Floral rewards in the tribe Sisyrinchieae (Iridaceae): Oil as an alternative to pollen and nectar?

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ORIGINAL ARTICLE

Floral rewards in the tribe Sisyrinchieae (Iridaceae): oil as an alternative to pollen and nectar?

Adriano Silvério · Sophie Nadot · Tatiana T. Souza-Chies · Olivier Chauveau

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Abstract Iridaceae is one of the few families in which floral oils are produced and collected by pollinators as a resource. Perigonal nectaries and trichomal elaiophores are highly unusual within the tribe Sisyrinchieae. Both structures occur mainly on the staminal column, while they are usually distributed on the tepals in the other tribes of the subfamily Iridoideae. Sisyrinchieae is the largest tribe of Iridaceae present on the American continent, and the diversity observed may be related to the exceptional development of trichomal elaiophores within the genus *Sisyrinchium*, but knowledge concerning the other types of nuptial glandular structures within the tribe is still limited, preventing us from estimating their implication for species diversity. Structural observations and histochemical tests were performed to identify and characterize glandular structures and pollen rewards within the flowers of the genera *Orthrosanthus*, *Sisyrinchium* and *Solenomelus*. Perigonal nectaries were detected only in *Solenomelus segethi*, and trichomal elaiophores were characterized only within *Sisyrinchium*. All species showed large amounts of additional resources available for pollinators in the form of pollenkitt and polysaccharides present in the cytoplasm of the pollen grains. The results are discussed in a phylogenetic context, with regard to pollinators and floral rewards reported for the tribe Sisyrinchieae.

Keywords *Orthrosanthus* · Perigonal nectaries · Pollenkitt · *Sisyrinchium* · *Solenomelus* · Trichomal elaiophores

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A. Silvério (✉) · T. T. Souza-Chies
Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre 91501-970, Brazil
e-mail: adriano.silverio@ufrgs.br

A. Silvério · S. Nadot · O. Chauveau
Laboratoire Ecologie Systématique et Evolution, UMR 8079, Université Paris-Sud, 91405 Orsay, France

Introduction

In flowering plants, resources sought by pollinators are predominantly pollen and nectar, which provide proteins and carbohydrates as a nutritional reward. However, a handful of families includes species that produce floral oils collected by specialized bees, which use these lipids to feed their larvae and build their nests (Michener 2007; Renner and Schaefer 2010). Iridaceae is one of the few families including both nectar-producing and oil-producing species, in addition to species providing only pollen as a resource for pollinators. Within Iridaceae, floral oil glands called elaiophores are exclusively observed among the American tribes of Iridoideae, except *Tritoniopsis parviflora* (Jacq.) G. J. Lewis, a species from South Africa belonging to Crocoideae (Goldblatt and Manning 2008). Oil foraging by specialized oil-collecting bees is a widely exploited pollination strategy in the Neotropics, and hundreds of Iridaceae secrete floral oils in the New World (Rudall et al. 2003). Based on the topology of the latest comprehensive phylogeny of the family (Goldblatt et al. 2008), the most parsimonious explanation for the distribution of oil glands is that they evolved once in the common ancestor of the Trimezieae and Tigridieae tribes, with at least six reversions, and twice within the genus *Sisyrinchium* L. (Sisyrinchieae) with at least one subsequent reversion (Renner and Schaefer 2010; Chauveau et al. 2011).

The tribe Sisyrinchieae is sister to the entirely New World tribes Trimezieae and Tigridieae. It includes four strictly American genera, namely *Olsynium* Raf. (c. 12 spp.), *Sisyrinchium* (c. 140 spp.), *Solenomelus* Miers (2 spp.) and *Tapeinia* Juss. (1 sp.) (Goldblatt et al. 2008; Goldblatt and Manning 2008), and 13 Australasian species within the genera *Orthrosanthus* Sweet (9 spp.) and *Libertia* Spreng. (16 spp.). *Sisyrinchium* with more than 77 % of the species richness of the tribe is the central genus of Sisyrinchieae. Beside pollen, oils are the second major reward produced by numerous *Sisyrinchium* species (Chauveau et al. 2011). Documented uses of floral lipids by oil-collecting bees from the Tapinotaspidini tribe (Apidae) show that females mix oils with pollen during their visit and subsequently use this mixture to feed their larvae (Vogel 1974; Cocucci and Vogel 2001; Truylio et al. 2002). These floral oils are secreted by trichomal elaiophores distributed either on the staminal column or on the adaxial side of tepals (Cocucci and Vogel 2001; Chauveau et al. 2011). Except for two species, nuptial trichomes among the genus are always glandular and secretory, but they do not always produce oils, and the secretions of numerous species from Central and North America have not yet been identified (Chauveau et al. 2011). Elaiophores have never been observed in any other genus of the tribe (Goldblatt and Manning 2008), although nuptial trichomes were identified on the filamental column of *Solenomelus pedunculatus* (Gillies ex Hook.) Hochr. and multicellular scales covered by secretions are present all along the column of *Solenomelus segethi* (Phil.) Kuntze (Chauveau et al. 2011). Within the tribe, sugar rewards produced by perigonal nectaries are known for only two species of *Olsynium* (*O. junceum* (E. Mey ex Presl) Goldblatt and *O. douglasii* (A. Dietr.) E. P. Bicknell), and nectar is probably secreted from cells of the swollen filamental column (Forcone et al. 1997; Cocucci and Vogel 2001; Rudall et al. 2003) and not from nuptial trichomes, which are entirely missing in *Olsynium*, *Libertia*, *Orthrosanthus* and *Tapeinia* (Cocucci and Vogel 2001; Rudall et al. 2003; Chauveau et al. 2011). Since neither elaiophores nor sugar nectaries have been identified within these genera, the only reward offered by their flowers to pollinators is probably the pollen itself (Rudall et al. 2003; Goldblatt and Manning 2006; Chauveau et al. 2011).

This study aims at identifying and characterizing pollination rewards within the tribe Sisyrinchieae. Floral secretory structures, pollen and pollenkitt contents were investigated intensively for the first time within the genera *Sisyrinchium*, *Solenomelus* and *Orthrosanthus*. The results allowed us to make hypotheses about the evolution of pollination rewards within the whole tribe, in a phylogenetic context.

Materials and methods

Plant material

Fresh flowers at anthesis were collected from plants grown in the botanical garden of the Université Paris-Sud (Table 1) and obtained mostly from seeds collected in the wild. A total of 22 *Sisyrinchium* species covering the different clades proposed in the literature (Chauveau et al. 2011) were sampled. In addition, two *Solenomelus* and one *Orthrosanthus* species were included to complete this sampling.

Inclusion and light microscopy

Fresh flowers at anthesis were dissected under a Zeiss Stemi SV6 stereomicroscope (Carl Zeiss AG, Göttingen, Germany) and mounted in glycerine-gelatine (Johansen 1940). For structural observations, the samples were fixed with 1 % glutaraldehyde and 4 % formaldehyde (McDowell and Trump 1976) in 0.1 M sodium phosphate buffer, pH 7.2; then washed in 0.1 M sodium phosphate buffer, pH 7.2, and lastly with distilled water. They were immediately dehydrated in a graded series of ethanol solutions and embedded in hydroxyethylmethacrylate (Gerrits and Smid 1983). Semi-thin sections (3–5 mm) obtained with a Leica RM 2165 microtome (Leica Microsystems, Nussloch, Germany) were stained for general histology using 1 % Toluidine Blue in 1 % sodium borate solution (Mercer 1963). Observations were carried out using a light microscope Reichert-Jung Polyvar MET (Polyvar Met, Reichert-Jung, Germany) equipped with a digital camera QImaging RETIGA 2000DC (QImaging, Surrey, Canada).

Histochemistry

The histochemical tests for lipids were performed on fresh material with Sudan Red 7B (Brundrett et al. 1991) and Nile Red (Greenspan et al. 1985; Diaz et al. 2008). Sudan Red 7B staining, which allows the detection of total lipids content, was used on fresh flowers sections including nuptial trichomes, contents being stained pink-red in the presence of lipids. Anthers were also dissected at anthesis for each species studied and stained with Sudan Red 7B to detect lipids in the pollenkitt. Nile Red staining, which allows the detection of lipids according to their hydrophobicity, was used to detect lipids in nuptial trichomes of fresh flowers at anthesis and to discriminate between polar and non-polar lipids. Nile Red yellow emission specific to non-polar lipids was observed with 450–500 nm excitation and 535 ± 20 emission filters; red emission detecting the presence of polar lipids was observed with 450–500 excitation and 610 long-pass emission filters. All observations

Table 1 List of species examined, with the distribution of nuptial trichomes and other floral secretory structures, sources of plant material and voucher informations

Taxon	Distribution			Source of plant material[a]	Voucher[b]
	Nuptial trichomes		Other nuptial secretory structures		
	Staminal column	Adaxial side of tepals			
Orthrosanthus monadelphus Ravenna	0	0	0	Mexico: Oaxaca	Chauveau and Pautz H09049 (UPS)
Sisyrinchium alatum Hook.	0	0	0	Brazil: Santa Catarina	Eggers and Souza-Chies 232 (ICN)
Sisyrinchium angustifolium Mill.	1	0	0	Cultivated in UPS Bot. Gard.	Chauveau H09002 (ICN)
Sisyrinchium chilense Hook.	1	0	0	Peru: Apumirac	Chauveau et al. H09054 (ICN)
Sisyrinchium claritae Herter	1	0	0	Brazil: Rio Grande do Sul	Eggers and Souza-Chies 267 (ICN)
Sisyrinchium commutatum Klatt	1	0	0	Brazil: Paraná	Eggers and Souza-Chies 265 (ICN)
Sisyrinchium cuspidatum Poepp.	0	1	0	Chile: Region IV	Chauveau and Aubert H09011 (ICN)
Sisyrinchium demissum Greene	1	0	0	USA: Arizona	Chauveau H09009 (ICN)
Sisyrinchium elmeri Greene	0	0	0	USA: California	Chauveau H09042 (ICN)
Sisyrinchium foliosum I. M. Johnst.	1	0	0	Brazil: Rio Grande do Sul	Eggers and Souza-Chies 281 (ICN)
Sisyrinchium idahoense E. P. Bicknell	1	0	0	Cultivated in UPS Bot. Gard.	Chauveau H09055 (ICN)
Sisyrinchium limarinum Ravenna	0	1	0	Chile: Region IV	Chauveau H09056 (ICN)
Sisyrinchium macrocarpum Hieron	1	1	0	Cultivated in UPS Bot. Gard.	Chauveau H09013 (ICN)
Sisyrinchium micranthum Cav.	1	1	0	Brazil: Paraná	Eggers and Souza-Chies 242-A (ICN)
Sisyrinchium palmifolium L.	0	0	0	Cultivated in UPS Bot. Gard.	Chauveau H09020 (ICN)
Sisyrinchium patagonicum Phil. ex Baker	1	0	0	Cultivated in UPS Bot. Gard.	Chauveau H09027 (ICN)
Sisyrinchium platense I. M. Johnst.	1	0	0	Brazil: Rio Grande do Sul	Eggers and Souza-Chies 187 (ICN)
Sisyrinchium setaceum Klatt	1	0	0	Brazil: Rio Grande do Sul	Eggers and Souza-Chies 214 (ICN)
Sisyrinchium striatum Sm.	0	1	0	Chile: Región Metropolitana	Chauveau H09018 (ICN)
Sisyrinchium tenuifolium Humb. & Bonpl. ex Willd.	1	0	0	Mexico: Veracruz	Chauveau and Pautz H09025 (ICN)
Sisyrinchium aff. *tinctorium* Kunth.	0	0	0	Cultivated in UPS Bot. Gard.	Chauveau and Eggers H09021 (ICN)
Sisyrinchium cf. *tofoense* Ravenna	0	1	0	Chile: Region IV	Chauveau H09057 (ICN)
Sisyrinchium sp. *nova* 05 (aff. *vaginatum*)	0	0	0	Brazil: Paraná	Eggers and Souza-Chies 383 (ICN)
Solenomelus segethi Kuntze	0	0	1	Argentina: Neuquen	Chauveau and Aubert H09001 (ICN)
Solenomelus pedunculatus (Gillies ex Hook.) Hochr.	1	0	0	Chile: Region V	Chauveau H09044 (ICN)

Character states are absent (0) or present (1)

[a] The geographical origin of each specimen is reported. Subsequently, they were cultivated in the greenhouse at Université Paris-Sud 11

[b] Voucher specimens are deposited in the following herbarium: Universidade Federal do Rio Grande do Sul (ICN)

were made using a Nikon AZ100 macroscope (Nikon France, Champigny-sur-Marne, France) equipped with digital Nikon DS-ri1. Achieving and compressing images with different focal planes was performed using the software Nikon NIS-Element D, version 3.0.

Semi-thin sections obtained with the microtome were submitted to the following histochemical tests to detect other classes of chemical compounds produced by floral glands or accumulated in pollen grains: Lugol solution was used to detect starch (Johansen 1940), periodic acid-Schiff (PAS reaction) for total polysaccharides (O'Brien and McCully 1981) and Ruthenium Red for mucilage and pectins (Johansen 1940); all images were captured using the light microscope. For all histochemical tests, appropriate controls were run simultaneously.

Results

Five species of *Sisyrinchium* (*S. alatum*, *S. elmeri*, *S. palmifolium*, *S.* aff. *tinctorium* and *S.* sp. *nova* 05, with affinities to *S. vaginatum*), as well as *Orthrosanthus monadelphus* and *Solenomelus segethi*, had flowers completely devoid of nuptial trichomes. The surface of the adaxial face of the tepals and of the staminal column was smooth (Fig. 1a), except for the staminal column of *S. segethi* (Fig. 1b). Within *Solenomelus*, the filaments were entirely fused, forming an elongated and cylindrical staminal column, which exhibited nuptial glandular structures significantly different for each species included in this small genus. The surface of the staminal column of *S. segethi* presented a regulate aspect decreasing from the lower half to the top (Fig. 1b). The dermal layer was distinctively covered by secretions in the lower half and composed of multicellular scales. The staminal column of *S. pedunculatus* was densely covered from the base to the top by numerous unicellular glandular capitate trichomes formed by a long and thin stalk distinctively overtopped by an enlarged part (Fig. 1c) devoid of any blister of secretion. Among the *Sisyrinchium* species bearing nuptial glandular trichomes, the distribution and anatomical features of trichomes were already described for most of the species included in this study (Chauveau et al. 2011), but two species (*S. limarinum* and *S.* cf. *tofoense*) and several new distinctive features concerning *S. macrocarpum* (Fig. 1f) were observed for the first time. In the two strictly Chilean species, *S. limarinum* and *S.* cf. *tofoense,* the staminal column was smooth and cylindrical, and nuptial unicellular and glandular trichomes overtopped by a blister of secretion were observed at the base of the adaxial side of tepals. A similar distribution pattern was observed in *S. cuspidatum* and *S. striatum* (Fig. 1d), in which the shape of the perianth was hypocrateriform, contrary to the flat perianth of the two former species. In *S. macrocarpum*, few unicellular glandular trichomes topped with a blister of secretion were detected on the inner side of the staminal column as well as on the adaxial side of the inner tepal, close to the base of the staminal column. The flowers of this South Andean species exhibited an unusual distribution pattern of trichomes, which were densely distributed all along the staminal column and on the free parts of the filaments (Fig. 1f). Various distribution patterns of trichomal elaiophores on the staminal column are illustrated in Fig. 1, showing variation in the size of the elaiophores and in the shape of the staminal column among *Sisyrinchium* species.

The floral chemical compounds identified in the nuptial trichomes and other putative floral glandular structures observed for each sample are reported in Table 2.

Nuptial glandular structures in *Sisyrinchium*

The structural observations and histochemical tests showed that nuptial glandular structures were always epidermal unicellular trichomes when they were present in flowers of *Sisyrinchium* species. Except for the North American species *S. angustifolium* (Fig. 1e), *S. demissum* and *S. idahoense*, as well as the Central American species *S. tenuifolium*, these trichomes were oil-secreting and possessed a dense, intensively staining and highly granular cytoplasm (Fig. 2b, d). Numerous vesicles lacking starch grains but often containing lipid droplets occurred within the cytoplasm, and the nucleus was not enlarged in most cases. Although a blister of secretion was always present in a subcuticular space at the tip of the trichomes observed, variations in size were observed and blisters of oil-producing trichomes (Fig. 2a–f) were always bigger than the blisters of trichomes that did not secrete oil (Fig. 2g, h), indicating that the amount of secretion accumulated between the cell wall and the cuticle was higher in the case of oil-producing trichomes. Oil accumulation within the subcuticular space of oil-producing trichomes was detected with Sudan Red 7B and Nile Red staining, the latter indicating that both polar and non-polar lipids were highly accumulated (Fig. 3a–d). The aspect of the cuticle surface and its thickness were variable among species. Among species with trichomal elaiophores, the trichome cuticle of *S. claritae* (Fig. 2a, f), *S. commutatum* (Fig. 1g), *S. foliosum* (Fig. 1h) and *S. setaceum* (Fig. 2c) was striate and thick, while for *S. chilense* (Fig. 1i), *S. micranthum* (Fig. 2d), *S. patagonicum*, *S. platense* (Fig. 2e) and *S. tenuifolium* (Fig. 2g), a Central American species bearing non-oil-secreting trichomes, striations were not observed, even though the thickness of the cuticle was comparable to the previous species. Thin cuticles without striations on the surface were observed for the non-oil-secreting trichomes

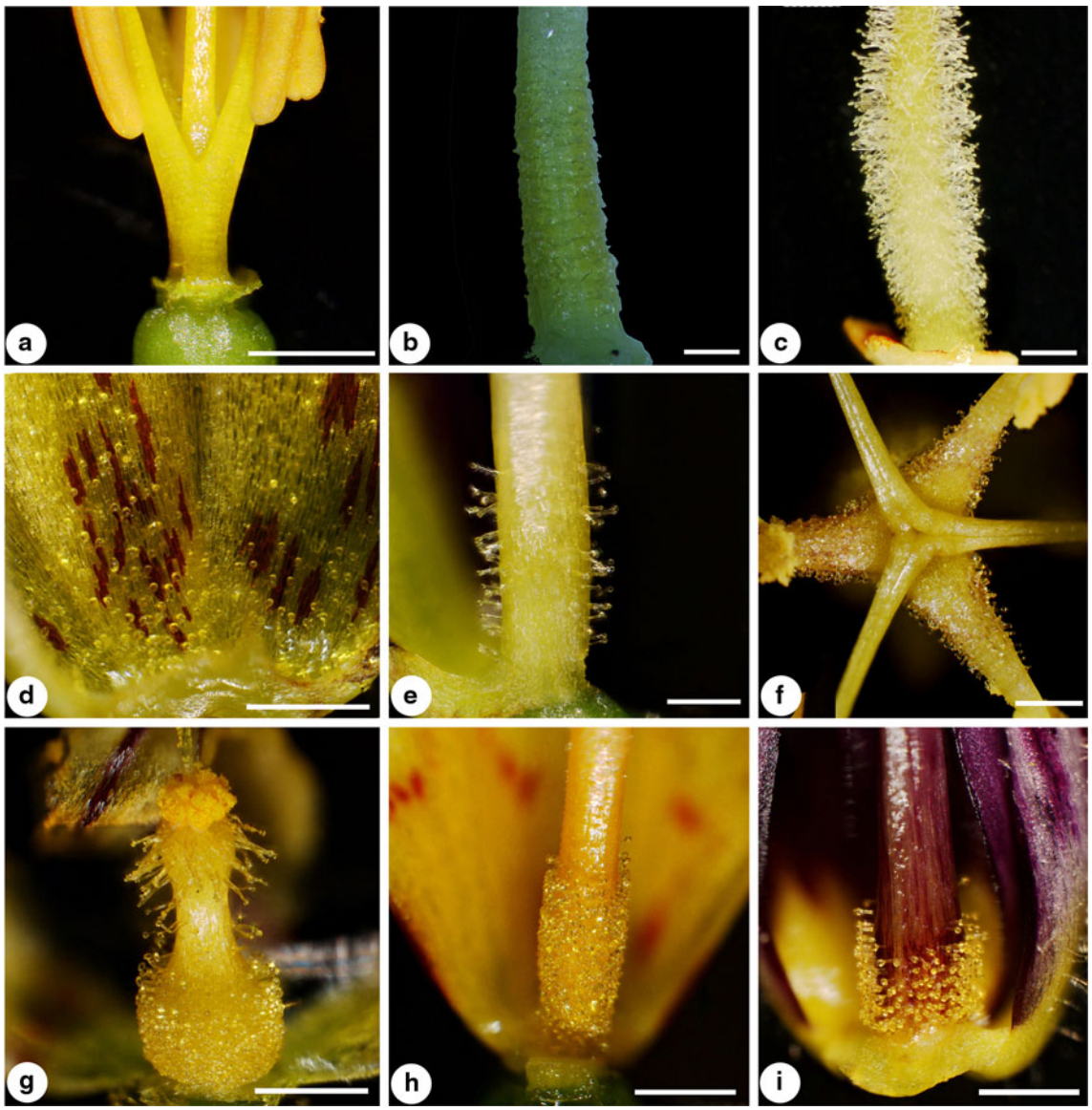


Fig. 1 Staminal column and distribution of trichomes among *Sisyrinchium* and *Solenomelus* flowers. **a** Staminal column devoid of trichomes: *Sisyrinchium elmeri*. **b** Staminal column of *Solenomelus segethi* covered with multicellular secretory scales. **c** Staminal column of *Solenomelus pedunculatus* entirely covered with unicellular non-secreting trichomes. **d** Close-up of a tepal of *S. striatum* showing the distribution of oil-glandular trichomes at the base of the adaxial surface. **e** Lower half part of the staminal column of *S. angustifolium* showing nuptial non-oil-secreting trichomes. **f** Top view of the andro-gynoecium of *S. macrocarpum*: oil-producing glandular trichomes are located all along the fused and free parts of the filaments. **g** Column of *S. commutatum* showing densely packed elaiophores on the lower half. **h** Cylindrical column of *S. foliosum* covered with densely packed elaiophores on the lower third part. **i** Column of *S. chilense* with elaiophores located on the lower quarter part. *Scale bars*: **a**, **d**, **g**, **h** and **i** = 1,000 μm; **b**, **c**, **e** and **f** = 500 μm

of the North American species (*S. angustifolium, S. demissum* and *S. idahoense*) and for the oil-producing trichomes of the South Andean species (*S. cuspidatum, S. limarinum, S. macrocarpum*, *S. striatum* and *S.* cf. *tofoense*).

The structural observations did not indicate the presence of secretory cells within the staminal column of the species sampled. Furthermore, no polysaccharides were detected within the staminal column for the species with or without nuptial trichomes, indicating that none of the species of *Sisyrinchium* sampled produced nectar.

Starch was never detected within nuptial glandular trichomes. Among species devoid of nuptial trichomes (Fig. 4a), as well as those with trichomal elaiophores (Fig. 4d), starch was only observed in the cells of the staminal column of *S. setaceum*, where starch grains were detected within the cytoplasm in the epidermal layer and the inner tissues (Fig. 4b). Numerous starch grains were

Table 2 Chemical compounds identified by histochemical tests in nuptial trichomes and other putative floral secretory structures

Taxon	Oil		Starch	Total polysaccharides	Acidic polysaccharides
	Sudan Red 7B	Nile Red	Lugol	Schiff-PAS	Ruthenium Red
Orthrosanthus monadelphus	− (0)	− (0)	− (0)	− (0)	− (0)
Sisyrinchium alatum	− (0)	− (0)	− (0)	− (0)	− (0)
S. angustifolium	−	−	−	−	−
S. chilense	++	++	−	−	−
S. claritae	++	++	−	−	−
S. commutatum	++	++	−	−	−
S. cuspidatum	++	++	−	−	−
S. demissum	−	−	−	−	−
S. elmeri	− (0)	− (0)	− (0)	− (0)	− (0)
S. foliosum	++	++	−	−	−
S. idahoense	−	−	−	−	−
S. limarinum	++	++	−	−	−
S. macrocarpum	++	++	−	−	−
S. micranthum	++	++	−	−	−
S. palmifolium	− (0)	− (0)	− (0)	− (0)	− (0)
S. patagonicum	++	++	−	−	−
S. platense	++	++	−	−	−
S. setaceum	+	+	−	−	−
S. striatum	++	++	−	−	−
S. tenuifolium	−	−	−	−	−
S. aff. *tinctorium*	− (0)	− (0)	− (0)	− (0)	− (0)
S. cf. *tofoense*	++	++	−	−	−
S. sp. *nova* 05 (aff. *vaginatum*)	− (0)	− (0)	− (0)	− (0)	− (0)
Solenomelus segethi	− (0)	− (0)	− (0)	++ (0)	++ (0)
Solenomelus pedunculatus	−	−	−	−	−

Species devoid of nuptial trichomes are indicated (0), and histochemical tests were performed for these samples on the epidermal cells of the staminal column. (−) is corresponding to the absence of a significant amount of each chemical compound tested. The detection of a significant amount of each chemical compound is reported with (+) and (++), (+) indicating a lower amount of the corresponding compound than (++)

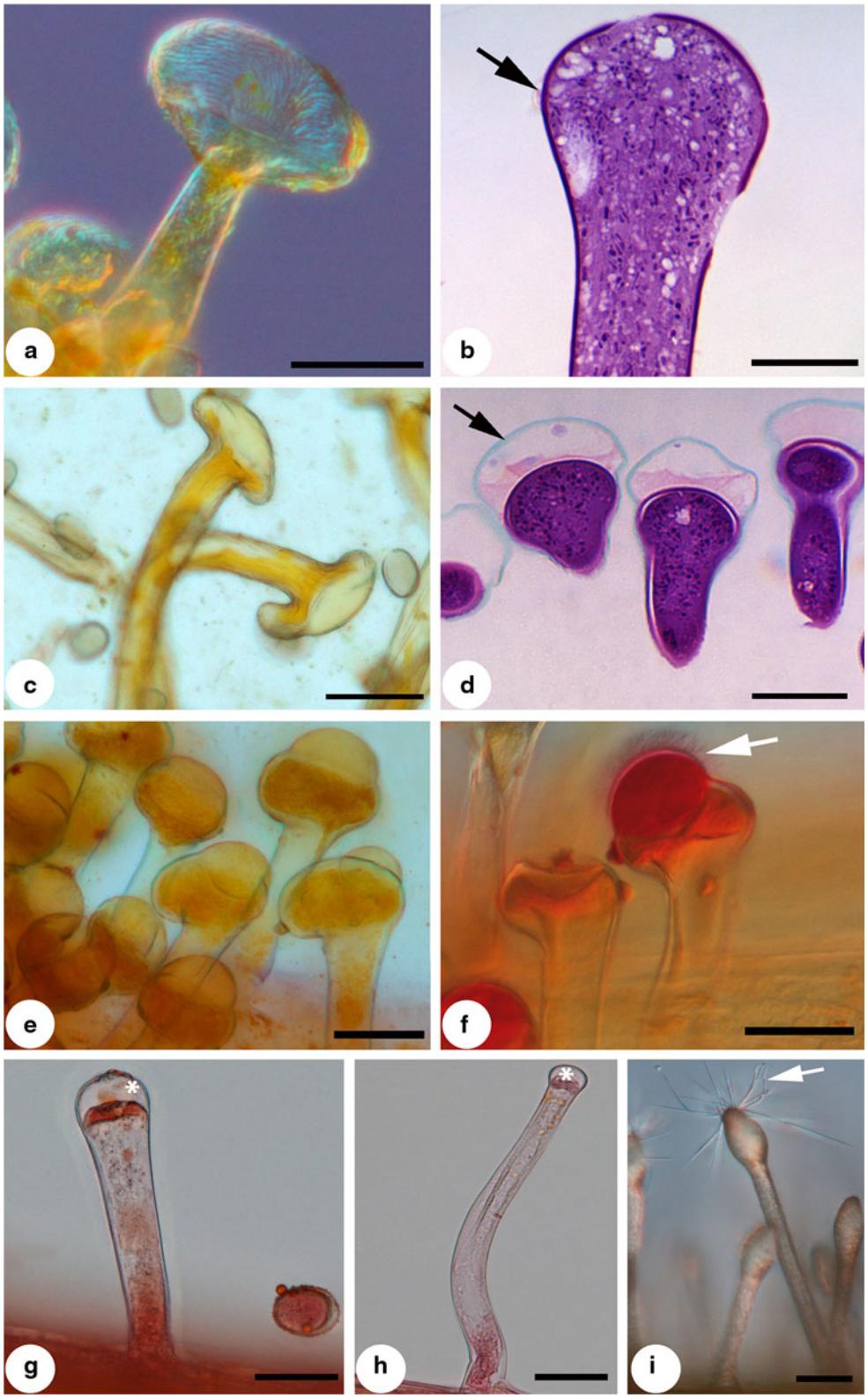
observed within the cytoplasm of cells mainly distributed around the vascularized tissues (Fig. 4c) of the staminal column in North and Central American species bearing non-oil-producing nuptial trichomes.

Nuptial glandular structures in *Orthrosanthus* and *Solenomelus*

Structural observations and results of each histochemical test were negative for the tissues of the staminal column of *O. monadelphus*, indicating the absence of nectaries or elaiophores.

Neither secretory cells nor polysaccharide production and accumulation were detected in the staminal column of *S. pedunculatus*, indicating no nectar production. Sudan Red 7B and Nile Red tests were negative for oil accumulation within trichomes and other tissues of the staminal column, and tests were also negative for starch accumulation. Structural observations of trichomes showed that

Fig. 2 Trichomal elaiophores of *Sisyrinchium claritae* (**a**), *S. macrocarpum* (**b**), *S. setaceum* (**c**) and *S. micranthum* (**d**) and the morphology of nuptial trichomes among Sisyrinchieae. **a** Differential interference contrast imaging (DIC) of a trichome located on the lower half of the staminal column, showing the oil blister covered with the striate cuticle. **b** Longitudinal section of a trichome located on the staminal column, showing the apical portion stained with Toluidine Blue. The cuticle was ruptured during the process; the arrow indicates a remaining portion. **c** Trichomes on the lower half of the staminal column showing the blister of oil secretion and the striate cuticle. **d** Longitudinal section of trichomes located on the staminal column stained with Toluidine Blue. The thick cuticle of the oil blister is indicated by the arrow. **e** Oil-producing trichomes on the staminal column of *Sisyrinchium platense*. **f** DIC imaging of oil-producing trichomes of *S. claritae* stained with Sudan Red 7B, showing the oil accumulated in the blister of secretion (arrow). **g** Trichome of *S. tenuifolium* stained with Sudan Red 7B (*blister without oil accumulation). **h** Trichome of *Sisyrinchium demissum* stained with Sudan Red 7B (*blister without oil accumulation). **i** DIC imaging of one trichome of *Solenomelus pedunculatus*; the *arrow* indicates the presence of very thin elongated structures on the enlarged apical part of the trichome. *Scale bars*: **a**, **c**, **e**, **f**, **g**, **h**, **i** = 50 μm; **b**, **d** = 20 μm



these cells were highly vacuolated and lacked any secretory cavity at anthesis (Figs. 2i, 5d). The cuticle had a striate surface, suggesting a reinforced resistance compared to a smooth surface.

The staminal column of *S. segethi* was covered by a layer of longitudinally elongated epidermal cells forming multicellular scales at the surface (Fig. 5a–c), mainly on the lower half of the column. PAS-Schiff and Ruthenium Red tests showed that

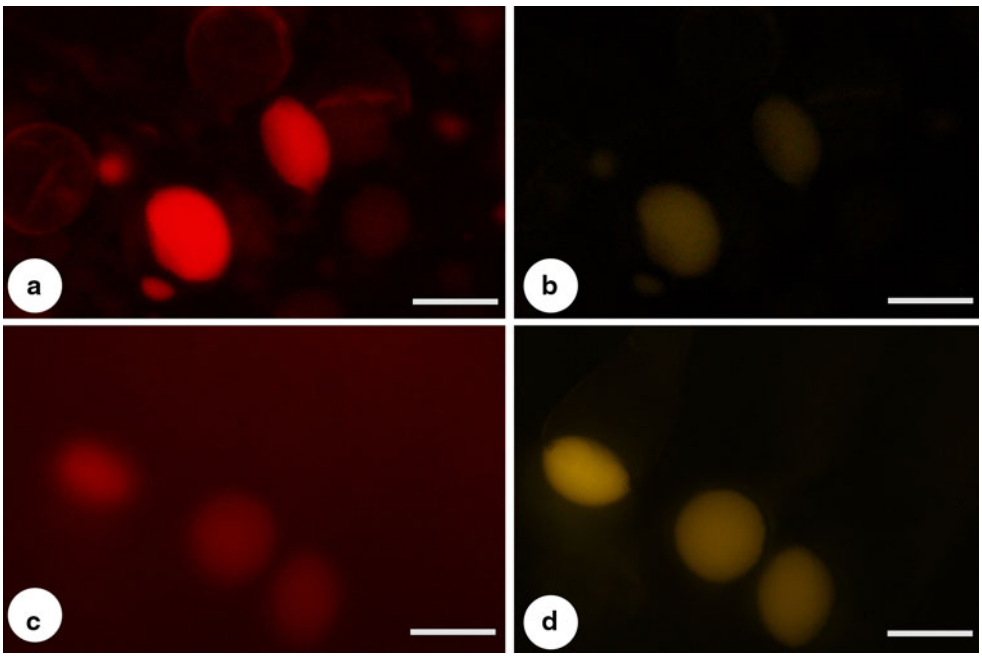


Fig. 3 Fluorescence micrographs of oil-producing trichomes stained with Nile *Red*. **a**, **b** *Sisyrinchium commutatum*. **c**, **d** *S. limarinum*. **a** and **c** *Red* emission showing the accumulation of polar lipids in the oil blister. **b** and **d** *Yellow* emission showing the accumulation of non-polar lipids in the same blisters of secretion. *Scale bars*: **a**–**d** = 50 μm (color figure online)

secretions of pectin mucilage mixed with polysaccharides were accumulated mainly within intercellular spaces between the outer epithelial layer and the subepithelial cells (Fig. 5b, c). The outer surface of the staminal column, with large epidermal cells secreting a high amount of polysaccharides, was characteristic of epithelial nectaries, nectar presumably accumulating in the subepidermal spaces in this region. Sudan Red 7B and Nile Red tests were negative for oil accumulation within epithelial cells and other tissues of the staminal column. No starch accumulation was detected with Lugol at anthesis.

Pollenkitt and pollen cytoplasm content

Observations on fresh material and Sudan red 7B histochemical tests showed that high amounts of pollenkitt were present in the pollen of each species sampled and numerous lipid droplets were always detected, often glued to the pollen grain external surface (Fig. 6a–f). Tests with PAS-Schiff on the pollen cytoplasm content revealed the presence of high amounts of dissolved polysaccharides within the cytoplasm for all samples (Fig. 6g–i). Starch grains were detected in the pollen cytoplasm of only two species *Sisyrinchium chilense* and *S. pedunculatus* (Fig. 6h, i).

Discussion

The genus *Sisyrinchium* is the most species rich genus among Neotropical Iridoideae, and numerous species have developed trichomal elaiophores related to specialized oil bee pollination (Goldblatt and Manning 2008). Chauveau et al. (2011) showed that these glandular structures probably evolved twice independently within the genus, leading to two distinct lineages. One of these lineages is distributed in the South Andean region, whereas the other one has its centre of origin and diversity in the Paranaensic region with subsequent dispersal events through South America towards North America. Among the study species, *S. cuspidatum*, *S. limarinum, S. macrocarpum*, *S.* cf. *tofoense* and *S. striatum* belong to the first lineage. *Sisyrinchium claritae*, *S. commutatum*, *S. setaceum* are included in one of the two major clades forming the second lineage, whereas *S. chilense*, *S. foliosum*, *S. micranthum*, *S. patagonicum* and *S. platense* are included in the other clade with the North American species *S. angustifolium*, *S. demissum* and *S. idahoense*.

The species belonging to the first lineage are distributed in the range area of the specialized oil bee pollinators of the genus *Chalepogenus* (Holmberg 1903), subgenus *Chalepogenus* (Apidae: Tapinotaspidini), which are the main known pollinators of the oil-producing species of *Sisyrinchium* within this range area (Cocucci et al. 2000; Cocucci and Vogel 2001). The species of *Chalepogenus* involved in *Sisyrinchium* pollination have specialized oil-collecting structures that are mainly formed of absorbing foreleg pads (Roig-Alsina 1997; Cocucci et al. 2000; Cocucci and Vogel 2001). The thin cuticle observed in species of the South Andean lineage of *Sisyrinchium* ruptures very easily during pollination.

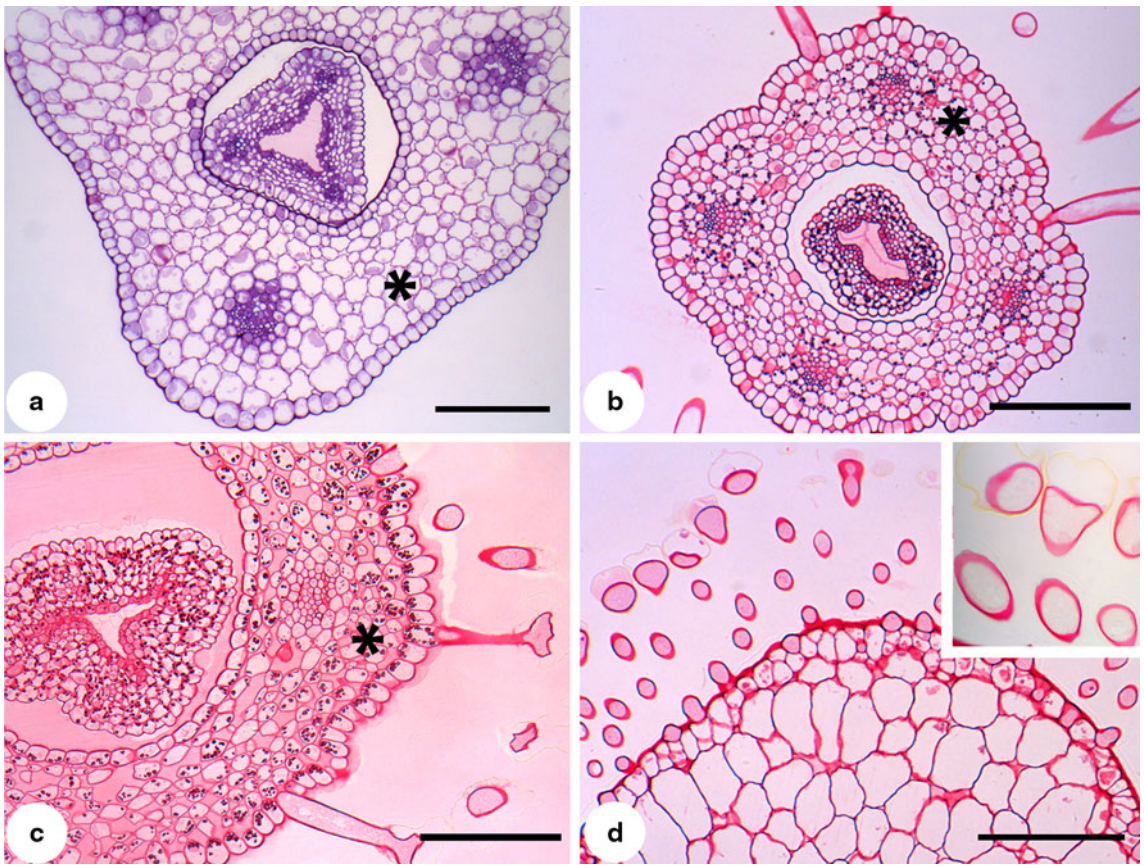


Fig. 4 Transverse sections of the staminal column of four *Sisyrinchium* species. **a** *S. elmeri*: Starch-less tissue (*asterisk*) of the staminal column devoid of nuptial trichomes stained with Toluidine Blue. **b–d** Staminal tissue stained with PAS-Schiff. **b** *S. setaceum*: starch was detected within the cytoplasm of the epidermal cells and the inner tissues of the staminal column (*asterisk*). **c** *S. angustifolium*: starch grains are present mostly around the vascular tissues (*asterisk*). **d** Transverse sections of the staminal column and the oil-producing trichomes of *S. platense*. No starch accumulation was detected in these tissues. Detail of elaiophores cells without oil content. *Scale bars*: **a–d** = 100 μm

Within the second lineage, several species sampled (*S. chilense*, *S. micranthum*, *S. platense*, *S. setaceum*) are almost exclusively pollinated by specialized oil bees of the genus *Chalepogenus*, subgenus *Lanthanomelissa* (Holmberg 1903), which were observed foraging oil only on *Sisyrinchium* species (Cocucci et al. 2000; Cocucci and Vogel 2001; Machado 2004). The bees of this subgenus have evolved abrasive and absorbing foreleg pads, potentially allowing them to rupture the thick cuticle of these species and then collect the oil released (Cocucci et al. 2000; Cocucci and Vogel 2001).

Neither structural observations nor histochemical tests detected nectaries or nectar within the staminal tissues of the study species, and compounds that were neither oil nor sugar were observed within the trichomal elaiophores of the North American species of the second lineage. It should be noted that the number and density of nuptial glandular trichomes on the staminal column, as well as the amount of compounds accumulated in the subcuticular space, were much lower than those observed in the South American species of the same lineage. These results, together with the fact that bees of the Tapinotaspidini tribe are absent in North America (Cocucci and Vogel 2001), suggest that the association with specialized oil-collecting pollinators was lost during the northward dispersion of the genus. The occurrence of few nuptial trichomes on the staminal column would then be a vestigial condition, and at least one major shift of pollination strategy would have occurred during the dispersal event. Likely the only reward available for pollinators among the North American species of this lineage is pollen, even though the chemical compounds accumulated in the blister of these trichomes still need to be characterized. Among the North American species, some are mainly pollinated by solitary bees of the family Halictidae that collect pollen, and even if insect pollination is generally thought to promote outcrossing, observations of their behaviour and the rate of self-compatibility observed for several North American *Sisyrinchium* species suggest that these pollinators may increase self-pollination (Cholewa and Henderson 1984; Montgomery 2009).

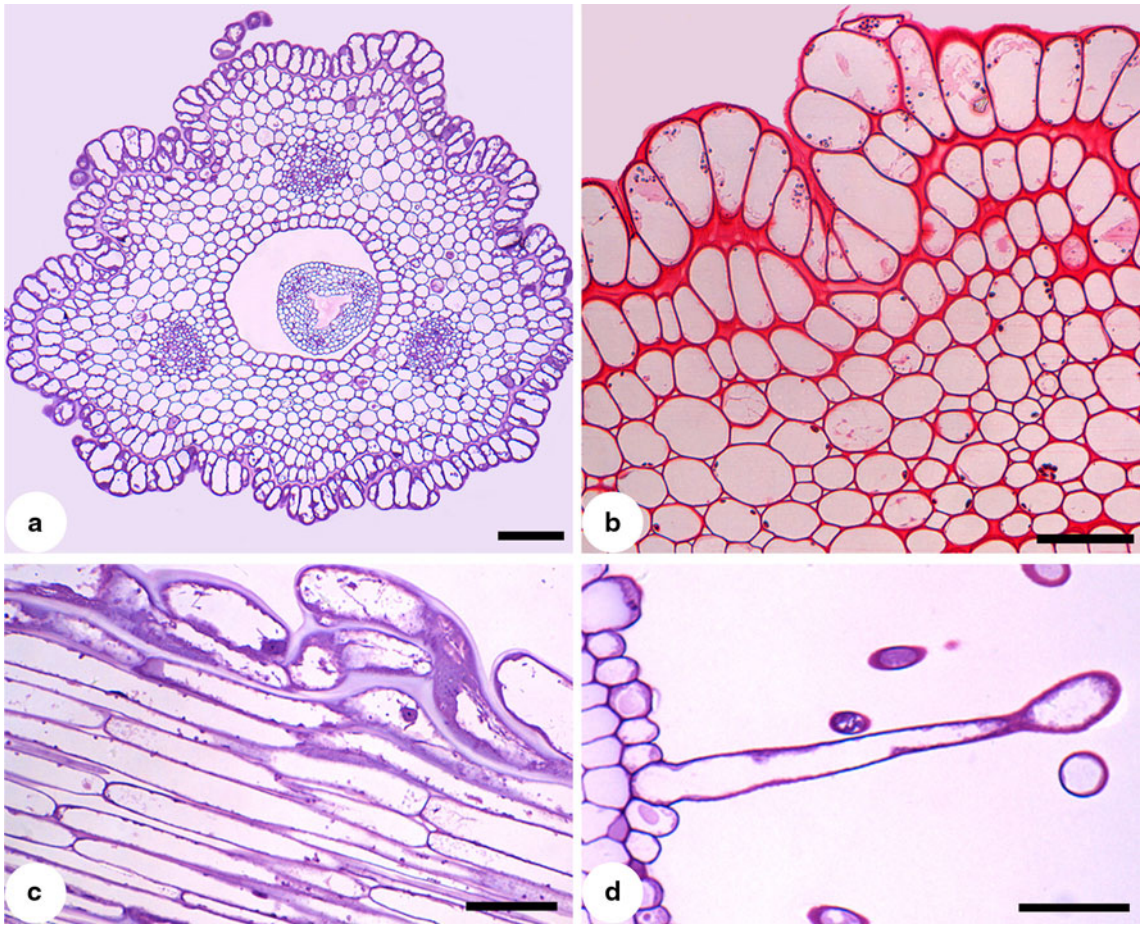


Fig. 5 Sections of the staminal column of *Solenomelus* species. **a–c** *S. segethi*. **a** Transverse section (TS) stained with Toluidine Blue, showing the outer epidermal layer of elongated secretory cells. **b** TS stained with PAS-Schiff, showing polysaccharides secretions (*red*) accumulated within the intercellular spaces and on the outer surface of the staminal column. **c** Longitudinal section stained with Ruthenium Red, showing the pectin mucilage (*purple*) covering the secretory cells and within the intercellular spaces below the outer epidermal layer. **d** TS of the staminal column of *S. pedunculatus* stained with Toluidine Blue, showing the highly vacuolated cytoplasm of the nuptial trichomes. *Scale bars*: **a** = 100 μm; **b–d** = 50 μm (color figure online)

Although most of the North American species studied are thought to be self-compatible, it has been shown that *S. idahoense* is nearly unable to be self-pollinated (Henderson 1976; Cholewa and Henderson 1984). Further investigations are needed concerning the pollination biology of these species of *Sisyrinchium* in order to determine the range of biotic vectors involved in their pollination, to estimate the relative extent of effective self-pollination and to determine whether pollen is indeed the only reward collected.

In *Sisyrinchium tenuifolium*, few nuptial trichomes located on the staminal column were also observed. This species, distributed from Mexico to Panama, is nested within a clade that includes only Central American species and where non-oil-secreting glandular trichomes evolved independently (Chauveau et al. 2011). Structural observations and histochemical tests conducted on this species gave similar results to those obtained for the North American species included in the second lineage, even though they are not closely phylogenetically related. Since the flowers of *S. tenuifolium* produce no oil and no nectar, it seems likely that the only reward offered to pollinators is pollen, and nuptial trichomes might therefore play another role in pollination.

No nuptial secretory structure and no accumulation of starch were detected within the staminal tissues of the different species of *Sisyrinchium* devoid of nuptial trichomes, suggesting that pollen is likely the only reward available for pollinators in these species.

The presence of starch is characteristic of many nectary and osmophore cells, generally at the presecretory stage. It has been suggested that starch could be a source of energy for intensive metabolic processes, or indirectly a nectar sugar component (Pacek and Stpiczyńska 2007). The difference observed in the presence/absence of starch between non-oil-producing trichomes and elaiophores could be

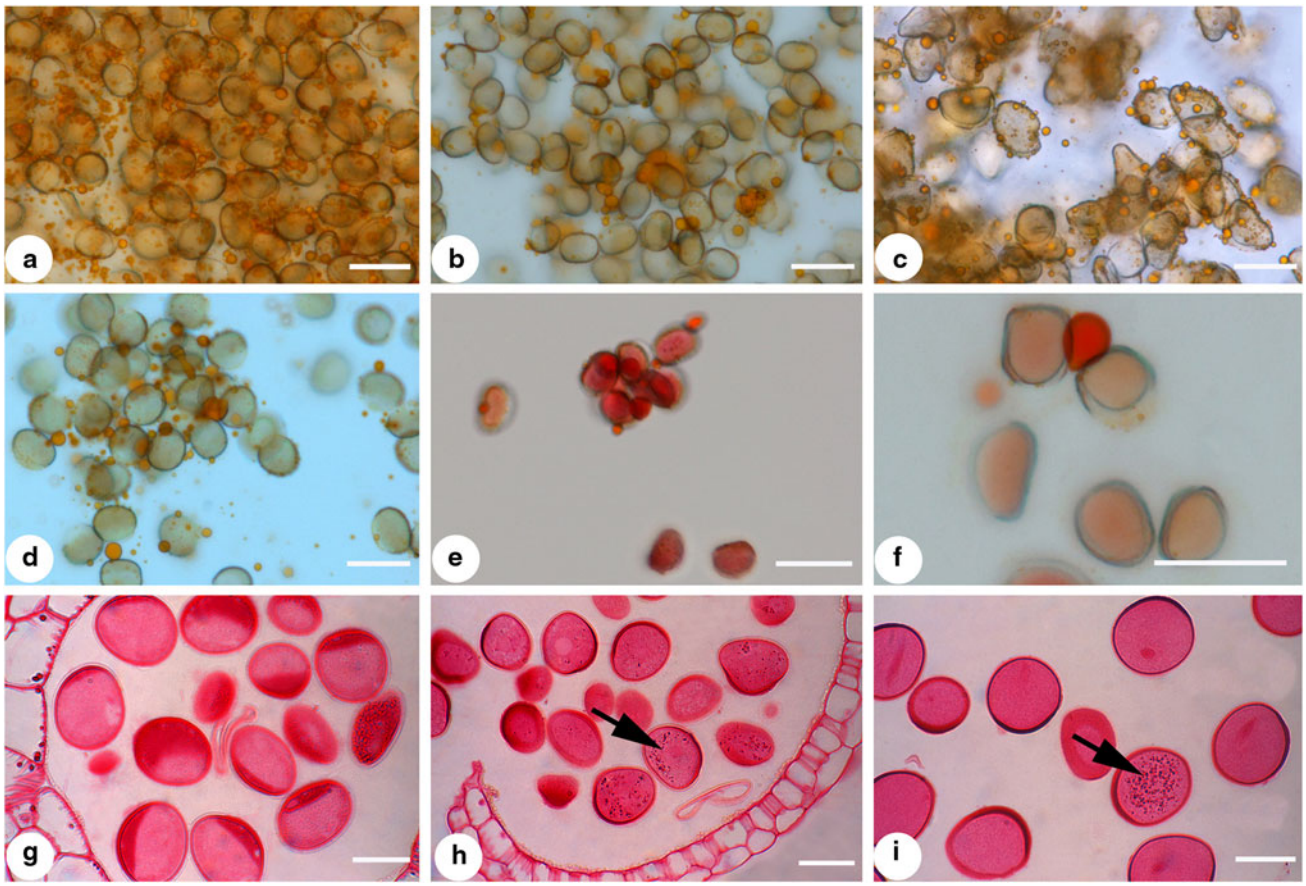


Fig. 6 Pollenkitt and pollen grain contents. **a–d** Light micrographs of fresh material. **a** *Sisyrinchium tenuifolium*. **b** *S. micranthum*. **c** *S. angustifolium*. **d** *S. limarinum*. **e–f** Light micrographs with Sudan Red 7B showing oil content in pollenkitt. **e** *S. limarinum*. **f** *S. tenuifolium*. **g–i** Pollen grains stained with PAS-Schiff showing the total polysaccharides content. **g** *S. elmeri*. **h** *S. chilense*. **i** *Solenomelus segethi*. *Arrows* indicate starch grains within the cytoplasm of few pollen grains of *S. chilense* and *Solenomelus segethi*. *Scale bars*: **a–f** = 50 μm; **g–i** = 20 μm

accounted for by a difference in starch use. At the anthesis stage, oil is already widely accumulated in the blister of secretion of trichomal elaiophores, and if starch was used during the metabolic process of production of lipids, it may explain why starch was not detected at this stage for species bearing trichomal elaiophores. The accumulation of starch observed for the North American species *S. angustifolium*, *S. demissum* and *S. idahoense* might be a vestigial condition related to the ancestral oil-producing state of the nuptial trichomes.

In the same way, the occurrence of few starch grains within the cytoplasm of the epidermal cells and the staminal column cells of *S. setaceum* might be related to the lower amount of oil secretions observed at the anthesis stage, compared to the amount of oil accumulation detected in the other species with trichomal elaiophores.

The results of the present study are in agreement with previous observations, suggesting that oil-producing trichomes are present in a wide range of species within *Sisyrinchium*, distributed almost only in the Neotropical region (Chauveau et al. 2011). We show here that these trichomes produce either exclusively oil or no reward for pollinators. In oil-producing trichomes, non-polar and polar lipids are accumulated in the subcuticular space. The higher intensity of specific fluorescence observed for polar lipids suggests that they are probably secreted in higher amounts than non-polar ones. Nectaries were not detected in the species sampled, suggesting that oils and pollen are likely to be the only rewards available for the biotic pollinators within the genus.

Pollenkitt abundance and pollenkitt lipid content were examined for each of our study species, together with the presence of total polysaccharides within the pollen grain cytoplasm. In starch-less pollen grains, starch is assumed to be totally hydrolysed during the final phase of maturation into glucose, fructose, sucrose, pectins and other types of cytoplasmic carbohydrates, and the pollen cytoplasm is therefore stained with PAS-Schiff (Pacini et al. 2006). The occurrence of these highly energetic compounds in the pollen of our study species suggests that pollen might play

a major role in the pollination strategies of the species of the tribe Sisyrinchieae. The role of pollenkitt in attaching pollen to pollinators and the ability of pollen-consuming insects to cue on pollen lipids have already been widely demonstrated in many Angiosperms, where pollen provides numerous other nutriments, including carbohydrates (Hesse 1979a, b, c; Dobson 1987, 1988; Pacini and Hesse 2005; Roulston and Cane 2000). Specialized oil bees involved in the pollination of species of *Sisyrinchium* bearing trichomal elaiophores also collect pollen (Cocucci and Vogel 2001; Truylio et al. 2002). Observations of the behaviour of the specialized oil bee *Chalepogenus betinae* (Urban 1995) on *Sisyrinchium micranthum* flowers show that pollen attracts primarily female bees (Truylio et al. 2002), suggesting an essential role of pollen in oil bee pollination. Truylio et al. (2002) showed that *Sisyrinchium micranthum* is also actively visited by pollen-collecting bees of the family Apidae, suggesting that several pollination systems may occur simultaneously among oil-producing species of *Sisyrinchium*.

Elaiophores and nectaries were not detected within the staminal tissues of the species of *Orthrosanthus* and *Solenomelus* observed, indicating that pollen might be the only resource available for pollinators in these species, except for *S. segethi* where nectaries were found. The results obtained for *O. monadelphus* are congruent with previous observations made on flowers of *O. laxus* (Endl.) Benth., a species distributed in Australia (Rudall et al. 2003). Nuptial trichomes were only observed on the staminal column of *S. pedunculatus,* but they were not secretory at the anthesis stage, and structural observations showed that the cytoplasm was highly vacuolated and presented none of the characteristics of secretory cells (dense, intensively stained and granular cytoplasm). The latest comprehensive phylogeny of the tribe showed that the nuptial trichomes of *S. pedunculatus* evolved independently from those observed in the genus *Sisyrinchium* (Chauveau et al. 2011), and our observations suggest that they are structurally and anatomically not related. Their function remains to be identified.

The staminal secretory structures reported for *Solenomelus segethi* are characterized as nectaries for the first time here. They are structurally different from those observed within the closely related genus *Olsynium* by Rudall et al. (2003). While nectar is secreted from cells forming scales on the outer epidermal layer of the staminal column in *S. segethi*, observations in *O. douglasii* suggested that nectar was secreted from highly vascularized tissue beneath the epidermal layer, and from anticlinally elongated epidermal cells on the inner and outer surfaces of a swollen part of the staminal column in *O. junceum* (Forcone et al. 1997; Rudall et al. 2003). Our observations combined with the results of a recent comprehensive phylogeny of the tribe (Chauveau et al. 2011) suggest that the absence of perigonal nectaries or elaiophores is the plesiomorphic condition for *Sisyrinchium* and it seems likely that the trichomal elaiophores identified in this genus did not evolved from perigonal nectaries.

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