The Genetic Basis of Pollinator Adaptation in a Sexually Deceptive Orchid

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Abstract

In plants, pollinator adaptation is considered to be a major driving force for floral diversification and speciation. However, the genetic basis of pollinator adaptation is poorly understood. The orchid genus Ophrys mimics its pollinators’ mating signals and is pollinated by male insects during mating attempts. In many species of this genus, chemical mimicry of the pollinators’ pheromones, especially of alkene double-bond positions, plays a key role for specific pollinator attraction. Thus, different alkene produced in different species are probably a consequence of pollinator adaptation. In this study, we identify genes that are likely involved in alkene biosynthesis, encoding stearoyl-acyl carrier protein (ACP) desaturases (SAD), in three closely related Ophrys species, O. garganica, O. sphegodes, and O. exaltata. Combining floral odor and gene expression analyses, two SAD homologs (SAD1/2) showed significant association with the production of (Z)-9- and (Z)-12-alkenes that were abundant in O. garganica and O. sphegodes, supporting previous biochemical data. In contrast, two other newly identified homologs (SAD5/6) were significantly associated with (Z)-7-alkenes that were highly abundant only in O. exaltata. Both molecular evolutionary analyses and pollinator preference tests suggest that the alkenes associated with SAD1/2 and SAD5/6 are under pollinator-mediated divergent selection among species. The expression patterns of these genes in F1 hybrids indicate that species-specific expression differences in SAD1/2 are likely due to cis-regulation, while changes in SAD5/6 are likely due to trans-regulation. Taken together, we report a genetic mechanism for pollinator-mediated divergent selection that drives adaptive changes in floral alkene biosynthesis involved in reproductive isolation among Ophrys species.

Introduction

Understanding the genetic basis of adaptation is of great interest to evolutionary biologists. For over a century, it has been debated whether adaptations are likely caused by a large number of mutations of small phenotypic effect or by very few genetic changes of large effect [1–3]. To address this question, it is necessary to identify the genetic basis of adaptive traits and their ecological significance in any given study system [4].

Pollinator-mediated selection on floral traits has been considered to be a major driving force of floral diversification and speciation in plants [4–9]. Closely related species featuring distinct floral traits, such as floral color, odor, or spur length, are widely thought to be a consequence of pollinator adaptation [4,6,8–11]. Furthermore, pollinator adaptation often conveys reproductive isolation [12,13], and thus may directly contribute to the origin of novel species. Therefore, floral traits associated with pollinator adaptation are of special interest for the understanding plant speciation and evolution.

Ophrys orchids mimic their pollinators’ mating signals and are pollinated by male insects during mating attempts with the flower. This pollination by so-called sexual deception is very specific, and each orchid species only attracts one or very few insect species [14,15]. Specific pollinator attraction has been reported to be the main reproductive barrier in Ophrys [15–17]. The key to specific pollinator attraction is the chemical mimicry of the insect female’s sex pheromone [11,18–22], usually a blend of cuticular hydrocarbons, namely alkanes and alkenes. Among these, alkenes with different double-bond position are particularly important for the key to specific pollinator attraction [11,20,22]. Thus, genes specifying alkene double-bond positions may be directly associated with pollinator adaptation.

In plants, alkene double-bonds are likely determined by desaturases such as stearoyl-ACP (acyl carrier protein) desaturases (SADs) [23–25]. At the onset of alkene biosynthesis, desaturases can insert a cis-double-bond into a saturated fatty acid (FA) intermediate, such as 16:0-ACP (C16:0 denotes a fatty acyl group of 16 carbons length with D double-bonds), to produce an unsaturated FA such as 16:1o-7-ACP or 16:1o-9-ACP (double-bond at position 3-7 or 3-9, counting from the aliphatic end). Unsaturated FA intermediates are elongated from the ACP end [26], leading to the production of 7-alkenes from 3-7 or 9-alkenes from 3-7 or 9-9 FA intermediates, respectively [23,24]. Since all alkenes in this study are presumed to be in the cis configuration, only double-bond...
Author Summary

In plants, the extraordinary floral diversity has been suggested to be a consequence of divergent adaptation. However, the genetic basis of this process is poorly understood. In this study, we take advantage of the high specificity of plant-pollinator interactions in the sexually deceptive orchid genus Ophrys. We leverage the available, ample evidence showing that floral odors, especially alkenes, are the key factor for specific pollinator attraction in certain species of these orchids. Further, we investigate the genetic basis of pollinator adaptation. By applying an inter-disciplinary approach, including chemical ecology, gene expression analysis, population genetics, and pollinator-behavioral tests, we show that genetic changes in different copies of a biosynthetic gene are associated with the production of different floral scents and with pollinator adaptation in these orchid species. Moreover, we found that both cis- and trans-regulatory factors are likely involved in controlling gene expression of these biosynthetic genes. These findings support the hypothesis that adaptation is mediated by very few genetic changes with large phenotypic effects, rather than requiring a large number of co-adapted genes.

Results

Stearoyl-ACP-desaturase homologs in Ophrys

We identified six SAD homologs, which we named SAD1–SAD6. Three of these (SAD1–3) have been described previously [24]; SAD4–6 were identified by homology from high-throughput transcriptome sequencing of O. sphaegodes flowers. Phylogenetic analysis of plant SAD genes indicated that all six Ophrys homologs evolved via gene duplication, forming three distinct lineages, SAD1/2, SAD3, and SAD4/5/6. The split of SAD1/2 from SAD4/5/6 was more recent than the split of these groups from SAD3 (Figure 1).

To test for the signature of selection, a codon-based analysis comparing the rates of synonymous and non-synonymous mutations was performed. It revealed a significant signal indicative of positive (ωs and ωn) and relaxed purifying selection (ωj, all \( p<0.001 \), Figure 1) after the split of SAD1 and SAD2, concordant with previous findings [24]. However, no indication of positive selection was found for any other SAD locus or clade. Purifying selection significantly stronger than the background rate was found on the SAD3 branch, as well as prior to the split of SAD1/2, and prior to the split of SAD4/5/6.

Expression of certain SAD homologs is correlated with alkene production during development

Among the six SAD homologs investigated for tissue- and floral developmental stage-specific expression, five (SAD1–5) were found to be expressed in the 11 tested greenhouse-grown individuals. Four homologs (all except SAD3) showed flower-specific expression (Figure S1). The expression levels of SAD2 and SAD5 were significantly associated with the presence of alkenes: SAD2 expression was significantly \( p<0.001 \) associated with both 12- and 9-alkenes, whereas SAD5 expression was significantly \( p<0.001 \) associated with 7-alkenes in O. exaltata across different tissues and floral developmental stages (Figure 2).

Allelic variation of SAD homologs among species

All SAD homologs except SAD3 and SAD4 showed species-specific patterns of allelic variation among the three studied orchid species (Figure S2). Two allele groups were found for each SAD1 (SAD1-A/B), SAD5 (SAD5-A/B) and SAD6 (SAD6-A/B), whereas four allele groups were found for SAD2 (SAD2-A/B/C/D). Among these SAD allele groups, biochemical activity assays suggest that SAD1-B and SAD2-C alleles do not encode functional desaturases, whereas one SAD2-A allele has been shown to be functional [24]. However, two further allele groups are unlikely to be functional: one group (SAD2-D) had a repetitive sequence insertion at the start of the coding sequence, and one (SAD6-B) contained significantly more stop and frame-shift mutations than expected by chance (Table S1).

Combining putative coding sequence functionality and biochemical activity data, we classified all alleles into three categories: (a) putatively functional and expressed, (b) putatively nonfunctional and expressed, and (c) non-expressed alleles (Figure S3). For SAD1/2/5/6, the distributions of these allele categories are significantly different between O. exaltata and the other two species (Figure 3). For SAD1/2, functional expressed alleles were significantly more common in O. garganica and O. sphaegodes than in O. exaltata. By contrast, SAD5/6 showed the opposite pattern, functional expressed alleles being more common in O. exaltata.

To estimate \( F_{ST} \) values for all expressed SAD homologs in all three species, an in silico resampling approach was employed (see Methods), treating all individuals as diploids based on flow cytometry data [17]. O. garganica was not included in this analysis due to the smaller sample size, and \( F_{ST} \) could not be calculated for SAD5 because it was only observed in O. exaltata. SAD2 and SAD6 showed significantly higher \( F_{ST} \), \( 0.44\pm0.02 \) and \( 0.32\pm0.006 \) respectively, mean \( \pm \) standard error, \( p<0.01 \) than SAD1, SAD3.
Allelic gene expression of \textit{SAD} is associated with alkene composition differences among species

Five \textit{SAD} copies (all except \textit{SAD3}) showed divergent species-specific gene expression (Figure S3). Among the alleles of \textit{SAD1}, \textit{SAD1-A} was highly expressed in \textit{O. sphegodes} and \textit{O. garranica} but not in \textit{O. exaltata} (Figure 4), whereas \textit{SAD1-B} was highly expressed in \textit{O. sphegodes} and \textit{O. exaltata} (Figure S3). Among the \textit{SAD2} allele groups, \textit{SAD2-A/B} were highly expressed in both \textit{O. sphegodes} and \textit{O. garranica}, whereas the expression of \textit{SAD2-C/D} was low in all study species (Figure S3). The expression of \textit{SAD4} was high in \textit{O. garranica} and \textit{O. exaltata}, but low in \textit{O. sphegodes}. All alleles of \textit{SAD5} and \textit{SAD6} were highly expressed mostly in \textit{O. exaltata}.

Natural F1 hybrids among \textit{O. sphegodes} and \textit{O. exaltata} (identified from AFLP data) showed a similar scent [17] and \textit{SAD} expression pattern to \textit{O. sphegodes} (Figure S3). For \textit{SAD1} and \textit{SAD2}, alleles (\textit{SAD1-A/B}, \textit{SAD2-A/B}) that were most likely inherited from \textit{O. sphegodes} were found to be highly expressed in these F1 hybrids, whereas none of the \textit{SAD5/6} alleles was expressed (Figure S3).

Statistical analysis of data from natural populations showed a strong correlation between the expression of specific \textit{SAD} allele groups and alkene production. The expression level of \textit{SAD1-A} was found to be significantly ($p<0.001$) positively correlated with 9-C$_{29}$, 12-C$_{27}$ and 12-C$_{29}$ alkenes; \textit{SAD2-A} was significantly ($p<0.001$) positively correlated with 9-C$_{27}$, 9-C$_{29}$, 12-C$_{25}$, 12-C$_{27}$, and 12-C$_{29}$ alkenes (Figure 2, Figure S4); \textit{SAD3-A/B} were significantly ($p<0.001$) correlated with all 7-alkenes (Figure 2, Figure S4).

**Alkene composition affects pollinator attraction**

To understand the driving force for allelic evolution of \textit{SAD} homologs in \textit{Ophrys}, we tested the effects of alkenes with different double-bond position – associated with different \textit{SAD} homologs – on pollinator behavior. We quantified pollinator responses to

\begin{align*}
\text{dW}_1 &= 0.056, \quad p = 0.004^* \\
\text{dW}_2 &= 0.029, \quad p = 0.003^** \\
\text{dW}_3 &= 0.052, \quad p = 0.005^* \\
\text{dW}_4 &= 0.029^* \\
\text{dW}_5 &= 0.071, \quad p = 0.002^* \\
\text{dW}_6 &= 0.055, \quad p = 0.006^* \\
\text{dW}_7 &= 0.059, \quad p = 0.005^* \\
\text{dW}_8 &= 0.022, \quad p = 0.0002^*** \\
\text{dW}_9 &= 1.37, \quad p = 0.0001^*** \\
\text{dW}_{10} &= 1.999, \quad p = 0.001^*** \\
\text{dW}_{11} &= 1.936, \quad p = 0.001^*** \\
\text{dW}_{12} &= 1.23
\end{align*}
control and manipulated flowers and scent extracts of O. exaltata and O. sphegodes. Addition of 9- and 12-alkenes (associated with \(SAD2-A\)) to O. exaltata labella reduced the attractiveness to its pollinator (the masked bee \(Colletes cunicularia\)) by about 40% for approach and contact \((N = 18, p = 0.023\) and \(p = 0.015\) respectively), Wilcoxon signed rank test, Figure 5A). Adding 7-alkenes (associated with \(SAD5-A/B\)) to floral scent extracts of \(O. \text{sphegodes}\) reduced the attractiveness to its pollinator (the mining bee \(Andrena nigroaenea\)) by about 30% and 60% for approach and contact respectively \((N = 17, p = 0.028\) and \(p = 0.045\), Wilcoxon signed rank test, Figure 5B).

**Discussion**

**Differences in alkene production are associated with \(SAD\) gene expression**

Our data indicate that alkene biosynthesis is associated with the expression of certain \(SAD\) homologs in \(Ophrys\). \(SAD2\) catalyzes the introduction of a double-bond at position 9 of 18:0-ACP and position 4 of 16:0-ACP [24], producing 18:1\(-9\)-ACP and 16:1\(-9\)-ACP, which should eventually lead to the production of 9- and 12-alkenes [23,24]. The significant correlation of \(SAD2\) expression with the amounts of certain 9- and 12-alkenes in different plant tissues, floral developmental stages, and natural populations lends further support to this hypothesis. Although biochemical assays suggested that \(SAD1\) (allele group \(SAD1-B\)) is not catalytically active [24], this may not be true for other \(SAD1\) alleles \((SAD1-A)\) found in both \(O. \text{sphegodes}\) and \(O. \text{garganica}\), \(SAD1-A\) and \(SAD1-B\) differing by 14% at the amino acid sequence level. Furthermore, \(SAD1-A\) expression was significantly correlated with some 9- and 12-alkenes [Figure S4]. This indicates that, like \(SAD2\), \(SAD1\) may also contribute to 9- and/or 12-alkene biosynthesis in natural populations.

The significant correlation of \(SAD5\) expression with the amount of 7-alkenes [Figure 2] suggests that \(SAD5\) is involved in 7-alkene biosynthesis. In addition, the high sequence identity (>95% at the amino acid level) of \(SAD5\) to \(SAD6-A\), which was highly expressed in one \(O. \text{exaltata}\) population \((\text{SPF})\), indicates that both may have the same enzymatic function. We hypothesize that \(SAD5/6\) introduces a double-bond at position 11 into 18:0-ACP, producing 18:1\(-9\)-ACP, or at position 9 of 16:0-ACP, producing 16:1\(-9\)-ACP. Further biochemical studies are required to test this hypothesis. These unsaturated intermediates could then be elongated to produce 7-alkenes. Therefore, changes in the expression of \(SAD5/6\) could directly lead to different amounts of 7-alkenes in different \(Ophrys\) species.

**\(SAD\) homologs are under pollinator-mediated selection**

Our data suggest that \(SAD\) homologs evolve under pollinator-mediated selection, considering that genetic drift is a less likely explanation given large effective population sizes in our study species [11]. The expression of \(SAD1/2\) was high in both \(O. \text{garganica}\) and \(O. \text{sphegodes}\), but was very low in most \(O. \text{exaltata}\) individuals [Figure 4, Figure S3]. In those very few individuals of \(O. \text{exaltata}\) that did highly express \(SAD2\), it either was rendered nonfunctional by a repetitive sequence insertion \((SAD2-D)\), population MDL), or had amino acid substitutions located on the surface of the protein \((SAD2-C)\) allele group), which have been suggested to reduce the activity of \(SAD2\) [24]. This indicates that disruptive selection might act on \(SAD2\) (in terms of gene expression or overall enzymatic activity) to maintain (in \(O. \text{sphegodes}\) and \(O. \text{garganica}\) or reduce (in \(O. \text{exaltata}\)) the production of 9- and/or 12-alkenes.
alkenes in *Ophrys* floral odor. Evidence of positive selection on SAD1/2 detected by dN/dS ratio tests is consistent with this hypothesis (Figure 1 and ref. [24]). Indeed, our pollinator-attraction assay suggests disruptive selection on 9- and/or 12-alkenes among species. This behavioral test demonstrated that adding 9- and 12-alkenes onto the floral labella of *O. exaltata* reduced its attractiveness to the pollinator by about 40% (Figure 5A). This indicates that while 9/12-alkenes act as the main attractants of *O. sphegodes* to its pollinator *A. nigroaenea* [18], these compounds actually reduce the attractiveness of the odor bouquet of *O. exaltata* to its pollinator *C. cunicularius*. Reduced responses of pollinator males to heterospecific odor blends may have evolved under sexual selection for maximum speed and accuracy of finding conspecific females [32]. Therefore, pollinator-imposed disruptive selection acts to change 9/12-alkene composition among these two *Ophrys* species by changing the expression or enzymatic activity of SAD1/2. However, for SAD5/6, the opposite pattern was observed. SAD5/6 were highly expressed in *O. exaltata*, but hardly expressed in *O. sphegodes* and *O. garganica*. A significantly higher frequency of frame-shifts or premature stop codons was found in SAD6 of *O. sphegodes* and *O. garganica* (Table S1), indicating that SAD6 alleles in these two species may be released from purifying selection such that loss-of-function mutations can accumulate. Neither positive selection nor purifying selection was detected on SAD5/6 using codon-based methods (Figure 1), suggesting that pollinator-mediated selection on 7-alkenes in *O. sphegodes* and *O. garganica* primarily acts on the expression level of SAD5/6. Indeed, selection against 7-alkenes in *O. sphegodes* was confirmed by behavioral tests with its pollinator, since addition of 7-alkenes to the floral scent of *O. sphegodes* resulted in a significant reduction in pollinator attraction (Figure 5B). Therefore, while 7-alkenes attract *O. exaltata*’s pollinator [19,31], they reduce the attractiveness of *O. sphegodes* to its pollinator. Hence, pollinator-mediated disruptive selection may also drive the evolution of 7-alkene quantity in these
two *Ophrys* species by changing the expression of *SAD5/6*. In contrast to these genes that are associated with alkene production, *SAD1* and *SAD4*, which were not significantly correlated with alkene occurrence, showed no significant sequence divergence among species (Figure 3, Figure S2), as would be expected for genes that are not targets of selection.

Overall, our data suggest that pollinator adaptation in *Ophrys* is achieved via reciprocal regulation or activity changes of desaturases involved in 7- and 9/12-alkene biosynthesis in response to disruptive selection by different pollinator preferences.

Both cis- and trans-regulatory elements may be involved in controlling species-specific alkene compositions

The changes in 7- and 9/12-alkene production that are linked to differences in *SAD* gene expression may be explained by the action of cis- or trans-acting elements. The expression of *SAD1*/*2*, which is associated with 9/12-alkene production, differed among *O. exaltata* (weak expression) and *O. sphegodes* (strong expression) (Figure S3). However, two putative F1 hybrids only expressed the alleles expected to be inherited from *O. sphegodes*, but not *O. exaltata* (Table S2; Figure S3). This indicates that down-regulation of *SAD1*/*2* expression in *O. exaltata* might be due to changes in a cis-regulatory element (such as a promoter or enhancer). In contrast, although differences in expression of *SAD5/6*, which are associated with 7-alkene production, were found between *O. sphegodes* and *O. exaltata*, the putative F1 hybrids did not express either allele expected from the parental species (Table S2; Figure S3). This suppression of expression of *SAD5/6* in F1 hybrids indicates that – while additional cis-regulatory changes cannot be ruled out – a trans-acting factor is likely involved in the different *SAD5/6* gene expression among species. This suggests the presence of a (dominant) suppressor of *SAD5/6* expression in *O. sphegodes* (e.g., a transcriptional repressor or a miRNA reducing *SAD5/6* mRNA levels) that is absent or inactive in *O. exaltata*. However, it is also possible that the dominant expression of *SAD5/6* genes in F1 hybrids is due to epigenetic changes upon hybridization [33,34].

In conclusion, our data based on multiple independent lines of evidence suggest that pollinator adaptation in the three studied *Ophrys* species is largely due to changes in *SAD1*/*2* and *SAD5/6*, in terms of gene expression and potentially also in terms of the function of their gene products, and that both cis- and trans-regulation of gene expression contribute to this process. Our data indicate that pollinator adaptation in plants with a specialized pollination system may be due to few changes in the genome, with a large phenotypic effect. Furthermore, because reproductive isolation among closely related *Ophrys* species is mainly a consequence of specific pollinator attraction, such adaptation to different pollinators can directly prevent gene flow and ultimately lead to speciation.

### Materials and Methods

#### Plant material

Population samples were collected in southern Italy (Table S3), at the same locations as described in Xu et al. [17], with three additional *O. exaltata* individuals from San Pietro in Fere (SPF) in southern Italy (N41°25′38″, E13°58′04″). Two F1 hybrids between *O. exaltata* and *O. sphegodes* were previously identified based on AFLP markers [17]. For each plant individual, one labellum of an unpollinated flower was used for floral odor extraction as described previously [17], and then immediately flash frozen in liquid nitrogen, and stored at −80°C until RNA extraction. For developmental stage- and tissue-specific analysis of hydrocarbons and gene expression, five *O. exaltata* and six *O. sphegodes* individuals grown in a greenhouse at the Botanical Garden of the University of Zurich were used and processed as described previously [24].

#### GC and GC/MS analysis

GC and GC/MS analysis, identification and quantification of compounds were performed as described previously [11,19] with modifications [17]. Discrimination of 11- and 12-alkenes was not possible with the GC parameters used, however, 12-alkenes were earlier determined to be the predominant isomers in *O. sphegodes* [35,36]. The absolute amounts of alkenes and alkanes with a carbon chain length from 21 to 29 were calculated based on an internal standard. For tissue/stage-specific samples, the relative amount of alkenes was calculated since the use of comparable amounts of tissue could not be ensured.

#### RNA extraction, cDNA synthesis, RACE, and RT–PCR

Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, USA) following the manufacturer’s protocol, and RNA quality and quantity were assessed by agarose gel electrophoresis and spectrophotometry on a NanoDrop ND-1000 (Witec AG, Littau, Switzerland). First strand cDNA was synthesized as described in [24]. To obtain the full-length coding sequence of candidate genes, 5’ RACE was performed as described in [37], with minor modifications [24], and 3’ RACE as in [38]. Gene-specific primers used for RACE are listed in Table S4. Advantage GC 2 DNA Polymerase (Clontech Laboratories Inc, Mountain View, USA) was used for RACE PCR with a touchdown program: 96°C 15 s; 3 cycles of [94°C 20 s, 68°C 3 min 30 s]; 7 cycles of [94°C 20 s, 67°C (1°C decrease per cycle) 30 s, 68°C 3 min 30 s]; 50 cycles of [94°C 25 s, 55°C 30 s, 68°C 3 min 30 s]; final extension at 68°C for 10 min. The amplified fragments were cloned into pDRIVE vector (Qiagen, Hilden, Germany), following the provided protocol. Desaturase homologs were amplified for all cDNA samples, using gene-specific primers containing attB adapter sequences (Table S4); RT–PCR was performed in 15 µl reaction volume containing cDNA template equivalent to 15 ng RNA as follows: 95°C 3 min; 33 cycles of [95°C 30 s, 58–60°C 30 s] (see Table S4 for annealing temperatures); 72°C 1 min 30 s; final extension at 72°C for 10 min, using REDTaq ReadyMix (Sigma-Aldrich, St. Louis, USA) mix supplemented with 0.6 units *Pfu* polymerase (Promega AG, Dübendorf, Switzerland). Three µl PCR product were loaded on an agarose gel to confirm amplification.

#### Cloning and sequencing

Amplified PCR products from each population of each species were pooled and then purified with Wizard SV Gel and PCR Clean-Up kit (Promega AG, Dübendorf, Switzerland), and recombined into Gateway cloning vector pDONR221 (Invitrogen, Carlsbad, USA) using the manufacturers’ protocols. Competent *E. coli* One Shot TOP10 cells (Invitrogen, Carlsbad, USA) were used for transformation. In order to recover all possible alleles, the number of clones picked and screened by PCR was at least three times the number of possible alleles in diploids, for each cloning library. Clones were PCR amplified, purified and sequenced as previously described [24]. All sequences were deposited in GenBank (accession numbers are listed in Table S3).

#### Sequence analysis and allele group assignment

Forward and reverse sequences of each clone were assembled and manually edited in SeqMan v7.1.0 (Lasergene DNASTAR, Wisconsin, USA). For each *SAD* homolog, the assembled sequences of each clone were aligned using Clustal W [39].

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Sequences with less than two nucleotide differences were considered to be the same allele with PCR or sequencing errors, and were merged into one consensus sequence. The consensus sequences and all singleton sequences, which differed by more than two nucleotides, were used for assignment to allele groups. To do so, given the low sequence divergence, a dendrogram was constructed for each SAD homolog in MEGA 4.0 [40], using a pairwise distance and the UPGMA method, with pairwise deletion of gaps, and a homogenous substitution pattern among lineages and sites. Bootstrap analysis was conducted using 1000 pseudoreplicates. Allele groups were assigned based on UPGMA tree topology with bootstrap support (Figure S2). The relationship replicates. Allele groups were assigned based on UPGMA tree and sites. Bootstrap analysis was conducted using 1000 pseudo-replicates. Allele groups were assigned based on UPGMA tree topology with bootstrap support (Figure S2). The relationship among alleles was also inferred by Bayesian analysis (Figure S5) in MrBayes (v3.2.1; for details see below) [41], which was congruent and largely confirmed the clusters from UPGMA analysis. The Bayesian analysis showed SAD2-C alleles to be nested in SAD2-B, and SAD6-A in SAD6-B, but was in agreement with our primary allele group assignment.

Measuring gene expression by semi-quantitative PCR
SAD gene expression was assessed by semi-quantitative RT-PCR with allele-specific primers (Table S4). PCR was performed in 10 μl reaction volume with CDNA from 12 ng total RNA as a template. Each PCR was performed as: 95°C 3 min; 29 cycles of [95°C 30 s, 58–60°C 30 s (see Table S4 for different primer annealing temperatures), 72°C 1 min 30 s]; final extension at 72°C for 10 min using REDTaq ReadyMix (Sigma-Aldrich, St. Louis, USA). For all RT-PCRs, the putative Ophrys housekeeping gene G3PDH [24] was used as control. Five μl of each PCR product were loaded on 0.8% agarose gel, recorded and quantified using ImageJ (1.42q) [42] as described in [24].

Bioassay for pollinator preferences on floral scents
Bioassays for pollinator preferences were performed between beginning of March to middle April 2011 at Capoiale (Southern Italy) and Charrat (Wallis, Switzerland) for O. exaltata and O. sphageodes, respectively. For both species, pollinator preferences on control and manipulated scent (for O. exaltata, 9/12-alkenes added; for O. sphageodes, 7-alkenes added) were tested (Table S6 and Table S7). The preference of O. exaltata’s pollinator, C. cunicularius, was assessed with whole inflorescences (bearing 2–3 flowers) assayed individually. Each inflorescence was placed on bushes where C. cunicularius males were abundant; pollinator responses were recorded for 10 minutes. Afterwards, a 9/12-alkene mixture mimicking natural blends occurring in O. sphageodes was added onto each floral labellum of the same inflorescence (see Table S6 and Table S7) and the pollinator responses monitored for a further 10 min. For each subsequent test, the plants were placed at a different position to avoid habituation effects often found after multiple subsequent testing at one location. In total, 18 inflorescences that had at least 2 flowers were tested. The preference of the pollinator of O. sphageodes, A. nigroaenea, was assessed in choice experiments on floral scent with black plastic beads. This different testing procedure was chosen because no natural plants were available at this testing location. The floral scent of floral labella was first extracted with 500 μl hexane [17]. For each choice experiment, 100 μl of floral extract was tested against 100 μl of floral extract of the same flower plus 7-alkene mixture (see Table S6 and Table S7). The dummy was placed on bushes where A. nigroaenea males were abundant. Pollinator responses were recorded for six minutes. In total, 17 replicates of this experiment were performed. For all pollinator behavioral tests, pollinator responses were classified into: approach (a zig-zagging or undulating approach towards the tested flowers or beads) and contact (either a short punching contact or landing on the tested flowers or beads) [19].

Bioinformatics and statistical analysis
All monocot SAD sequences were taken from the plant SAD homolog data set of [24]. Sequences were re-aligned based on amino acid sequence using Muscle 3.8.31 [43]. Phylogenetic analysis was performed in MrBayes 3.1.2 [41] (burn-in 13 out of 40 million generations) using the GTR+I+G model, which was estimated to be the best model by MrModeltest (2.3; AIC criterion) [44]. All sequence data were partitioned by codon positions, and MrBayes analysis performed using one cold and three heated chains, trees sampled every 1000 generations, and combined into a 50% majority rule consensus tree. The signature of selection on selected branches was tested using PAML 4.4 [45], as previously described [24]. The significance of different amounts of floral odor and gene expression among species was assessed by ANOVA after normality testing of the data distribution by the Shapiro test [46]. Differences in allele distribution among different species were assessed by χ²-testing. To estimate the pattern of divergence for each SAD homolog, we first genotyped each Ophrys individual by allele-specific RT-PCR; then we randomly sampled two sequences from the allele groups based on species and population information. This procedure was repeated 100 times for m silico resampling. FST was calculated using Arlequin (v.3.5.1.3) [47]. The association between floral scent and gene expression in natural populations was assessed using a generalized linear model (GLM) and a linear mixed-effect model (LME) with population as random factor. These models were simplified by stepwise removal of factors using the stepAIC method [48]. For the tissue/stage-specific dataset, the relative amount of each floral scent compound was used (arcsine square-root transformed) as described by Schütler et al. [24], since the size of floral labella varies in different developmental stages. For the population data set, the absolute amount of each floral scent compound was used. The significance of the presence of nonfunctional alleles in different allele groups was tested using Fisher’s exact test. All statistical analyses were performed in R 2.11.0 [49].

Supporting Information

Figure S1 Gene expression and floral odor in different plant tissues. (A, B and C), relative amount (as proportion of hydrocarbons) of different alkenes in floral labella (4), sepals & petals (Bf, and leaf (C) tissue of O. sphageodes or O. exaltata. (D, E and F), normalized gene expression of the five SAD homologs in floral labella (SAD6 was not expressed in these individuals) (D), sepals & petals (E), and leaf (F) tissue of O. sphageodes and O. exaltata. Error bars indicate the standard error. Asterisks indicate significant differences between species (p<0.05, one-way ANOVA). (TIF)

Figure S2 Dendrograms of SAD1, SAD2, SAD3, SAD4, and SAD5/6 produced using the UPGMA method in MEGA (v. 4.0). Symbol color refers to the species from which sequences were obtained, while symbol shape indicates the source population. Blue, O. sphageodes; red, O. exaltata; green, O. garganica; pink, F1 hybrids of O. sphageodes and O. exaltata; black, consensus sequences from at least two species. Numbers on branches are bootstrap values. An asterisk (*) indicates stop codon or frame-shift mutations in the sequence. Sequences included for PAML analysis shown in Figure 1 are marked with “#”. (TIF)
Figure S3  Allelic gene expression of six SAD homologs in natural populations of the study species. The height of each bar indicates mean normalized expression of each allele, and error bars indicate standard error. Letters on each bar indicate statistical significance comparing among species within each allele group (p<0.05, one-way ANOVA). (TIF)

Figure S4  Statistical summary of associations among gene expression of SAD homologs and each alkene. GLM and LME indicate different statistical methods, Generalized Linear Model and Linear Mixed-Effects model, respectively. (A) Relative expression of SAD1–SAD3 versus relative amount of each alkene among different floral tissues/stages. Relative amount of alkenes was used after $f(x) = x$ transformation. (B) Allelic expression of SAD1–SAD6 versus absolute amount of each alkene among species/populations. Absolute amount of alkenes (in ng) was used after $f(x) = ln (x+0.01)$ transformation in the population dataset. (TIF)

Figure S5  Phylogenetic tree of SAD1, SAD2, SAD3, SAD4, and SAD5/6 using Bayesian inference in MrBayes (v3.2.1). Sequence data were partitioned by codon positions. The analysis used one cold and three heated chains, trees were sampled every 1000 generations, and combined into a 50% majority rule consensus tree, discarding trees from the ‘burn-in’ period. Symbol color refers to the species from which sequences were obtained, while symbol shape indicates the source population. Blue, O. sphaegodes; red, O. exaltata; green, O. garganica; pink, F1 hybrids of O. sphaegodes and O. exaltata; black, consensus sequences from at least two species. Numbers at branches are posterior probability values. An asterisk (*) indicates stop codon or frame-shift mutations in the sequence. Sequences included for PAML analysis shown in Figure 1 are marked with ‘#’. (TIF)

Table S1  Number of sequences in a given allele group in which frame-shift or stop codon mutations were found. All sequences were classified into two groups: putatively functional and nonfunctional, based on whether frame-shifts/stop codons were observed. Asterisks indicate the proportion of nonfunctional sequences where significantly higher than expected, using Fisher’s exact test [p cut-off value 0.05]. (XLS)

Table S2  Allelic gene expression in different species. The number in each cell refers to the number of individuals that showed expression of a certain allele group. N refers to the total number of individuals assessed. E, G and S refer to O. exaltata, O. garganica and O. sphaegodes, respectively. E×S refers to F1 hybrids among O. sphaegodes and O. exaltata (identified by Xu et al. [17], according to molecular maker data; direction of the cross unknown). (XLS)

Table S3  Plant samples collected in this study. Numbers in cells refer to the number of individuals. Hybrid refers to F1 hybrids between O. sphaegodes and O. exaltata as assigned based on AFLP data in previous study [17]. Location information for each population is given in the main text. (XLS)

Table S4  Oligonucleotides used in this study. For primers compatible with Gateway (Invitrogen) cloning, the full attB sites were introduced as described in Invitrogen’s manuals. TA refers to the annealing temperature used for PCR reactions. (XLS)

Table S5  Accession numbers of all sequences obtained in this study. (XLS)

Table S6  Alkene compositions of O. exaltata, O. sphaegodes, and alkene mixtures used for pollinator tests. For O. sphaegodes and O. exaltata, mean ± SE absolute amounts in individual compounds per 100 μl odor extract (in ng) are shown. For alkene mixtures that were used for odor manipulation per 100 μl odor extract, absolute amounts (in ng) of the compounds are shown. (XLS)

Table S7  Relative amounts (%) of 7-alkenes and 9/12-alkenes in control and manipulated floral odor bouquets used for pollinator behavior tests. Mean ± standard error for each compounds are listed. (XLS)

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Author Contributions

Conceived and designed the experiments: SX PMS UG FPS. Performed the experiments: SX. Analyzed the data: SX PMS. Contributed reagents/materials/analysis tools: PMS UG FPS. Wrote the paper: SX PMS.
Ophrys

13. Kay KM, Sargent RD (2009) The role of animal pollination in plant speciation: integrating ecology, geography, and genetics. Annual Review of Ecology Evolution and Systematics 40: 637–656.

14. Kullenberg B (1961) Studies in Ophrys pollination. Zoologiska Bidrag Uppsala 34: 1–346.

15. Paulus HF, Gack C (1990) Pollinators as prepollinating isolation factors - evolution and speciation in Ophrys (Orchidaceae). Israel Journal of Botany 39: 43–79.

16. Schiestl FP, Ayasse M (2002) Do changes in floral odor cause speciation in sexually deceptive orchids? Plant Systematics and Evolution 234: 111–119.

17. Mant J, Braund C, Vereecken NJ, Schulz CM, Francke W, et al. (2005) Cuticular hydrocarbons as sex pheromone of the bee Colletes cunicularius and the key to its mimicry by the sexually deceptive orchid, Ophrys exaltata. Journal of Chemical Ecology 31: 1763–1787.

18. Stökl J, Schiestl FP, Stuessy TF, Paulus HF, Fraberger R, et al. (2009) Speciation in sexually deceptive orchids: pollinator-driven selection maintains discrete odour phenotypes in hybridizing species. Biological Journal of the Linnean Society 98: 439–451.

19. Stökl J, Twiele R, Erdmann DH, Francke W, Ayasse M (2007) Comparison of the flower scent of the sexually deceptive orchid Ophrys circularis and the female sex pheromone of its pollinator Andrena moria. Chemoecology 17: 231–233.

20. Stökl J, Paulus H, Dafini A, Schulz C, Francke W, et al. (2005) Pollinator attracting odour signals in sexually deceptive orchids of the Ophrys fuciflora group. Plant Systematics and Evolution 254: 105–120.

21. Stökl J, Twele R, Erdmann DH, Francke W, Ayasse M (2010) Biological origins of normal-chain hydrocarbons: a pathway model based on cuticular wax analyses of maize silks. Plant Journal 64: 618–632.

22. Schlöter PM, Xu S, Gagliardini V, Whittle E, Shanklin J, et al. (2011) Stearoyl-acyl carrier protein desaturases are associated with floral isolation in sexually deceptive orchids. Proceedings of the National Academy of Sciences of the United States of America 108: 5696–5701.

23. Devey DS, Bateman RM, Fay MF, Hawkins JA (2006) Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid genus Ophrys. Annals of Botany 101: 385–402.

24. Delorme P (2006) Orchids of Europe, North Africa, and the Middle East. Penent L, Collin C, translator. London: A&C Black. 640 p.

25. Thompson JD, Higgins DG, Gibson TJ (1994) Clustal-W - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

26. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

27. Yang ZH (2007) PAML 4: Phylogenetic analysis by maximum likelihood. Molecular Biology and Evolution 24: 1586–1591.

28. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

29. Post-Beittenmiller D (1996) Biochemistry and molecular biology of wax production in plants. Annual Review of Plant Physiology and Plant Molecular Biology 47: 405–430.

30.background of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

31. Thompson JD, Higgins DG, Gibson TJ (1994) Clustal-W - improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

32. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs for population genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

33. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

34. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

35. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

36. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

37. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

38. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

39. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

40. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

41. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

42. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

43. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

44. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

45. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

46. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

47. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

48. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

49. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.

50. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599.