Heterostyly in *Primula nutans* ssp. *finmarchica* (Jacq.) Á. Löve & D. Löve (Primulaceae) from three northern coenopopulations (Kandalaksha Bay, White Sea)

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Summary: Heterostyly is widely spread in *Primula nutans* ssp. *finmarchica* and is characterized by different positions of styles, anthers as well as morphology of stigma and pollen grains as in other heterostylous primroses. Two types of anther position can be found, at the mouth and at the middle of the corolla tube. Position of styles in the short-styled flowers is variable. So, we have divided this type into three groups. A great number of short-styled forms in which the stigma is directly below the anthers or just opposite them show that there is a weak trend from heterostyly to homostyly. The revealed differences in the structure of pistils and pollen grains are not as significant as in some other heterostylous primroses. The style-morph ratio of long-styled and short-styled plants as well as flowers in two studied coenopopulations were nearly 1:1 (with a slight predominance of short-styled individuals, 0.8 and 0.9, respectively), in the smallest third one – nearly 3:1. Thus, according to alternative features, the two studied coenopopulations are completely stable having an almost equal ratio of both morphotypes. The number of ovules in long-styled and short-styled flowers lies in the same range, which indicates the potential equal probability of obtaining the same ratio of different plant forms in the next generation.

Keywords: *Primula nutans* ssp. *finmarchica*, heterostyly, homostyly, long-styled flowers, short-styled flowers, ratio of heterostylous plants

The genus *Primula* is well known for the phenomenon of heterostyly which is widespread among its species (Gilmartin 2015). Heterostyly occurs when a species has two or more different positions of anthers and stigmas in its flowers; in primrose, there are more often two types of flowers (distyly): 1) long-styled (or 'pin'; hereafter LS) – in which the style ending in the stigma is long and exposed at the mouth of the tube, the anthers are only half of the tube length, 2) short-styled (or 'thrum'; hereafter ShS) – in which the anthers are positioned at the mouth of the corolla tube and the style is short and located nearly half-way down the corolla tube. Heterostyly helps to avoid self-pollination that in evolutionary terms leads to a higher frequency of cross-pollination and the genetic heterogeneity and stability of populations (Deschepper et al. 2018). The presence of distyly in *Primula* has long been known, since experiments by Ch. Darwin (Darwin 1862). Darwin investigated *P. veris* extensively over many years (Weller 2009). The floral morphotypes in distylous species are normally self-incompatible and at the equilibrium they occur at a ratio of 1:1 (Ganders 1979; Richards 1986; Barrett 1992). Further it has been suggested that such a polymorphism is controlled by a super-gene with several major components (Wedderburn & Richards 1992), determining style and anther length, papilla size and pollen size, which effectively comprises the two alleles S and s (Boyd et al. 1990; Richards 1997; Li et al. 2007). The dominant allele gives rise to the ShS morph, the recessive homozygote, ss produces LS phenotypes. If all crosses are legitimate, one would expect a LS-ShS ratio fluctuating around 1:1 in large, undisturbed populations of primroses (Boyd et al. 1990; Richards 1997). In most heterostylous taxa, however, self-compatible homostylous phenotypes occur, particularly

in association with ecologically or geographically marginal environments (Charlesworth & Charlesworth 1979; Richards 1986).

Much information about heterostyly is known for *Primula veris* (Brys & Jacquemyn 2009) and *P. vulgaris*. Our interest was the northern primrose, *P. nutans* ssp. *finmarchica*. It is a subspecies of *Primula nutans* Georgi (Siberian primrose), isolated from the type subspecies by a distance of more than 1000 km (Kreivi et al. 2011). Siberian primrose is most often found on low-lying shores, where the soil is a mixture of clay and sand and the plants may be submerged for short periods by the rising sea water or the flooding of rivers. Siberian primrose prefers open habitats, where it starts to grow earlier in spring than its competitors (Mäkinen & Mäkinen 1964; Björnström 2006). In northern Europe, the Siberian primrose subspecies *finmarchica* has a disjunctive, tripartite distribution. It grows in the northern part of the Bothnian Bay in Finland and Sweden, in the Barents Sea part of the Arctic Ocean, in northern Norway and Russia, and on the western shores of the White Sea in Russia (Kreivi 2009).

We have studied *P. nutans* ssp. *finmarchica* on the White Sea coast, where it forms separate local populations in order to describe reproductive organs regarding heterostyly and to find out the ratio of different morph types. We wanted to answer the question: do populations exhibit biased style-morph ratios? It was also interesting to test the hypothesis that in the north the transition from heterostyly to homostyly in some *Primula* species could be observed.

Materials and methods

Field studies were carried out in 2016. We have investigated three natural coenopopulations of *P. nutans* ssp. *finmarchica* on the coast of Kandalakha Bay at the White Sea in the vicinity of Kandalaksha State Nature Reserve (67°06'35" N, 32°41'02" E; about 1 km from the Luvenga village, Kandalaksha district, Murmansk oblast). The primroses grew on a moist matted coastal meadow with domination of *Juncus gerardii* ssp. *atrofuscus*, *Agrostis straminea*, *Carex subspathacea*, *Triglochin palustre*, *Eleocharis uniglumis* ssp. *septentrionalis* and *Atriplex praecox.* The locality is situated between littoral clays with sparse populations of *Tripolium vulgare* and *Triglochin maritimum* which are flooded by sea water each tide, as well as at a birch forest margin with *Empetrum hermaphroditum* and *Lathyrus maritimus.*

The number of different plants and flower morphotypes in coenopopulations was counted and as a result their ratio was composed. Some plants were dug out and their life form was analyzed. For morphological descriptions and determining the size of anthers, pollen grains and stigmas, light microscope and scanning electron microscope (SEM) were used. For light microscopy, fully matured anthers were removed from the fresh flowers. Pollen was placed directly on slides, mounted in glycerin and observed. To study surface morphology, dehydrated anthers and stigmas were critical point dried. To isolate individual pollen grains, dried anthers were macerated using a syringe needle and mounted onto aluminum stubs with double-sided adhesive tape. Stubs were sputter-coated with platinum, and material was examined and imaged using a CamScan in the electron microscopy laboratory of the Biological faculty MSU.

Results

Primula nutans ssp. *finmarchica* is a perennial rosette, hemicryptophyte, vegetative short-lived plant. It has a rosette of 4–5 leaves and adventive roots on the basal nodes of the stem (Fig. 1)

and a leafless flowering part of the shoot, ending by the inflorescence of umbrella-type. The umbrella consists of rays, beginning from the axil of bracts. Renewal is due to an axillary bud usually originating in the axils of the lower leaves; this bud gives life to a daughter ramet that can completely replace the mother plant, which dies off after fruiting. If there are more than one bud, vegetative propagation is possible (although this situation was not detected in the studied populations). However, propagation is more often by the seed set.

The majority of the studied plants were heterostylous. The position of styles in the studied ShS flowers proved to be highly variable. In connection with the revealed heterogeneity, the type of the flower is more correct to be determined by the position of the anthers: in the ShS forms, they are located at the mouth of the corolla tube, and in the LS forms at the middle of the corolla tube. In true ShS flowers, the length of the style has to be such short that the stigma should be located near the middle of the flower tube. In true LS flowers, the style with the stigma extends beyond the flower tube and it is therefore clearly visible when examining the flower from above.
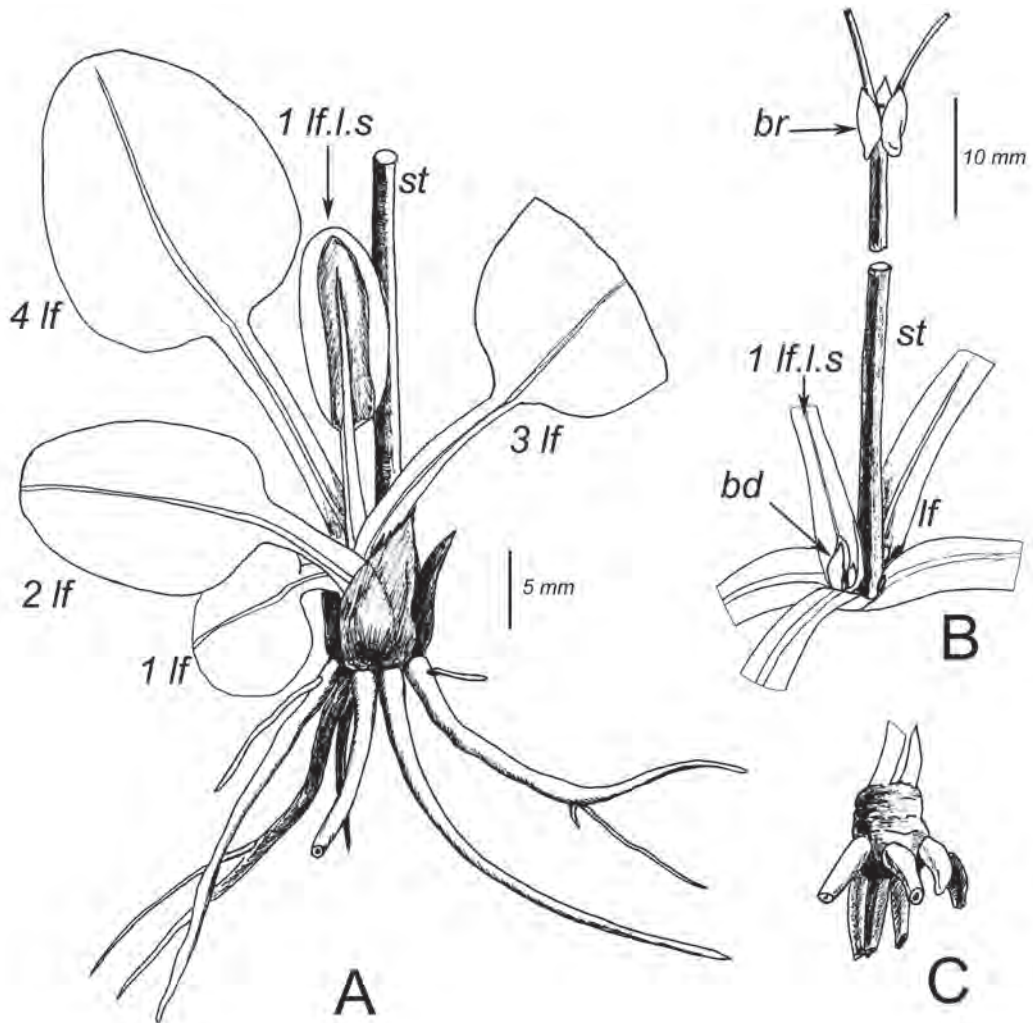

Figure 1. Line drawings of *Primula nutans* ssp. *finmarchica*. A – vegetative organs; B – the lower part of the plant with axillary bud of resumption and the upper part with bracts; C – short rhizome with adventive roots. Abbreviations: bd – bud, br – bract, lf – leaf, lf.l.s – leaf of the lateral shoot, st – stem.

Table 1. The ratio of different plant and flower morphotypes in three studied cenopopulations.

Coenopopulation	Sum. Plants	LSP	ShSP							ShSP-ShAP	Ratio LSP:ShSP	Sum. Flowers	LSF	ShSF	Ratio LSF:ShSF	Number of plants with flowers					
			m0	m1	m0+m1	m0+m1+m2	m0+m2	m1+m2	m2HS							1	2	3	4	5	6
I	81	36	44							1	0.82	219	98	120	0.83	4	18	57	2	0	0
			3	32	0	0	0	7	1												
II	33	25	8							0	3.13	96	70	26	2.70	0	7	23	2	1	0
			1	1	5	1	0	0	0												
III	108	52	56							0	0.93	325	161	164	0.98	1	14	80	12	1	1
			14	32	8	0	2	0	0												

Abbreviations. LSP – long-styled plant; ShSP – short-styled plant; ShSP-ShAP – plant with short style and short stamens; LSF – long-styled flower; ShSF – short-styled flower; m0 – the stigma is located in the middle of the flower tube; m1 – the stigma is directly under the anthers or somewhat below them, but much higher than the middle of the flower tube; m2 – the stigma is opposite the anthers (in all cases the anthers are located at the mouth of the corolla tube); + is used when different types of flowers are combined in one plant.

However, the length of the style in the ShS is most often to such extent that the stigma is located directly under the anthers, and in some cases even opposite them. In order to take into account the numerical ratio of different forms, we divided different intermediate morphotypes of ShS flowers into groups: m0 – the stigma is located in the middle of the flower tube, m1 – the stigma is directly under the anthers or somewhat below them, but much higher than the middle of the flower tube and m2 (nearly homostylous form) – the stigma is opposite the anthers; in all cases the anthers are located at the mouth of the corolla tube. Since usually several flowers are on the plant, there may be different combinations of ShS types. The summary data for the three coenopopulations are presented in Table 1 and in two diagrams.

Analysis of the structure of the reproductive organs with a light microscope yielded the following results (Table 2). Stigma diameter is larger in LS than in ShS-m1, although different morphotypes
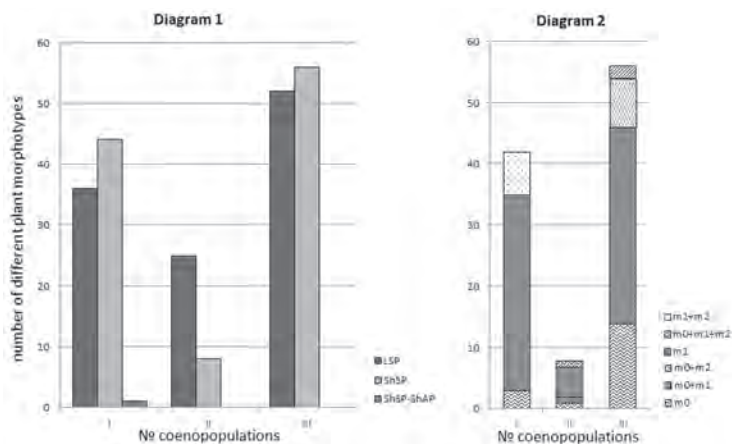
Diagrams. The number of different plant morphotypes (Diagram 1) and different flower types among ShS (Diagram 2) in three studied coenopopulations (abbreviations are the same as in Table 1).

Table 2. Dimensions of the reproductive organs in *Primula nutans* ssp. *finmarchica* in studied coenopopulations.

Feature	LS	ShS
Dimension of anthers (length × width, mm)	1.40 ± 0.08 × 0.70 ± 0.07	1.45 ± 0.05 × 0.81 ± 0.08
Pollen grain diameter (equatorial, µm)	17.4 ± 1.02	23.39 ± 1.81
Stigma diameter (on the side, mm)	0.56 ± 0.09	0.45 ± 0.05
Papillae length (µm)	41.90 ± 4.90	46.80 ± 7.23
Length of the protruding part of the papillae (µm)	19.90 ± 7.16	22.75 ±4.71
Width of apical part of papillae(µm)	8.73 ± 1.58	10.26 ± 1.15

sometimes overlap in size. In the LS, papillae of the stigma are not much smaller than in ShS (Fig. 2). The number of ovules per flower is 66 (42–86) in LS and 64 (34–94) in ShS. Anthers in two morphs are nearly of the same size. The shape of pollen grains (in glycerin without special treatment) is nearly spheroidal, the diameter (equatorial) of pollen grains is larger in ShS.

Pollen grains of *P. nutans* ssp. *finmarchica* on a scanning electron microscope (Fig. 3) are (according to ERDTMAN's classification (1971)) small, isopolar, radially symmetric, 6-zonocolpate, and showing difference in size between LS and ShS-m1. Equatorial diameter of LS pollen is
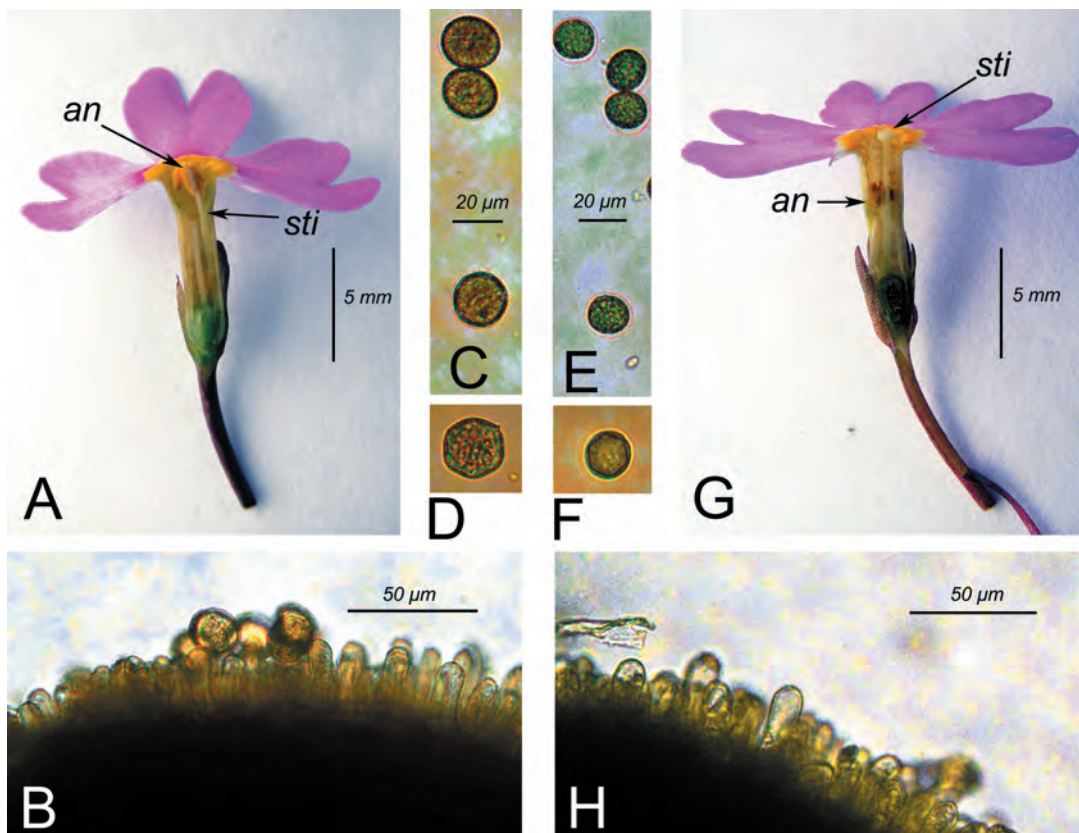

Figure 2. The structure of flowers, stigma papillae and pollen grains (optical microscope) of two morphs. A – the flower of the ShS type; B – the stigma surface with papillae (ShS type); C, D – large pollen (ShS); E, F – small pollen (LS); G – the flower of the LS type; H – the stigma surface with papillae (LS). Abbreviations: an – anthers, sti – stigma.

12.1–12.5 µm and of ShSF pollen 15.8–17.5 µm. Pollen grains in polar view are circular; along the perimeter of the equator they have long (0.58–0.83 µm in LS; 1.25 µm in ShS) and narrow meridian located colpi that do not meet at the poles. The pollen grains have a reticulate exine ornamentation. Pits in ShS pollen are naturally larger than those in LS pollen in the case of
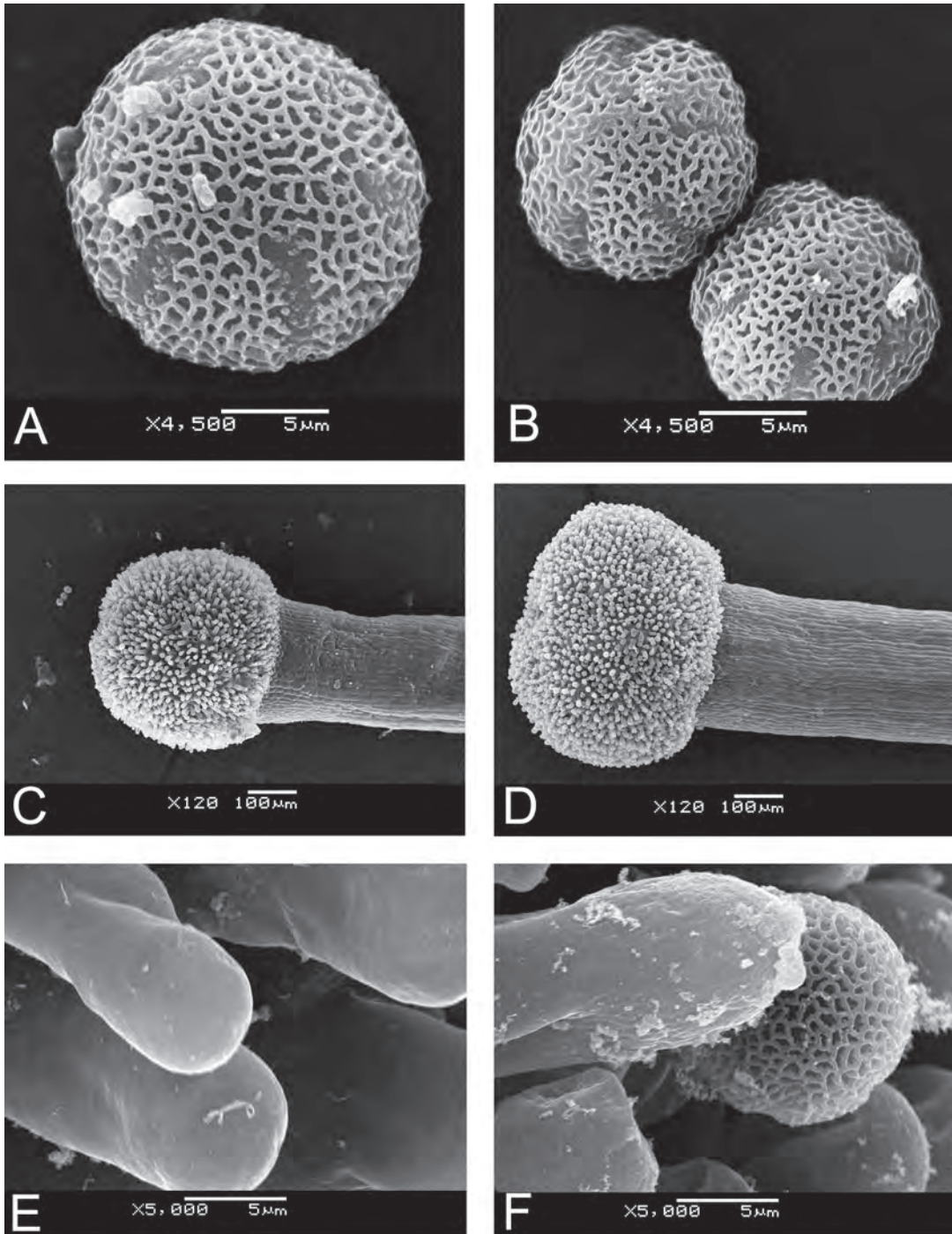

Figure 3. The structure of reproductive organs (SEM) of two morphs: pollen grains: A – large in ShS, B – small in LS, the stigma with papillae: C – in ShS, D – in LS, papillae on the stigma: E – ShS, F – LS.

larger pollen grains in ShS. The distance between the ends of the colpi on the apocolpium is 2.9–3.4 µm (LS), 3.3–3.75 µm (ShS). The mesocolpium width is 4.2–5 µm (LS) and 5.0–6.6 µm (ShS) respectively. The membrane of the colpi is granular. The pattern of the surface on the apocolpium and mesocolpium is usually the same.

Discussion and conclusion

In populations of *P. nutans* ssp. *finmarchica*, two reproductive morphs with reciprocal placement of anthers occur. Here, heterostyly includes different positions of styles, anthers as well as of morphology of pollen grains and stigmas as in other heterostylous primroses.

Thus, if we do not take into account the diversity of flowers according to the position of the style in the group of ShS, it can be noted that in two more or less large coenopopulations, where the number of counted individuals is about 100, the style-morph ratios of LS and ShS plants is close to 1:1. In the small coenopopulation, significant deviations from equal morph frequencies was exhibited, – i.e. LS plants were 3 times more than ShS plants. Very similar patterns have also been found in populations of some other *Primula* species (Aronne et al. 2014); morph frequencies were equal in large populations (>100 plants) and symmetric deviations were noticeable in smaller populations (Endels et al. 2002; Jacquemyn et al. 2002; Kéry et al. 2003). The equilibrium of style-morph ratios consistently found in populations of some primeroses, for example in *P. mistassinica* (Larson & Barrett 1998), suggest that self-fertilization occurs rarely, the same can be applied to the two populations of *P. nutans* ssp. *finmarchica*. Though we didn't investigate the physiology of reproduction of the studied species, the obtained ratio results suggest that in two coenopopulations of *P. nutans* ssp. *finmarchica* alternative morphs are usually self-incompatible and cross-incompatible with individuals possessing the same reproductive morph, as pointed for many other primroses (Darwin 1877). Darwin's studies of distylous *Primula* showed that heterostylous morphology and function were similar across *Primula* species. Despite this, Wedderburn & Richards (1992) noted that in many primrose species some compatibility can be found in both morphs. So, in subgenus *Sphondylia* (Duby) Rupr. including the distylous *P. gaubeana* and *P. edelbergii* all species are self-fertile and with an average of at least 60% of the ovules developing into seed (Al Wadi & Richards 1993). Pollination experiments in *P. nutans* ssp. *finmarchica* have also revealed that the species is not fully self-incompatible (Degerman-Fyrsten 2001, cited by Kreivi 2009).

Returning to the third small population, where the LS plants prevailed indeedly, discrepancies in the morph ratio have been found to be much larger in small than in large populations (Kéry et al. 2003). What are the reasons? This imbalance could arise because of demographic stochasticity, the lower intra-morph incompatibility of the LS morph and niche differentiation between morphs (Kéry et al. 2003). In some primroses, LS × LS crosses occur at a low rate, thus favouring an increase of LS and this could result in a LS-bias of morph frequencies in small populations (Kéry et al. 2003). In *P. veris*, LS plants could have a reproductive advantage over ShS plants, because they are more fertile when paired with the same morph. On the other hand, the heterozygous ShS might have a minor advantage over LS in certain microclimates. Besides, chance effects (due to demographic stochasticity) may produce dominance of one of both phenotypes in very small populations (Endels et al. 2002). Differential mortality or clonal growth could bias morph frequencies in a population if one morph is favoured at a concrete habitat (Kéry et al. 2003). It

is not known, whether these processes occur in small populations of *P. nutans* ssp. *finmarchica*. In whole, there is little information about the factors influencing morph frequencies in populations of distylous species (Endels et al. 2002; Jacquemyn et al. 2002).

Our study revealed that style length varies considerably relative to corolla length, so that some individuals exhibit a reverse herkogamy, while others are effectively non-herkogamous. So, if we consider all the variations in the position of the styles in ShS, it can be seen that the largest number of flowers has relatively long styles, their stigmas are usually located under the anthers or slightly below them (group m1). True ShS flowers, in which the style with a stigma is located half the length of the tube or only slightly higher, are rare. The situation of combining flowers with different style lengths on one plant (groups m0 + m1, m0 + m1 + m2, m0 + m2, m1 + m2) is quite common. In some cases, the different position of the stigma in flowers depends on the length of the flower tube, the intercalary extension of which can change the position of the non-growing parts inside the flower. The growth of the flower tube itself occurs quite late and the position of the main reproductive organs especially the stamens, which can be used to determine the morphotype of the flower, is well expressed in the buds and in the reduced flowers. Individual modifications in the position of stigma in *P. nutans* ssp. *finmarchica* are very similar to that observed in some flowers of monomorphic *P. verticellata* (Ernst 1955). The situation of stigmas variability has often been stated as an evolutionary way of the transition to heterostyly. So, Darwin hypothesized that great variability in the length of the pistil and stamens, or of the pistil alone was the first step in the origin of heterostyly. Darwin assumed that more regular crossfertilization would occur in a population with variable pistil and stamen length, (Weller 2009).

The tendency to get the same position of stigmas and anthers at the upper edge of the flower tube, is most pronounced in the first coenopopulation; here also deviating morphotypes are marked, including the secondarily homostylous flower and the transitional type, when the stigma almost reaches the anther level (m1 + m2). In the same coenopopulation, another interesting type of flower is noted, in which the position of the anthers nearly half-way down the corolla-tube corresponds to the LS type, and the position of the style, also in the middle of the tube (just below the anthers), is as in the ShS type. However, owing to the fact that the rest of the flowers on these plant were all LS, the homostylous condition in thise case is the result of accidental abnormality and not of real homostyly, an event caused by crossovers in the S-gene and well-known from several distylous primroses (for example, in *P. palinuri* Petagn. (Aronne et al. 2014)). Charlesworth & Charlesworth (1979) stated that homostyly within populations of *Primula* is rare, they are generated rather infrequently (on the order of 10^{-3}) through crossovers within the supergene controlling heterostyly. Nevertheless, the phenomenon of homostyly in accordance to *P. nutans* ssp. *finmarchica* coenopopulations is interesting because many studied ShS plants had relatively long styles, and there is a tendency to locate the stigma closer to the anthers; the question arises – is it really a way to secondary homostyly? Abnormal plants which show combinations of some ShS and some LS characters have been found in many species and are described as short and long homostyle plants (Dowrick 1956). Long homostyle plants of *Primula vulgaris* combine the male characters of the ShS flower with the female characters of the LS flower. They are self-fertile and produced by a third allele which is dominant to s and recessive to S (Boyd et al. 1990). Homostyle *P. vulgaris* are also able to fertilize heterostyles with the LS morph. If this is also true for *P. nutans* ssp. *finmarchica* is unknown. It is believed that self-fertile

homostyles have a mating advantage over heterostyles and are favoured in alpine-arctic conditions, where seed-set is ensured even during a short and unreliable summer (WEDDERBURN & RICHARDS 1992). In relation to ShS flower morphology of *P. nutans* ssp. *finmarchica*, it is more likely that in addition to the genetic definition of the features of heterostyled plants, certain traits, most of all concerning the length of the style, may change during the development of flowers.

As for the number of flowers per plant in the studied coenopopulations, the most common number is 3 flowers (about 70% of all plants), then 2 flowers (about 22% in two coenopopulations and about 13% in the third one), 1, 4, 5 and 6 flowers per plant are much less common. The ratio of flowers of different types (without taking into account the different units in the ShS group) is almost identical with the ratio of the plants themselves.

Thus, according to alternative features, two from three studied coenopopulations are completely stable having an almost equal ratio of both morphotypes. The number of ovules in LS and ShS flowers lies in the same range, which indicates the potential (only at the earliest stages of development) equal probability of obtaining the same ratio of different types in the next generation. Though, only the data on the real seed production as well as on the rate of germination and survival of seedlings can confirm this assumption.

From the obtained results, it is not exactly clear how the special way of resumption of *P. nutans* ssp. *finmarchica* influences the ratio of different plant morphotypes. One can only assume that an equivalent replacement of a parent plant of the concrete morphotype by a daughter ramet of the same type slightly distorts the original ratio characteristic of *P. nutans* ssp. *finmarchica* natural coenopopulations consisting of primary genes. As showed by KREIVI et al. (2011), clonal reproduction really does not seem to have an effect on genetic structure of *P. nutans*. In this case it is also important to know the length of the whole life cycle and the rate of individuals' survival. In some primroses, ShS suffered the greatest mortality and has the shortest longevity (BOYD et al. 1990)

The morphology of reproductive organs of *P. nutans* ssp. *finmarchica* is similar in most features to other heterostylous primroses, which ShS plants have flowers with short styles, high anthers and large pollen, while the LS have long styles, low anthers and small pollen (MAČUKANOVIĆ-JOCIĆ et al. 2010; DOWRICK 1956). In our case the greatest differences are noted in the diameter of the pollen grains. It shows non-overlapping distributions, in the ShS plants pollen grains are larger. The revealed differences in the structure of pistils and pollen grains, however, are not as significant as, for example, in some others primroses: *P. elatior* (SCHOU 1983), *P. veris* (MOROZOWSKA & IDZIKOWSKA 2004), *P. vulgaris* (HESLOP-HARRISON et al. 1981) or other heterostylous species (HERMANN et al. 1999). In most distylous Primulas, LS of this species have stigma papillae about several times the length of those of ShS (PANDEY & TROUGHTON 1974; ARONNE et al. 2014), but in our study the mean of the papillae length of LS were somewhat less than in ShS, the values of this parameter were overlapping and did not differ significantly. Apparently, this is due to the fact that the most common ShS-m1-type with a relatively long style is indeed an intermediate morph, in which the contrast of features is smoothed.

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