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## The effect of different chiral morphs on visitation rates and fruit set in the orchid *Spiranthes spiralis*

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**Background:** The arrangement of flowers on inflorescences is important for determining the movement of pollinators within the inflorescence and, consequently, the overall mating success and fruit set of a plant.

**Aims:** *Spiranthes spiralis* is an orchid that has a spiralled inflorescence. The species has two chiral forms that show opposite coiling directions (clockwise and anti-clockwise). We tested if this arrangement of inflorescence influences pollinator attraction and behaviour.

**Methods:** We surveyed two natural populations, analysed the reproductive compatibility of the two morphs and estimated pollination success in natural and experimental populations.

**Results:** We found that the two morphs were not isolated by pre- or post-mating barriers, occurred with a similar proportion in natural populations and showed similar levels of pollination success both in natural and artificial populations. However, we found a different pattern of pollination success along the inflorescences. In the two morphs, lower flowers experienced a higher pollination rate and this rate decreased along the inflorescence faster in anti-clockwise than in clockwise individuals.

**Conclusions:** This finding suggests that pollinators visit the flowers sequentially from the lower part of the inflorescences and leave the anti-clockwise individuals more rapidly than the clockwise ones. However, this pollinator behaviour is not detrimental for the pollination success of either of the two morphs.

**Keywords:** bee pollination; chirality; pollinator behaviour; pollination success; reproductive barriers

### Introduction

In animal-pollinated plants, the flower has traditionally been the evolutionary unit of pollination biology investigations aiming at understanding plant–insect relationships (Harder et al. 2004; Prusinkiewicz et al. 2007). However, many studies have highlighted how also the plant inflorescence is subject to strong pollinator-mediated selection as its shape strongly determines pollinator behaviour and duration of visits (Ohashi and Yahara 2001; Sletvold and Ågren 2010; Iwata et al. 2012). Flower size and number, density of flowers on an inflorescence, three-dimensional arrangement of flowers on the inflorescence have all been found to be important for determining the attractiveness of individual plants, the movement of pollinators within the inflorescence and the overall mating success of the plant (Fishbein and Venable 1996; Harder et al. 2004; Ishii et al. 2008). Indeed, Jordan and Harder (2006) and Ishii et al. (2008) have demonstrated that inflorescence architecture (i.e. the arrangement of flowers on an inflorescence) affects the numbers of flowers probed by bumble bees and that bees discriminate between inflorescences with different energetic foraging costs based on their architecture.

Most vascular plant species are characterised by a unique genetically predetermined arrangement of flowers along the inflorescence and of floral organs inside flowers (Endress 1999; Costa et al. 2005). However, chirality, i.e.

where a symmetric organism has two mirrored morphs, has been seldom reported. The most common cases of chirality deal with the arrangement of leaves on the plant stem (phyllotaxis) and the coiling direction of climbing plants (Fredeen et al. 2002). More rarely, chirality has been reported also for flower morphs and inflorescences (Davis 1964; Davis and Ramanujacharyulu 1971; Kihara 1972; Jesson and Barrett 2000; Diller and Fenster 2014, 2016). Although the vast majority of plants are fixed for one coiling direction (Edwards et al. 2007), the cause for this directionality is known in only a few cases (Vermeij 1975; Jesson and Barrett 2000, 2002). In particular, Hashimoto (2002) and Ishida et al. (2007) discovered that SPIRAL1-like genes are involved in twisted growth of various plant species.

The orchid *Spiranthes spiralis* (L.) Chevall. has flowers which are bilaterally symmetrical, but produces chiral inflorescences because the flowers coil around a central axis in a clockwise or anti-clockwise fashion, or, very rarely, uncoiled (Jacquemyn and Hutchings 2010; Iwata et al. 2012). Often, chirality is a developmental consequence and stochastically determined. For instance, in *Arabidopsis thaliana* (L.) Heynh. phyllotaxy is determined where a new organ meristem is formed relative to the position of existing older organ meristems, on the flank of the central shoot apical meristem (Hashimoto 2002). However, little is known about the development of

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chirality in angiosperms in general, and orchids in particular. In *Spiranthes sinensis* (Pers.) Ames somewhere during the development of the leaves, the stalks of the individual leaves start to turn (Koriba 1914), but it is unknown in which developmental stages this happens exactly. Regardless of the developmental mechanism, inflorescence chirality may have an effect on pollinator behaviour if they show a preference for right- or left-sided plants, a behaviour called handedness (Kells and Goulson 2001). Indeed, brain lateralisation acting as side bias for motor output, perception and/or information processing is being detected in a growing range of animals including bees. Individuals of the bumblebee species *Bombus lapidarius* L., *B. lucorum* L. and *B. pascuorum* Scopoli have a tendency to circle flowers arranged in rings on a vertical raceme by either moving clockwise or anti-clockwise, rarely switching between the two strategies (Kells and Goulson 2001). Anfora et al. (2011) described lateralisation of learning in the brain of *Bombus terrestris* L. in which learned responses to olfactory cues are biased towards information from the right antenna. A similar result was found in the honeybee *Apis mellifera* L. by Letzkus et al. (2008), Anfora et al. (2010) and Frasnelli et al. (2010). The drivers for the evolution of lateralisation in associative learning of social bees are not yet known. It is hypothesised that shared directionality in a population might arise as an evolutionary strategy driven by living in a social group, whereby an individual within a group benefits from acting according to the behaviour of the majority of the group individuals (Anfora et al. 2011).

*Apis mellifera*, *B. lapidarius*, *B. pascuorum* and *B. terrestris* have all been recorded as main pollinators of *S. spiralis* (Claessens and Kleynen 2011; Duffy and Stout 2011). When foraging on *S. spiralis* inflorescence, bees and bumblebees land on a flower and climb upwards from flower to flower, along the axis of the inflorescence (Van der Cingel 1995; Claessens and Kleynen 2011). These bees, when climbing up, can individually prefer to either rotate clockwise or anti-clockwise along the inflorescence. However, it is difficult to predict whether the preference of individual pollinators can affect the pollination success and fecundity of individual plant chiral morphs.

If pollinators of *S. spiralis* have a preference for one chiral morph over the other, then this chiral morph will be selectively preferred and experience higher fecundity. This is because more flowers will be pollinated, and result in an overall male and female reproductive success of the preferred morph. In the most extreme scenario, the two chiral morphs can be partially or totally reproductively isolated if pollinators selectively discriminate between the two morphs and move pollen only within their preferred morphs. Further, if one of the two chiral morphs has an advantage over the other morph, then the amount of clockwise or anti-clockwise individuals in the field is not expected to be equal.

Here, we test whether the two chiral morphs of *S. spiralis* have a different frequency in natural populations and whether they show some degree of reproductive

isolation either through pre-pollination or post-pollination barriers. Then we estimate pollination success in natural and artificially manipulated populations and the frequency of pollination events along the inflorescences in order to understand if chirality has an effect on pollinator behaviour and on the overall plant reproductive success.

## Materials and methods

### Species studied and study sites

*Spiranthes spiralis* is a perennial orchid species widely distributed ranging from Britain and Ireland, through mainland Europe, to southern Europe and the Mediterranean (Jacquemyn and Hutchings 2010). This species produces nectar and pollinators, mainly bumblebees and bees, necessary for successful pollination and fruit set (Van der Cingel 1995; Claessens and Kleynen 2011, 2016; Duffy and Stout 2011). Flowers are twisted spirally in the inflorescence, either clockwise (hereafter referred to as C) or anti-clockwise (hereafter referred to as A), around the axis, or more rarely all turned to one side. There is no correlation between the coiling direction of basal leaves and that of inflorescences (G. Scopece, personal observation).

In this study, we surveyed a natural population growing in the surrounding of the University campus of Monte Sant'Angelo (Naples, Italy; hereafter referred to as MSA) and a natural population growing in a pine forest on Mount Vesuvius (Naples, Italy; hereafter referred to as VES).

### Assessment of spiral direction

In each sampling year and population, we categorised individuals in C and A based on the direction of the spiral of flowers (Figure 1). To prevent confusion about the terms clockwise and anti-clockwise, the direction of coiling of flowers around the axis of the inflorescence was defined as clockwise (C) if the flowers are placed from west to east when the position of the flowers is followed



Figure 1. *Spiranthes spiralis* inflorescences with opposite coiling direction: (a) anti-clockwise, (b) clockwise.

from the bottom of the inflorescence towards the top. Anticlockwise (A) was defined as the opposite of this pattern. Plants are considered uncoiled when all flowers are placed on the same side of the inflorescence. We assessed spiral direction in three years in both sampling populations (MSA 2009, 2015, 2016; VES 2009, 2013, 2015).

#### *Pollination success*

In three years in both of the natural populations (2011, 2015 and 2016 in MSA, 2009, 2013 and 2015 in VES), for each sampled individual we counted the number of flowers and, at the end of the flowering season, the number of fruits produced as estimate of pollination success. We used these data to calculate the absolute pollination success as the number of fruits produced and the relative pollination success as the ratio among fruits and flowers produced by individuals of each morph. In two years in MSA (2015 and 2016) and in one year in VES (2013), for each investigated individual, we recorded the position of the inflorescence the flowers developed into a fruit. This enabled us to calculate the probability of setting fruits for the different positions of flowers along the inflorescence as the number of fruits produced at position *n*/total number of flowers at position *n*.

During autumn 2015, we also established six artificial plots, close to the MSA population, in order to compare pollination performances between A, C and mixed AC experimental populations. Plots were comprised of six individuals. In mixed plots, we selected A and C individuals in order to avoid significant differences in number of flowers. We established four intra-morph plots (two including six individuals C and two including six individuals A) and two inter-morph plots (each including three individuals C and three individuals A).

#### *Reproductive barrier estimates*

To estimate premating isolation due to phenological isolation, flowering data were collected in VES in 2013 and in MSA in 2013 and 2015. Individuals were marked and visited periodically to record the number of flowers open on each sampled individual.

In 2013, to estimate premating isolation due to pollinator isolation, we established two experimental plots using fluorescent dyes as pollen analogues following

methods described by Waser and Price (1982) in order to understand whether different morphs exchange pollen. Each plot was made of six plants, three A and three C. Inflorescence of a single plant per morph in each plot was stained with a fluorescent dye (Radiant Color®, Magruder Color, Richmond, CA, USA) using a brush and, after one day unstained plants were inspected under UV lamp in order to see whether they received fluorescent dye grains.

Finally, for estimating post-mating (pre- and postzygotic) isolation due to crossing compatibility between chiral morphs, we performed intra- and inter-morph hand pollinations following the methods of Scopece et al. (2007). After crossing, individuals were bagged until fruits were ripe. A subset of ripe fruits was collected and seeds were observed under an optical microscope with 100× magnification and were assigned to two mutually exclusive categories, viable and unviable seeds, based on the presence or absence of viable embryos.

#### *Data analysis*

In each sampling year and in each sampled population, we calculated the relative proportion of the two chiral morphs (A, C) and we tested significant departures from the expected 50:50 using a two-tailed binomial test. Then in each population and in each year, we compared the number of flowers, the absolute and the relative pollination success between the two chiral morphs using a Mann–Whitney U test. We also conducted this analysis combining the data from all years of the two natural populations (MSA and VES) in a single data set. We used the non-parametric Kruskal–Wallis test with Mann–Whitney U tests for multiple comparison.

We calculated a confidence interval (CI) of  $\pm 5\%$  on the phenological data and on the probability of setting fruits for flower position along the inflorescence and we considered differences to be significant where the CI of data-points were not overlapping. All analyses were performed using SPSS version 21 (SPSS Inc., Chicago, IL).

#### **Results**

In the VES population, A morphs were more abundant, though not significantly, compared with C morphs over the three sampling years (2009, 2013, 2015; see Table 1). In the MSA population, A morphs were

Table 1. Different proportions of *Spiranthes spiralis* individuals with opposite coiling directions (A = anti-clockwise and C = clockwise) in the sampling populations of Vesuvius (Province of Naples, Southern Italy, VES) and Monte Sant’Angelo (Naples, Southern Italy, MSA).

Population	Year	Number of C	%C	Number of A	%A	Total number of individuals	Two-tailed binomial test
VES	2009	163	45.79	193	54.21	356	0.124
VES	2013	34	40.00	51	60.00	85	0.082
VES	2015	34	47.89	37	52.11	71	0.813
MSA	2009	138	53.08	122	46.92	260	0.352
MSA	2015	106	43.98	135	56.02	241	0.071
MSA	2016	108	40.30	160	59.70	268	0.002

significantly more abundant in one year (2016) and not significantly different from C morphs in the other years (2009 and 2015; see Table 1). The average amount of non-spiralled plants in the natural populations was minute (~3%); hence, these individuals were excluded from the analyses.

The number of flowers produced did not differ between A and C morphs in the natural populations and across all years, except for the MSA population in 2016 ( $U = 6383.5$ ;  $Z = -3.632$ ;  $P < 0.001$ ; Figure 2). Absolute pollination success was not significantly different between A and C morphs in the natural populations across sampled years, except in the MSA population in 2016 where C morphs produced significantly more fruits than A morphs ( $U = 6179.5$ ;  $Z = -3.960$ ;  $P < 0.001$ ; Figure 2). Relative pollination success was significantly higher in A morphs in the VES population in 2009 ( $U = 102.5$ ;  $Z = -2.233$ ;  $P = 0.024$ ) and in C morphs in the VES population in 2015 ( $U = 411.0$ ;  $Z = 1077.0$ ;  $P = 0.018$ ) but was not significantly different in all the other sampling populations and years (Figure 2).

Overall, combining data from the three years, we found no significant differences in the MSA population in terms of number of flowers produced ( $U = 31,206.5$ ;  $Z = -1.333$ ;  $P = 0.183$ ), absolute pollination success ( $U = 30,261.0$ ;  $Z = -1.884$ ;  $P = 0.060$ ) and relative pollination success ( $U = 30,340.0$ ;  $Z = -1.841$ ;  $P = 0.066$ ) in C morphs compared to A morphs. In the VES population, we found no significant differences in the number of flowers produced ( $U = 3130.0$ ;  $Z = -1.434$ ;  $P = 0.152$ ) and absolute pollination success ( $U = 3376.0$ ;  $Z = -0.665$ ;  $P = 0.506$ ) yet a significant higher relative pollination success ( $U = 2951.0$ ;  $Z = -1.990$ ;  $P = 0.047$ ) in C morphs compared to A individuals.

Experimental plots with manipulated frequency of the two chiral morphs showed no significant differences for absolute pollination success (Kruskal–Wallis test: Chi-square = 2.586;  $df = 2$ ;  $P = 0.274$ ) and relative pollination success (Kruskal–Wallis test: Chi-square = 1.735;  $df = 2$ ;  $P = 0.420$ ) (Figure 3).

We did not find any evidence of premating and post-mating isolation between the two morphs.

Phenology was largely overlapping in the three sampled years (Figure 4) and experimental plots with stained individuals showed intra-morph movements of fluorescent dye. We recorded one movement from A to C and three movements from C to A. We did not record any intra-morph movement.

Intra- and inter-morph crosses led to high levels of fruit formation (Intra-morph crosses:  $n = 27$ ; fruit formation =  $0.93 \pm 0.27$ . Inter-morph crosses:  $n = 24$ ; fruit formation =  $0.85 \pm 0.34$ ) and seed viability (Intra-morph crosses:  $n = 4$ ; seed viability =  $0.79 \pm 0.02$ . Inter-morph crosses:  $n = 3$ ; seed viability =  $0.72 \pm 0.14$ ). Intra- and inter-morph hand pollinations did not differ in fruit set ( $U = 282.5$ ;  $Z = -1.311$ ;  $P = 0.190$ ) or in their seed viability ( $U = 4.0$ ;  $Z = -0.707$ ;  $P = 0.629$ ).

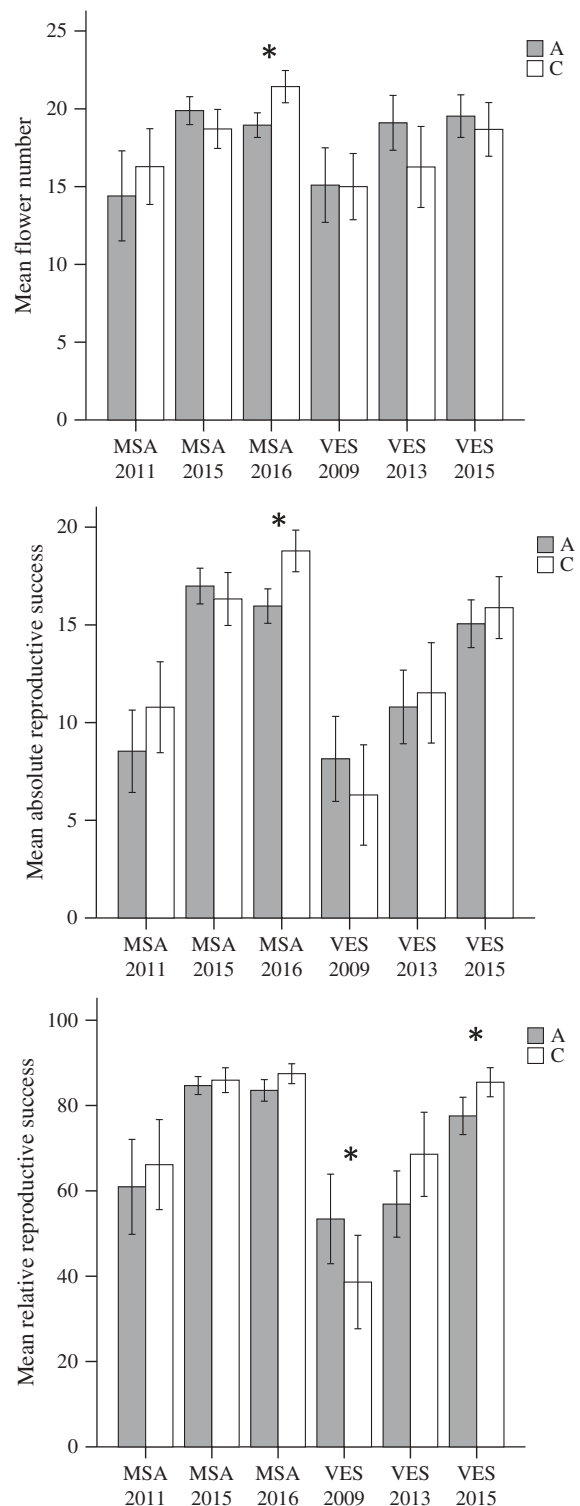


Figure 2. Comparison of mean flower number, mean absolute pollination success and mean relative pollination success of *Spiranthes spiralis* individuals with opposite coiling directions (A = anti-clockwise: grey bars; C = clockwise: white bars) in the populations at Mount Vesuvius (Province of Naples, Southern Italy, VES) in 2009, 2013, 2015 and at Monte Sant'Angelo (Naples, Southern Italy, MSA) in 2011, 2015, 2016. Error bars represent standard error. Asterisks indicate significant differences after Mann–Whitney U test.



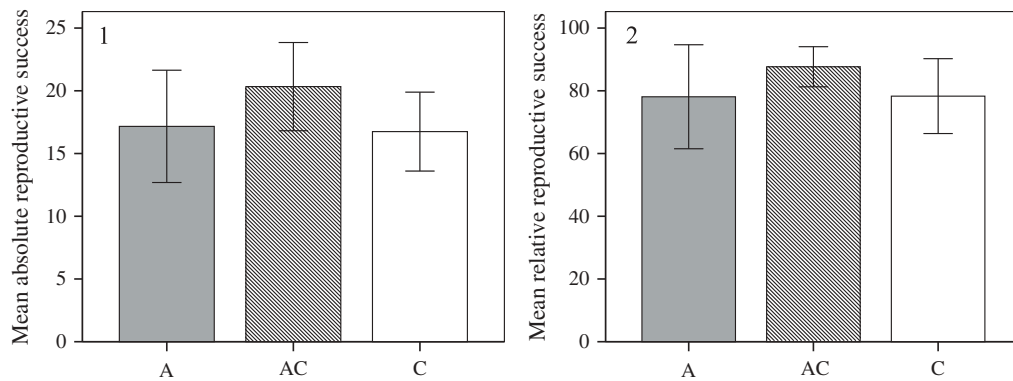


Figure 3. Mean absolute (1) and relative (2) pollination success in experimental plots containing *Spiranthes spiralis* individuals with a single (intra-morph) or both (inter-morph) chiral morphs.

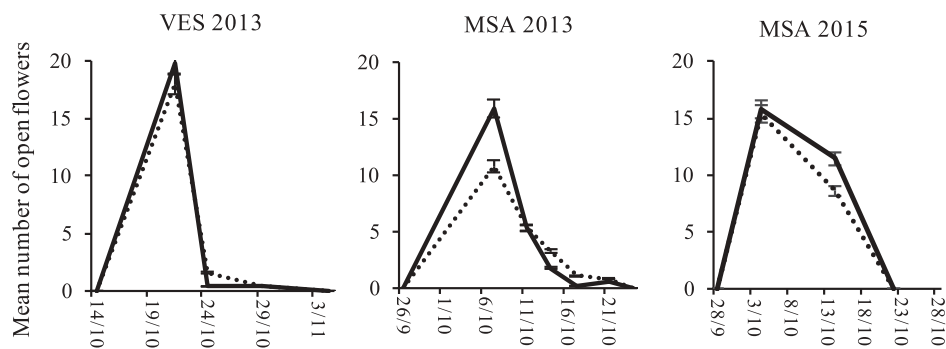


Figure 4. Phenology of *Spiranthes spiralis* individuals with anti-clockwise (A: dotted line) and clockwise (C: solid line) chiral morphs in the sampled populations at Mount Vesuvius (Province of Naples, Southern Italy, VES) and at Monte San'Angelo (Naples, Southern Italy, MSA).

The probability of setting fruit along the inflorescences showed different patterns in A and C morphs. In the first (lowermost) flowers (up to flower 10 from the bottom of the inflorescence), the proportion of flowers pollinated was similar in C and A morphs (Figure 5). However, after this position in the inflorescence, fruit set was higher for C morphs than A morphs (Figure 5). This pattern was found in each year of the study.

## Discussion

In this study, we analysed pollination success (in terms of fruit set) of two chiral morphs of the orchid *Spiranthes spiralis*. Overall, we have found that the two morphs are not reproductively isolated and that they show similar levels of pollination success. However, the two morphs can differ in probability of setting fruit depending on the flower position along the inflorescence. Although we never directly observed pollinators on the two chiral morphs, this finding suggests that different pollinator behaviour can affect fruit set in the two morphs, depending on the preferences of the local pollinator community.

Chiral morphs can be partially or totally reproductively isolated if chirality directly affects inter-morph mating. For instance, in snails, rare individuals with anti-clockwise shell coiling are unable to mate with

individuals with clockwise shell coiling, so directionality is maintained by frequency-dependent sexual selection (Schilthuizen et al. 2007). In our case study, chirality does not represent an isolating barrier as pollinators move pollen (as estimated by pollen-analogue fluorescent dyes) between the two morphs, and hand pollinations confirmed that the two morphs are fully compatible. The two morphs also show an almost complete overlap in flowering time (Figure 4). Thus, overall, they do not show any degree of pre- or post-mating reproductive isolation.

Nevertheless, different chiral morphs could still affect pollinator visits with consequences for plant pollination success and relative frequency of the two morphs in natural populations. The two chiral morphs do not differ both in terms of phenology and flower number so that different inflorescence orientation represents the major visual factor discriminating between the flower display of the two morphs. With few exceptions, we did not find significant difference in absolute and relative pollination success of the two morphs both in natural populations and in artificial plots where the ratio between morphs has been artificially manipulated.

However, our data suggest that chirality affects pollinator foraging behaviour. The frequency of pollinated flowers along the inflorescence confirms the

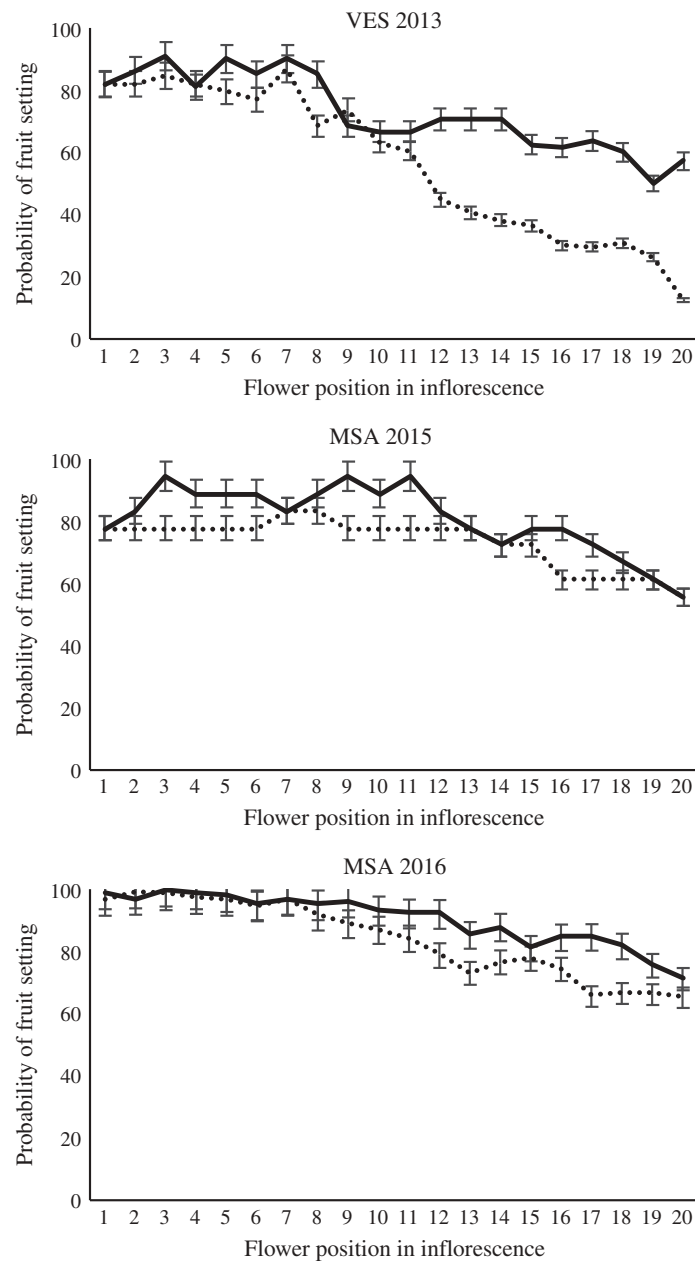


Figure 5. Probability of setting fruits in *Spiranthes spiralis* individuals with anti-clockwise (A: dotted lines) and clockwise (C: solid lines) coiling direction. Error bars represent 5% confidence intervals of the mean. Graphs are cut after 20 flowers.

upward movement of the bees along a vertical inflorescence (Darwin 1862). Indeed, we found that the probability of being fertilised progressively decreases from lower to upper flowers of the inflorescences, with the uppermost flowers often unvisited. This suggests that pollinators preferentially start foraging from the basal flowers but often stop probing at one point along the inflorescence axis. The twisting of the inflorescence probably reduces the number of successive probes within inflorescence and the time length of foraging path. A similar pattern was found in *Spiranthes sinensis* where a negative correlation between the size of

helical angle between two successive flowers and skip behaviour (number of skips of neighbouring flowers per visit) has been found and hypothesised as a potential mechanism for reducing geitonogamy (Iwata et al. 2012). The probability of setting fruits for the different positions of flowers along the inflorescence suggests that frequency of pollinator probing differs between clockwise and anti-clockwise morphs, being shorter in the latter and, in particular, that the probability that a pollinator leaves the inflorescence (thus reducing the number of pollinated flowers) increases after the first 10 flowers (roughly corresponding to a first

inflorescence turn). However, the probability of being pollinated decreases, from the bottom to the top, faster for flowers on the A morphs than on the C morphs. Whether biases in learned responses of main pollinators result in a preference for visiting particular inflorescence morphs as found here for clockwise *S. spiralis* needs further experimental validation but it might offer an evolutionary explanation for the presence of chirality in these plants.

Across three years and in the two populations we often found a corresponding slightly higher pollination success for C morphs (both for absolute and relative pollination success). Overall, this difference in pollinator visitation rates does not seem to be detrimental for absolute pollination success of A morphs. However, when analysing all years together, this difference seems to confer a slight advantage to C morphs (at least in VES population) in terms of relative pollination success, an issue particularly relevant when dealing with perennial plants as these orchids are. This trend was however contradicted in VES population in 2009, where relative pollination success was higher for A than C plants suggesting that other, maybe stochastic, factors may sometime alter the general trend we found and supporting the absence of any evident selective advantage for the C plants. Accordingly, in the investigated natural populations, the two morphs have similar frequency with slight variation around a 50:50 ratio of clockwise and anti-clockwise morphs. This finding is in general agreement with similar results on clockwise and anticlockwise arrangement of the leaves in several wild herbaceous and woody plants (Allard 1951).

In extensive studies on the related species *Spiranthes sinensis*, clockwise and anti-clockwise inflorescences were found to grow in similar proportions and, in single individuals producing two inflorescences, the two chiral morphs showed CC, AC, AA combinations in a ratio 1:2:1, indicating that the direction of spirals was not predetermined but likely it was determined by chance (Koriba 1914). As the frequency of the two different morphs are not apparently selected by pollinators, this may explain their similar occurrence in natural populations of *S. spiralis* with the few reported cases of skewed distribution that are probable due to local demographic/stochastic processes typical of orchids (during seedling establishment and dormancy) rather than to the action of selective agents. Nevertheless, inflorescence chirality is widespread in the genus *Spiranthes* (Dueck et al. 2014). This suggests that, rather than some adaptive advantage, the occurrence of chirality in *S. spiralis* may also be the consequence of a phylogenetic constraint.

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## Disclosure statement

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## Notes on contributors

Giovanni Scopece is a Researcher with a special interest in plant–insect interactions and in their impact on plant evolution.

Barbara Gravendeel research focuses on genomics and museomics for answering biological questions.

Salvatore Cozzolino is a Professor. His main research interests span from plant ecology to plant genetics with a particular focus on evolutionary dynamics of Mediterranean plants.

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