancy between the two species [16]. Raquin et al. [19] compared the relative efficiency of germination of *F. excelsior* and *F. angustifolia* (with seed samples from one population of each species), applying three different treatments and suggested that germination tests can be potentially used as a first test to separate the two species. The embryo/seed size ratio gives a first measure of the underdevelopment of the embryo, and this character is known to be involved in germination ability and evolution of seed dormancy [1,2,12]. Thus, multiple tests including physiological responses may be a more powerful and straightforward approach for identifying individuals of closely-related species.

The first aim of this study was to develop repeatable molecular markers: we obtained three SCARs from RAPD loci [6] and a microsatellite-derived marker. Then we assessed the utility of two of them, together with germination tests of *intact untreated* seeds and embryo/seed length ratios measurements, for distinguishing between these two closely-related ash species at the population level.

2. MATERIALS AND METHODS

2.1. Molecular markers

2.1.1. Marker development

For all molecular tests, DNA was extracted with a DNeasy® 96 Plant Kit (Qiagen) from dried leaf material issued from 50 mg of fresh adult tree leaves (almost one leaflet per tree). Two individuals of each species on the Orsay campus, described in Morand-Prieur et al. [10], were used to perform the primary RAPD amplifications. DNA was amplified following the method described by Jeandroz et al. [6] in a MJ Research PTC-200 thermocycler, with primers OpL03 and OpH04 (Qiagen/Operon). Three *F. angustifolia*-specific bands were excised from a 1.5% agarose gel after Ethidium Bromide staining: OpH04 1600 bp and OpL03 750 bp described in Jeandroz et al. [6], and a new one, OpH04 300 bp.

Fragments were purified using a QiAquick® Gel Extraction Kit (Qiagen) and cloned in a pDrive Cloning Vector®, before sequencing using M13 universal probes at Qiagen laboratories (Hilden, Germany). Homology searches were performed using the BLAST algorithm at http://www.ncbi.nlm.nih.gov (National Center for Biotechnology Information), with programs blastn, tblastn and tblastx. New primers were redefined on the three sequences FaH299, FaL757 and FaH1549 (Genbank Accession Numbers AY760060, AY760061 and AY760062, respectively) with Primer3 software [22]. Preliminary PCR tests were performed with 10 individuals of each species in 20 μl solution containing 0.5 units of Q-BIOgene *Taq* DNA Pol, 100 μM of each dNTP, 1.5 mM MgCl$_2$, 50 mM KCl, 20 mM Tris-HCl pH 8.3 (Q-BIOgene) and 0.5 μM of each primer. Amplifications were carried out using the following program: 94 °C for 3 min; 45 cycles (1 min denaturation at 94 °C, 30 s annealing, 1 min 30 s extension at 70 °C); 72 °C for 5 min (final extension). The annealing temperature was chosen at 58 °C, 57 °C and 58 °C for FaH299, FaL757 and FaH1549 respectively, depending on the T_m of each primer (Tab. I).

Second, primers were defined on the flanking regions of the nuclear microsatellite locus Femsatl 19 (GenBank AN: AF020400 [7]) with Primer3 software, and the same PCR tests were carried out with an annealing temperature of 55 °C.

2.1.2. Tests on population samples

Markers that showed frequency differences between the two species were tested on mature leaves of a total of 656 individuals from: eight *F. angustifolia* populations (mean number of individuals per population: 17.9), 21 *F. excelsior* populations (mean number of individuals per population: 19.1) and five populations (mean number of individuals per population: 25.4) from three areas of sympatry between these two species (Tab. II). A bi-marker phenotype was assigned to each individual (0 for the absence and 1 for the presence of the dominant marker). Four phenotypes were possible: 10, 11, 00 and 01. A Simple Correspondence Analysis (SCA) on a two-way contingency table was performed on the phenotypic composition of each population using procedure CORRESP in SAS (SAS Institute, Cary, NC, USA).

2.2. Seed characteristics

Seeds were harvested on adult trees separated by 30 to 50 m. We had previously carried out a study in 2004 on samples from one population of each species (12 trees per population) to verify the stability of the results within a population (data not shown). These preliminary data conducted us to evaluate germination and embryo size patterns at a larger scale: three provenances of each species were chosen for the experiment in 2005, from which seeds of sufficient quantity and quality were available (*F. angustifolia*: three populations from La Mole-Cogolin, Cuxac d'Aude and Cazouls-lès-Béziers from Southern France, *F. excelsior*: three populations from Orsay campus natural vegetation, Dourdan forest and Alençon seed orchard from North-Western France). Three mother trees were chosen in each population. In addition, seeds were harvested on three mother trees in two populations from zones of sympatry in France (St-Dyé-sur Loire, Loire valley and Tavaux, Saône valley) to evaluate germination ability and embryo/seed length ratio variation in comparison with the populations of the two species.

Table I. Sequence and characteristics of primers used.

Name[2] and sequence (5'→3')		Position[1]	Size (mers)	T_m (°C)	GC (%)
FaH299F	GGAAGTCGCCCTTAGACTTTG	1-21	21	59.8	52.4
FaH299R	GCCAAATGGCCTTACACAAC	273-292	20	57.3	50.0
FaL757F	CAGCTTGGTGAGCGATCC	5-22	18	58.2	61.1
FaL757R	CCAGCAGCTTCACAAGGTTA	738-757	20	57.3	50.0
FaH1549F	GAAGTCGCCGCTATCAAGTAA	2-22	21	57.9	47.6
FaH1549R	GGAAGTCGCCCTCTTTTGC	1531-1549	19	58.8	57.9
F19LgF	TTGATATAGGAATGCCAGAGC	3-23	21	57.0	42.9
F19LgR	CAGATACTCGCGTATGATGGTC	436-457	22	59.6	50.0

[1] Position in the SCAR-RAPD sequence.

[2] F and R represent forward and reverse primers, respectively.

For germination tests, 160 seeds per mother tree were sown directly without any prior treatment in 40×40 cm boxes, containing a 1:1 mixture of leaf mould (Floradur®, Floragard) and chalk-less sand. Boxes were placed in the greenhouse under natural light conditions (Mean temperature: 18.5 °C during winter 2005). Germinated seeds were counted every week during 16 weeks.

For embryo size measurements, 16 seeds per mother tree were rehydrated and sterilized as described in Raquin et al. [19], and embryo length was measured under a Wild M5 stereomicroscope at magnification 6×. After verifying that the variable "embryo/seed length ratio" (E:S ratio) did not deviate from a normal distribution within samples of each species we compared E:S ratios between the two species using procedure NESTED in SAS, with mother tree nested within population nested within species (the samples from sympatry were not included in the analysis of variance). When analysing balanced data, this procedure allows determination of the amount of total variation explained by each level (factor) on the observed data [29].

3. RESULTS

3.1. Molecular markers

No homology with any sequence from nucleotide or translated databases was found along the three SCAR sequences. Of the three SCAR markers (Tab. I), only FaL757 was used further because FaH299 and FaH1549 were both present in all individuals tested in preliminary tests (data not shown).

The two markers FaL757 and that corresponding to the entire nuclear microsatellite Femsatl 19 locus (amplified with the primers described in Tab. I, named F19Lg) were complementary, i.e. they were not present at high frequency in the same species (Tab. II). Among all samples, FaL757 was present at high frequency in *F. angustifolia* populations (70.6% of presence in the total sample), whereas it was almost absent from the *F. excelsior* populations (10.6% frequency in the total sample). Its frequency was 20% in the populations from zones of sympatry. On the other hand, F19Lg was present at very high frequency in *F. excelsior* populations (95%), but was rare in the *F. angustifolia* populations (15%). In sympatric populations, this marker had a frequency of 55% (Tab. II). The SCA

(Tab III) allowed populations to be assigned to one of the two species: the proportion of the extreme phenotypes (10 and 01) determined the first axis (which explained 73.65% of the observed variation), while the second axis represented the proportion of intermediate phenotypes (00 and 11) and explained only 16.32% of the observed variation (Fig 1). The populations from the zone of sympatry were distributed between the two groups, and the population of St-Dyé (S5) was a good example of a mixed population.

3.2. Seed characteristics

The percentages of germination obtained for populations of the two species and from the zone of sympatry (Tab. II) reinforced the results obtained by Raquin et al. [19] with depericarped, rehydrated and sterilized seeds. Germination was observed only for seeds of *F. angustifolia* (for all progeny arrays) and St-Dyé and Tavaux seed lots. Moreover, the mean germination rate was two-fold higher for *F. angustifolia* seeds than for seeds from zones of sympatry.

The E:S ratio did not deviate significantly from normality for either species (Cramer-von Mises test: W-sq = 0.06, $P > 0.25$ for *F. excelsior*, W-sq = 0.09, $P = 0.15$ for *F. angustifolia*). Values of E:S ratio were significantly higher in *F. angustifolia* populations (Tab. II): the level "species" explained 77% of the total observed variation ($F_{1,4} = 43.42$, $P = 0.0027$), the level "population" explained 1% of this variation ($F_{4,12} = 1.30$, $P = 0.33$, n. s.) and the level "mother tree" explained 12% ($F_{12,270} = 19.99$, $P < 0.0001$).

These results were in agreement with the preliminary data analysis on seeds of 2004 that were structured in only two levels, with the population and species levels being confounded (results not shown).

4. DISCUSSION

The two molecular markers we developed can be used under any laboratory conditions, contrary to the RAPD markers developed earlier [6, 10]. Not surprisingly FaL757 and

Table II. Description of the study populations (sample size, origin, latitude, longitude). Absolute frequencies of bi-marker phenotypes are given, as well as mean percentage of germinations in population samples and mean E:S ratios.

Population	N^a	Country	Code	Lat.	Long.	Bi-marker phenotypes[b]				% germ.[c]	E:S ratio[d]
						10	00	11	01		
F. excelsior											
Farchau	17	Germany	E1	53°41'	3°47'	0	1	0	16		
Hoge Bos	15	Belgium	E2	50°50'	2°57'	0	2	1	12		
Abetone	20	Italy	E3	44°11'	10°38'	0	1	0	19		
Cadore	14	Italy	E4	46°30'	12°28'	0	1	0	13		
Monti Lessini	20	Italy	E5	45°40'	11°13'	0	2	0	18		
Valle Pesio	20	Italy	E6	44°15'	7°39'	0	2	0	18		
Kasiadorys	20	Lituany	E7	54°51'	24°25'	0	2	0	18		
Zeimelio	20	Lituany	E8	56°16'	24°00'	0	3	1	16		
Rabstejn	18	Czech R.	E9	50°50'	14°20'	0	0	0	18		
Donadea	16	Ireland	E10	53°20'	6°45'	0	0	0	16		
Enniskillen	20	Ireland	E11	54°14'	7°28'	0	0	5	15		
Currachase	17	Ireland	E12	52°36'	8°53'	0	1	6	10		
Wytham	15	UK	E13	51°47'	1°20'	0	0	4	11		
Settrington	12	UK	E14	54°01'	0°69'	1	0	3	8		
La Romagne	20	France	E15	49°40'	4°19'	0	0	0	20		
Athis	20	France	E16	49°01'	4°07'	0	2	3	15		
St-Gatien	19	France	E17	49°21'	0°11'	0	1	3	15		
St-Paul-de-S.	21	France	E18	45°08'	2°31'	0	0	3	18		
Dourdan	20	France	E19	48°31'	2°00'	0	0	0	20	0 (0)	0.50 (0.07)
Orsay	8	France	E20	48°42'	2°11'	0	0	1	7	0 (0)	0.51 (0.10)
Alençon	24	France	E21	48°29'	0°07'	0	0	9	15	0 (0)	0.46 (0.07)
Overall	376					1	18	39	318	0 (0)	0.49 (0.08)
F. angustifolia											
Alter do Chao	20	Portugal	A1	39°12'	7°40'	14	4	1	1		
Boujan-sur-L.	23	France	A2	43°23'	3°14'	14	4	5	0		
Grabels	18	France	A3	43°39'	3°47'	7	7	2	2		
Mas-Larrieu	24	France	A4	42°36'	3°02'	17	4	3	0		
Cogolin	10	France	A5	43°12'	6°28'	6	3	1	0		
La Mole	10	France	A6	43°25'	6°53'	5	1	3	1	4 (3)	0.69 (0.09)
Cuxac d'Aude	24	France	A7	43°16'	2°59'	10	11	2	1	18 (14)	0.71 (0.08)
Cazouls-lès-B.	24	France	A8	43°25'	3°07'	17	6	1	0	20 (11)	0.80 (0.09)
Overall	153					90	40	18	5	14 (12)	0.73 (0.10)
Zone of sympatry											
Hohenau	9	Austria	S1	48°36'	16°55'	0	3	0	6		
Rigny	30	France	S2	47°28'	5°38'	1	15	0	14		
Tavaux	20	France	S3	47°02'	5°24'	1	5	2	12	2 (2)	0.59 (0.08)
St-Pryvé	20	France	S4	47°53'	1°52'	0	3	3	14		
St-Dyé-sur-L.	48	France	S5	47°39'	1°29'	14	15	5	14	12 (10)	0.70 (0.11)
Overall	127					16	41	10	60	7 (8)	0.65 (0.11)

[a] Sample size for molecular marker analysis.
[b] The first number represent the phenotype presence (1) / absence (0) of FaL757, and the second number that of F19Lg.
[c] Mean percentage of germinated seeds (standard deviation).
[d] Mean embryo/seed length ratio (standard deviation).

F19Lg are not strictly species-specific, since these closely-related species sometimes hybridize and thus still exchange genes [5, 8]. Nevertheless, they allow discrimination between the two species at the population level. In zones of sympatry, it is preferable to use them concomitantly with the study of seed characters. For example, the Tavaux population (Saône valley) exhibits a large proportion of F19Lg, indicating *F. excelsior*-like provenance, but some germinations were observed and E:S ratios were intermediate, indicating the presence of *F. angustifolia*-like genotypes. Indeed, this population has been shown to be mainly composed of morphologically *F. excelsior*-like individuals, but a small amount of introgression by *F. angustifolia* gene pool was detected (Fernandez-Manjarrés et al., submitted). On the other hand, the St-Dyé stand (Loire valley) can be robustly characterised as an admixed population as it exhibits both molecular markers in similar proportions, and intermediate germination rates and high E:S ratios. In an intensive local scale study, we demonstrated that this hybrid zone population was composed in a major part of *F. angustifolia* gene pool, and that parental species and different
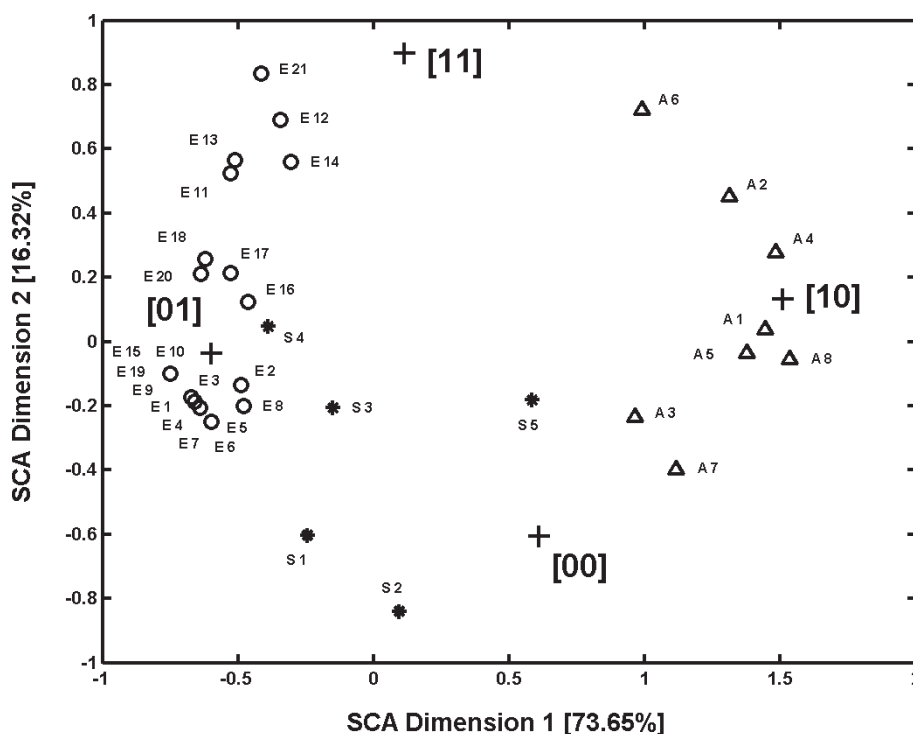
Figure 1. Simple Correspondence Analysis (SCA) on bi-marker phenotypes. Circles represent *F. excelsior* populations, triangles represent *F. angustifolia* populations and stars represent populations from zones of sympatry (codes are listed in Tab. II). Crosses are plotted at the coordinates of the four phenotypes in the first two dimension of the SCA (the first number in the brackets represent the phenotype presence (1)/absence (0) of FaL757, and the second number that of F19Lg). The percentages of the total inertia provided by the first two dimensions are shown in brackets on each axis.

Table III. Inertia and chi-square decomposition of the Simple Correspondence Analysis (SCA).

Singular value	Principal inertia	Chi-square	%	Cumulative %
0.79839	0.63742	418.150	73.65	73.65
0.37581	0.14123	92.648	16.32	89.97
0.29464	0.08681	56.948	10.03	100.00
Total	0.86547	567.746	100.00	d.f.[a] = 99

[a] Degrees of freedom.

types of hybrids were present (Gérard et al., submitted). By extension, other combinations of marker frequencies and seed characteristics could signal other types of admixed or hybrid populations, since hybrid segregation usually produces a mosaic of morphological and molecular markers that can vary among hybrid zones, particularly relative to varying selection regimes [21].

The germination test is a simple way to detect the presence of narrow-leaved ash seeds, but the sample size needs to be sufficient and the results may depend on the damaging degree of the seed lot. We chose to conduct the test on intact samaras (seeds within the fruit), first because we wanted to know the ability of both species to germinate without any treatment at all, and second to be able to provide a straightforward means for managers to rapidly detect the presence of *F. angustifolia* seeds. But it is sometimes difficult to detect in-

fected seeds without removing the pericarp [17]. Only empty samaras were removed from the samples. We also acknowledge that the number of sampled populations for the two seed experiments is not very large, although it covers a large geographic area. Nevertheless, germinations were observed in all *F. angustifolia* seed lots and no germination whatsoever was observed for any *F. excelsior* seed lots: it seems that stratification is absolutely necessary to remove first-year dormancy in *F. excelsior* seeds. These same germination patterns were also found across a broader range of *F. angustifolia* and *F. excelsior* provenances and similarly E:S ratios differed between the two species (Gérard and Raquin, unpublished results).

This investigation on ash seeds showed particular developmental characters of the species. The embryo made up a larger proportion of the seed in *F. angustifolia* and this species produces more vigorous seedlings during the first years of growth (Dufour, unpublished results). This could represent a better ability for colonizing newly open forest gaps or open habitats, and thus explain the replacement of common ash by narrow-leaved ash in some regions [9]. Embryo size also affects germination and dormancy. Morphological dormancy is due to underdevelopment of the embryo at the seed dispersal stage and is considered ancestral in seed plants [1, 2]. Common ash seeds with their small embryos seem to exhibit morphophysiological dormancy [12] while narrow-leaved ash seeds appear to retain only physiological dormancy [16]: the embryo usually does not seem to be underdeveloped. The evolution

towards large embryos in the seeds of this latter species may have been possible by changes in developmental timing (i.e. heterochrony [2]) possibly selected in *F. angustifolia* as an adaptation to Mediterranean ecological and climatic conditions. Indeed in a wide-scale study of these two species (some populations were the same as in the present study) and their hybrids, we showed that their range distributions are linked to environmental features such as altitude, number of frost days and annual mean temperature (Fernandez-Manjarrés et al., submitted).

In conclusion, we developed a set of robust and powerful tools for rapidly detecting the presence of *F. angustifolia* in the field or in seed lots, which could be easily applied by forest managers. The present set of markers allows us to distinguish the two species adequately and this discrimination improves with increasing sample sizes, which was not the case for earlier studies. Moreover, the present study points out possibly different ecological strategies of the two species that can be linked with their colonizing abilities (see [9] for *F. excelsior*). This can be useful for studies of hybridization in zones of sympatry and/or of putative replacement of common ash by narrow-leaved ash, for example in the context of global change.

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