SHORT TERM SCIENTIFIC MISSION (STSM)

FINAL SCIENTIFIC REPORT

Assessing the degree of reciprocity in *Primula carniolica* Jacq.

This report is submitted for approval by the STSM applicant to the STSM coordinator

THE MAIN PURPOSE OF THE STSM

The main purpose of the proposed STSM was to assess the degree of reciprocity of an endemic Slovenian species Carniolian primrose (*Primula carniolica*) in order to better understand its' reproductive biology.

Carniolian primrose (*Primula carniolica*) is restricted to the small area in southwestern part of Slovenia (Figure 1). Due to its narrow endemism and attractive appearance, which can lead to overexplotation, it was included in the list of protected species in Slovenia. Additionally, with entering EU, Slovenia suggested its inclusion to the II Annex of the Habitat directive, which created the basis for establishing conservation sites within the Natura 2000 network. Like the majority of species in genus *Primula* (Mast et al., 2006), *P. carniolica* is a heterostylous species. Heterostyly is a genetically defined flower polymorphism where flowers can produce two (distily) or three (tristyly) different flower morphs with stigma and stamens positioned at reciprocal heights (Barrett, 1990). Perfect reciprocal hercogamy in a population is achieved when the anthers of one morph are positioned at the same level as the stigmas of the other. However, normally different degrees of reciprocity can be found in natural populations (Sánchez et al., 2008). P. carniolica is a distylous species, where the long-styled (pin) form has short stamens positioned inside the corolla tube and long style positioned in the mouth of the corolla tube and the short-styled (thrum) form with reciprocal position of stamens and style (Darwin, 1877). Distyly is genetically inherited, usually by the Mendelian mechanism, where long-styled morphs are homozygous (ss) and short-styled ones are heterozygous (Ss). Disassortative mating system between morphs helps to maintain a high genetic diversity of populations and maintain equal morph ratios in sufficiently large populations (Lewis & Jones, 1992). Geographical occurence of P. carniolica has been intensively investigated in the past years (Accetto, 2010; Dakskobler et al., 2004; Dolinar & Vreš, 2012; Dolinar et al., 2013;) and there is a very good insight in the plants' distribution. However, apart from some preliminary reproductive assessments (Toplak, 1969), its reproductive biology remained mainly unknown.

P. carniolica is well protected from direct human threats by its inaccessible habitat, however it grows in very confined habitats with specific microclimatic conditions, where climatic changes could have a severely negative impact on this species and its interactions with already scarce pollinators. If these interactions are broken, numbers of individuals would quickly decrease. In order to be able to protect this species in the future (*in situ* and *ex situ*), it is important not only

to know its distribution and population sizes, but also to understand the reproductive biology of the species.

Figure 1: Population of *Primula carniolica* in Hleviše.

This study aims to contribute to better understanding the reproductive biology of this narrow endemic species and coupled with the information from population genetic structuring (that will be investigated in near future), will yield significant insights into importance of the floral form and function in plant reproductive strategies.

In order to achieve our aims, we tested two hypotheses:

1. The population size has a strong impact on the ratio of long- and short-styled morphs.

2. In populations where ratio of long- and short-styled morphs has changed from the 50:50 equilibrium, the stigma-stamen position is less reciprocal than in populations where there is equal presence of long- and short-styled morphs.

DESCRIPTION OF WORK CARRIED OUT DURING THE STSM

The first step of our study was to assess the size of the studied populations. We used the data collected during the fieldwork in spring 2016. We selected 16 populations with the largest number of collected flowers, 7 population from Southern part of Slovenia and 9 populations from the Northern part (Figure 3). The populations with less than 70 individuals identified were considered as small population (Table 1). The only exception is Zala waterfall (SZ), which is one of the biggest populations, however, at the time of our visit most of the individuals were not flowering, so morph forms of only 46 individuals could be recorded. We calculated the percentage of each morph in the populations. When more than 60% of specimens belonged to only one of the morphs, population was considered as unequal.

In order to test the second hypothesis, we dissected flower material collected in spring 2016 and preserved in 1:1 70% ethanol:glycerol. Approximately 20 individuals (10 for each morph) per each of the 16 populations were dissected. In some cases, the number of available flowers per morph was smaller, due to of the small population size. Dissected flowers were photographed with Olympus TG camera (Microscope setting) and following characters were measured using Image J Software: 1) length of stigma (from the base of ovary, to the top of stigma), 2) length of stamens (from ovary base to the top of the highest anther) and 3) the length of anther (Figure 2). To get the central position of the anthers, the anther length was divided by 2 and deducted from the stamen length. After the dissection, flowers were conserved for further investigation of stigma surface and pollen size of each morph.

Results of the measurements were graphically presented in boxplots (Figure 4). The mean length of stigma and anthers in L position and in S position was calculated and tested how well stigmas and anthers in one position (L or S) overlap.
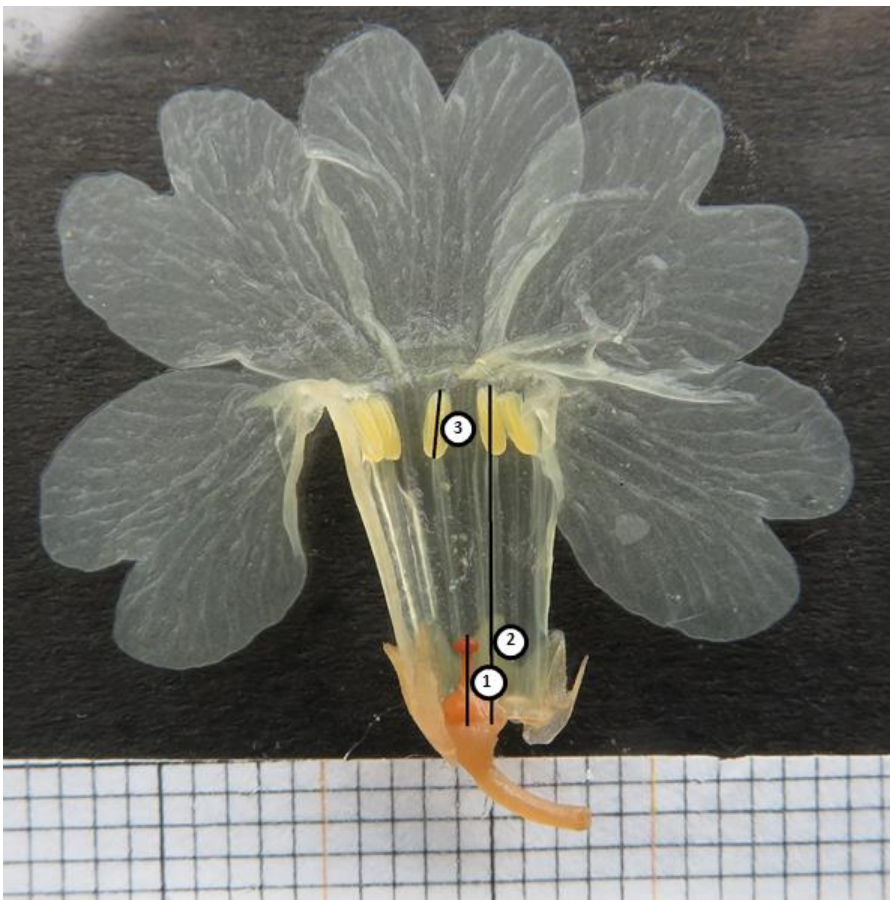

Figure 2: Characters measured for each flower: 1) stigma length, 2) stamens length and 3) anther length.

Stigma-stamen reciprocity index was calculated according to Sànchez et al. (2013 - R2; 2008 - R1). The index compares stigma-stamen height gaps for all the potential crosses in the population. The two indexes were calculated using Excel macro RECIPRO 2.0. Values of the R1 index span between 0 (prefect reciprocity) and 1 (no reciprocity), and the opposite for R2, where value 1 means perfect reciprocity.
For cartographical representation of the R2 index, software ESRI ARC GIS ver. 10.1 was used.

DESCRIPTION OF THE MAIN RESULTS OBTAINED

After calculating the percentage of both morphs in populations, we found there was no significant correlation between the population size and the ratio of both morphs within the population.

The difference between the position of stigmas and anthers is significant in all populations, which means that the anthers and stigmas within populations are well separated and their position is not overlapping, which is also evident from the boxplots in Figure 4.

We also tested how well stigmas and anthers in one position (L or S) overlap. In 9 populations (GS, PKP, SZ, CP, P5S, DRN2, KBP, MED, PRV) the mean value of the position did not differ significantly. In the other 7 populations (SKC, ISCP, KBL, CEP1, HL, RAS, PAR) the differences between stigma in higher (L) or lower (S) positions were significant (Table 1). However, this difference was not corelated neither with size of the population nor, with the morph ratio within the population.

Stigma-stamen reciprocity index was calculated according to Sànchez et al. (2013 - R2; 2008 - R1). The populations with the highest reciprocity index are SZ (R1=0.012, R2=0,88), PKP and KBP (R1=0.015, R2=0.85) and PRV, PAR (R1=0.018, R2=0.82) (Figure 3). Populations with lowest values are HL (R1=0.037, R2=0.63) and SKC, KBL, MED (R1=0.036, R2=0.64) (Figure 3). Value of the reciprocity index was not in correlation with size, nor with the morph ratio of the population.
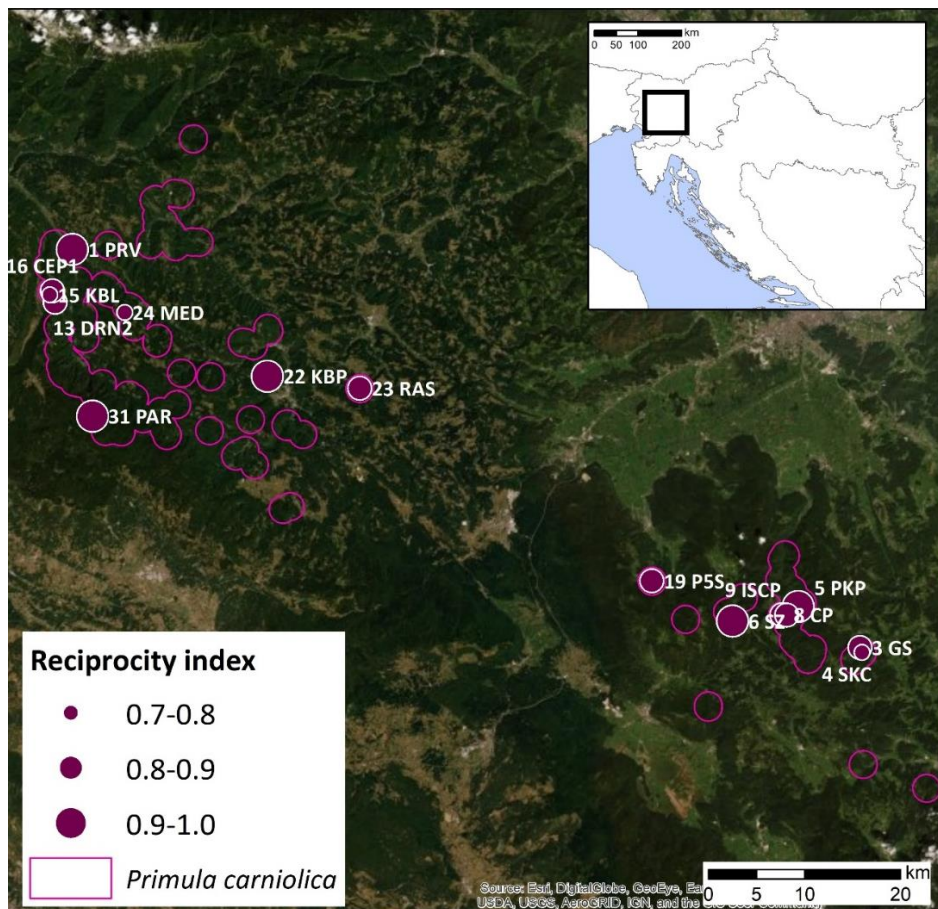

Figure 3: Population samples included in the analysis with the graphical representation of the Stigma-stamen reciprocity index values (R2) calculated by Sànchez et al. (2013).

Stigma-stamen reciprocity index (R1) according to Sànchez (2008) has already been tested for several other *Primula* species form different sections. The highest reciprocity was detected in the section *Primula* by Keller et al. (2012) for *P. veris* (R1=0.003-0.012), and somewhat lower for *P. elatior* (R1=0.005-0.030) and *P. vulgaris* (R1=0.007-0.024). However, some differences have been detected depending on the habitat in which the species lives. Brys and Jacquemyn (2015) have recorded different values for populations of *P. veris* growing in the forest (0.035) and in grassland (0.012).

Species in the section *Proliferae* also have low reciprocity index, which shows to high stigma-stamen reciprocity: *P. anisodora* (0.009-0.012), *P. baeesiana* (R1=0.007-0.015), *P. bulleyana* (0.007-0.014), *P. poissonii* (0.008-0.016) and *P. secundiflora* (0.0105) (Liu et al., 2016; Li et al., 2018).

In the section *Auricula*, to which *P. carniolica* belongs, stigma-stamen reciprocity is less pronounced. R1 values for *P. marginata* and *P. allionii* were relatively high (R1=0.042-0.043 and R1=0.043 respectively) (Casazza et al., 2017), lower values were recorded in *P. pallinuri* (R1=0,020) (Aronne et al., 2014).

Compared to other *Primula* species from this section, stigma and stamen position in *P. carniolica* are more reciprocal. Mean value for R1 stigma-stamen reciprocity index in *P. carniolica* is 0.0248, which indicates distyly (values < 0.05). The highest reciprocity was recorded in population Zala waterfall (R1=0.012), which is the lowest one so far for this section, and the lowest reciprocity was detected in population Hleviše (R1=0.037). This range between the highest and lowest values in our study is very big, however it has been recorded previously for other *Primula* species (Brys & Jacquemyn, 2015).

In populations with such high values of R1 index as in Hleviše, the function of distyly is becoming less efficient and the availability of compatible pollen load is lower in such cases. This could have long-term consequences on the population fitness, which could lead to disappearance of individual populations.

This STSM project was a preliminary study of the degree of stigma-stamen reciprocity in *P. carniolica* populations. It gave us important insights into this species reproductive biology, and this information in mind, we can now more easily determine, which are the populations we should focus on in the upcoming population genetic study. However, due to the small sample number in each population, the study should be repeated on a bigger sample in the future.

Table 1: Origin of the sampled material, position of the location within Slovenia, number of individuals with morph form investigated at the location site, number of measured individuals per morph and the mean height for the stigma and stamen positions (L – higher position, S – lower position) and values of the stigma-stamen reciprocity indexes according to Sànchez et al..

Location		Coordinates N	E	Position of the location	Total # of individuals	S / L morph in pop (%)	Measured individuals per L / S morphs	Mean height of stigma/anthers in L position (mm)	Mean height of stigma/anthers in S position (mm)	R1 value	R2 value
3 GS	Gspanova stena	45°50'15,69''	14°33'51,32''	S Slovenia	88	51 / 49	10/11	8.68/9.24	3.49/3.15	0.022	0.78
4 SKC	Kobilji curek waterfall	45°50'04,3''	14°33'58,2''	S Slovenia	15**	73 / 27†	4/8	7.98/9.28•	3.22/3.48	0.036*	0.64*
5 PKP	Krvava peč	45°51'52,40''	14°30'23,47''	S Slovenia	67**	60 / 40	10/10	9.57/9.32	3.18/3.18	0.015*	0.85*
6 SZ	Zala waterfall	45°51'17,8''	14°26'42,2''	S Slovenia	46	52 / 48	10/10	8.65/8.91	3.23/3.30	0.012*	0.88*
8 CP	Črni potok	45°51'32,8''	14°29'28,4''	S Slovenia	17**	71 / 29†	5/10	8.56/9.44	3.39/3.16	0.023*	0.77*
9 ISCP	Črni potok and Iška confluence	45°51'32,4''	14°29'44,5''	S Slovenia	75	47 / 53	10/10	9.17/10.24•	3.39/3.07	0.028*	0.78*
19 P5S	Pekel gorge	45°52'52''	14°22'11''	S Slovenia	64**	53 / 47	12/11	9.30/10.07	3.64/3.69	0.027*	0.73*
13 DRN2	Drnulk 2	46°03'44,7''	13°48'41,6''	N Slovenia	85	52 / 48	11/11	8.17/8.92	2.82/3.04	0.023*	0.77*
15 KBL	Kobilica hill	46° 4'2.18''	13°48'23.72''	N Slovenia	40**	48 / 52	10/11	8.16/9.22 •	2.98/3.58 •	0.036*	0.64*
16 CEP1	Čepovan 1	46° 4'11.35''	13°48'32.24''	N Slovenia	81	54 / 46	11/11	8.0/9.10 •	2.86/3.06	0.027*	0.73*
21 HL	Hleviše	45°59'10,5''	13°59'39,8''	N Slovenia	133	50 / 50	10/10	8.36/9.35 •	2.94/4.23•	0.037	0.63
22 KBP	Kobalove plateau	46°00'51,7''	14°00'36,5''	N Slovenia	107	66 / 34†	9/10	7.98/8.00	2.93/3.15	0.015*	0.85*
23 RAS	Raskovec gorge	46° 0'23.35''	14° 5'47.70''	N Slovenia	62**	61 / 29†	11/10	8.56/9.51•	2.82/3.16	0.021*	0.79*
24 MED	Vojsko	46° 3'20.98''	13°52'35.96''	N Slovenia	125	50 / 50	11/10	7.43/6.81	2.98/3.36	0.036*	0.64*
1 PRV	Prvejk	46°05'48,34''	13°49'39,84''	N Slovenia	73	62 / 38†	3/4	8.84/9.77	3.58/3.58	0.018*	0.82*
31 PAR	Paradana	45°59'18.5''	13°50'47.8''	N Slovenia	51**	45 / 55	13/11	8.30/8.42	3.08/2.72•	0.018*	0.82*

* p value < 0.05

• difference between the mean height of stigma and anthers in L or S position is significant (p value < 0.05)

** Populations considered as small (less than 70 individuals could be investigated at the location site)

† Population with unequal morph ratio (if more than 60% of individuals belong to only one of both morphs)
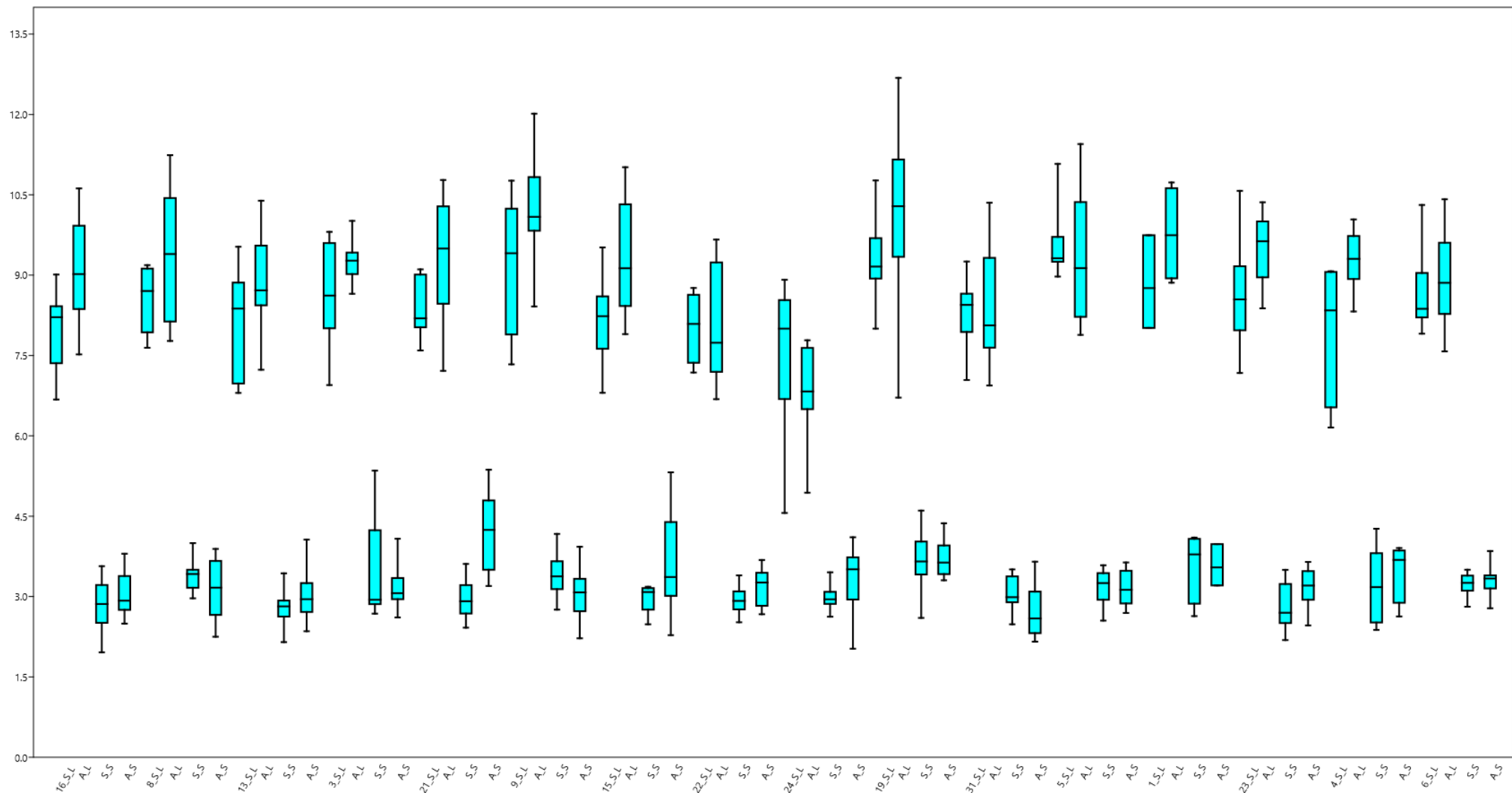
Figure 4: Boxplots for each of the 16 investigated populations S_L – log-styled stigma, S_S – short-styled stigma, A_L – anthers from short-styled morph, but corresponding to the position of long-styled stigma, A_S - anthers from long-styled morph, but corresponding to the position of short-styled stigma. Populations presented are: 16-Čepovan 1, 8- Črni potok, 13- Drnulk 2, 3- Gspanova stena, 21- Hleviše, 9- Črni potok and Iška confluence, 15- Kobilica hill, 22-Kobalove plateau, 24- Vojsko, 19- Pekel gorge, 31-Paradan, 5- Krvava peč, 1- Prvejk, 23- Raskovec gorge, 4- Waterfall Kobilji curek and 6-Zala waterfall.

FUTURE COLLABORATIONS WITH THE HOST INSTITUTION

During this STSM, the collaboration between both institutions (UP FAMNIT and Botanical Institute of Barcelona) was improved and will continue in the future. In the future, bigger sampling is planned for the populations with limited number of individuals, in order to get enough samples. Population genetics study will be made in the next months and this autumn, seeds from different populations of *Primula carniolica* and *Primula auricula* will be collected in order to start determining the germination protocol for both species, and later start with investigations of their reproductive biology. So far, 100 individuals of *Primula carniolica* (Population Vojsko) and 5 of *Primula auricula* are growing in a private garden in Slovenia that are available for our experiments. However, to investigate their reproductive biology and mechanisms behind we would need more individuals of both species from several populations. Pollen counts of studied populations will be performed at Botanical Institute of Barcelona this autumn. In future a major focus on pollinators and pollination networks should be addressed as well.

The results of this study have the potential to be included in the publication resulting from an extensive study of *Primula carniolica* reproductive biology.

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