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Stochastic variation of flower structure in *Trientalis europaea* L.

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Summary: The unique natural population of *Trientalis europaea* L. with extremely high variation of flower structure was studied by RAPD analysis. Using 20 semi-specific primers complementary to plant exon-intron boundaries, we found 9 molecular markers for DNA polymorphism analysis in *T. europaea*. The studied population displayed low DNA polymorphism: we found only 5 polymorphic variants and 3 of them were represented by single plants. These data suggest that the studied population was mostly clonal. So, high variation of flower structure in this population was not mediated by genotypic diversity. In our previous studies, we demonstrated that the structure of *T. europaea* flowers was also not determined by plant ontogeny. Moreover, various local deviations in *T. europaea* flower structure were shown to fit the Poisson distribution. These data strongly support the idea that the key role in the studied fluctuations is played by stochastic events.

Keywords: stochastic variation, *Trientalis europaea*, flower development, RAPD analysis, natural populations

The phenotype of an organism is traditionally considered to be a result of the interplay between the genotype, environment and stage of development. However, even under clear control of all three factors, remarkable phenotypic variation can be obtained. This phenomenon is known in many species and is usually referred to as incomplete penetrance (Lutova et al. 1997; Bourgeois et al. 1998; Raj et al. 2010), variable expressivity (Bourgeois et al. 1998; van Wyk et al. 2015) or fluctuating asymmetry (Kozlov 2015; Reeves et al. 2016). Thus, an additional factor participating in phenotype establishment should exist. Multiple data suggest that this factor is the stochastic nature of molecular processes underlying gene expression and gene product functioning, especially at the critical stages of ontogeny, when even slight molecular deviations can result in considerable differences in the phenotype (for a review see Tikhodeyev 2013; Ruvinsky 2016). Indeed, the role of molecular stochasticity in phenotype establishment has recently been proven to be crucial both in prokaryotes and eukaryotes (Tchuraev 2006; Raj & Van Oudenaarden 2008; Kulkarni et al. 2013).

One of the most useful models to study stochastic variation in plants is natural fluctuation in flower organ numbers (for a review see Kitazawa & Fujimoto 2014). In particular, *Trientalis europaea* L. (= *Lysimachia europaea* (L.) U. Manns & Anderb.), a small pseudo-annual angiosperm common in the boreal zone of Eurasia, displays natural fluctuation in the number of sepals, petals and stamens (Tikhodeyev & Tikhodeyeva 2001, 2002; Tikhodeyev 2012). Depending on their structure, all *T. europaea* flowers can be subdivided in two groups:

I) regular flowers (R): they possess equal number of sepals (S), petals (P) and stamens (St). 5 variants of such flowers, namely, pentamerous (R_5), hexamerous (R_6), heptamerous (R_7), octamerous (R_8) and nonamerous (R_9) have been described so far, and R_7 are usually most abundant (Charlier 1913; Hiirsalmi 1969; Grivlova & Vahrameeva 1990; Tikhodeyev 2012).

II) irregular flowers (Ir): they possess unequal number of S, P and St. Up to now, more than 40 variants of such flowers have been described. The overwhelming majority of them are the results of either single lacking (–1) or single extra (+1) flower organ; more complex alterations also occur (Tikhodeyev 2012).

Based on statistical analysis, R_7 have been considered as a norm, and two types of developmental events leading to variation in *T. europaea* flower structure have been supposed (Tikhodeyev & Tikhodeyeva 2001). First, the merosity of three outer whorls in the normal (R_7) floral meristem undergoes simultaneous alteration to 5, 6, 8 or 9, thus producing R_5, R_6, R_8 or R_9, respectively. Second, either –1 or +1 local deviation affects a single whorl; as a result, the corresponding Ir is produced. Several local deviations may independently arise in the same floral meristem, but such cases are rare and cannot explain appearance of R_5, R_6, R_8 and R_9. Thus, the structure of a certain *T. europaea* flower depends on two autonomous parameters: the merosity of three outer whorls in the floral meristem and the local deviations arisen in the meristem. These parameters will be further designated as M (from merosity) and LDs (from local deviations).

Using the abovementioned suggestions, it is possible to reconstruct the origin of each Ir (Tikhodeyev & Tikhodeyeva 2001). For example, a flower with 7 S, 8 P and 7 St is considered as Ir_{7+1P} (1 extra P in a heptamerous flower). Similarly, a flower possessing 6 S, 8 P and 8 St is considered as Ir_{8-2S} (2 lacking S in an octamerous flower). In more complicated cases, the origin of a certain flower is suggested based on the minimal required number of LDs.

Figure 1. An example of structural variation among the flowers produced by the same *T. europaea* plant.

A *T. europaea* plant can produce two or even more flowers, which are usually opened sequentially. Although these flowers possess the same genotype, they can differ in their structure (Fig. 1), and the sequence of flower opening is not significant for such difference (Tikhodeyev 2012). So, the main factors underlying natural variation in *T. europaea* flower structure are likely to be neither genetic nor ontogenetic.

The rate of Ir in natural *T. europaea* populations is usually low, about 5–10% (Tikhodeyev & Tikhodeyeva 2002). However, 7 years ago, a unique population with an extremely high rate of such flowers (about 60%) was described (Tikhodeyev 2012). Statistical analysis of the two-flowered plants in this population had shown that different types of LDs fit the Poisson distribution and thus were likely to be conditioned by stochastic events (Tikhodeyev 2012).

In this article, the unique population of *T. europaea* is studied by RAPD (Random Amplified Polymorphic DNA) analysis. We demonstrate that DNA polymorphism in this population is low: only five polymorphic variants were found, and three of them were represented just by single plants. So, the studied population is mostly clonal. This fact significantly supports our suggestion that the key origin of natural variation in *T. europaea* flower structure is stochastic.

Materials and methods

Object of study. *Trientalis europaea* (common names: chickweed wintergreen and arctic starflower; Primulaceae) is a small herbaceous clonal angiosperm common in the Eurasian boreal zone (Hegi 1908). Its reproduction is predominantly vegetative: the mother plant produces one or several tubers and dies off in late autumn, while the tubers retain in the soil till spring providing a pseudo-annual life cycle (Warming 1918; Grivlova & Vahrameeva 1990; Piqueras & Klimeš 1998). In northwest Russia, the growing season of *T. europaea* starts in May and ends in September, flowering usually takes place in June. The mature plants display significant ecological plasticity (Poljanskaja 2010). While flowering, the plant produces one or several (up to 5) actinomorphic flowers with 1 pistil and predominantly 7 S, 7 P and 7 St (Charlier 1913; Matthews & Roger 1941; Hiirsalmi 1969). However, the number of flower organs in *T. europaea* is variable, and this variation can be found even within the same plant (Tikhodeyev 2012). The seed set in *T. europaea* is rather low and seedling recruitment is also rare (Hiirsalmi 1969; Grivlova & Vahrameeva 1990).

Study area. We carried out the present study in natural populations of *T. europaea* on Konevitsa island (southwest of Ladoga Lake, Russia). This island arose about 2000 years ago due to the lowering of the Ladoga Lake level; so, its plant communities are comparably young (Verzilin & Kalmykova 2000). 80% of Konevitsa island are covered with multiple types of forests from pure *Pinus sylvestris* L. to pure *Picea abies* (L.) Karst. (Lebedeva & Tikhodeyeva 2003; Lebedeva et al. 2006). Forests with *Betula pendula* Roth, *Alnus incana* (L.) Moench and *Populus tremula* L. are also present. We studied 3 following populations of *T. europaea*:

I) Population K-1. This population was located in the *P. abies* forest at the southeast coast of the island, approximately 4 m above the lake level. In addition to *P. abies*, single mature *P. sylvestris* and *Sorbus aucuparia* L. trees were present, accompanied predominantly by *Vaccinium myrtillus* L. and *Calamagrostis arundinacea* L. with presence of *Pteridium aquilinum* (L.) Kuhn. In moss cover, *Pleurozium schreberi* (Brid.) Mitt. and *Pohlia nutans* (Hedw.) Lindb. were the most

common species. This population was large: it included more than 1000 *T. europaea* plants and occupied a territory about 4000 m^2. In our previous studies (Tikhodeyev 2012), this population displayed low rate of Ir (5–10%); that is typical of *T. europaea*.

II) Population K-2. This population was located in the *P. sylvestris* forest in the internal part of the island, approximately 10 m above the lake level. In addition to *P. sylvestris*, rare young plants of *S. aucuparia* L. were also present. Shrub layer was represented predominantly by *V. myrtillus*, *V. vitis-idaea* L. and *Calluna vulgaris* L. Moss cover was formed by *P. schreberi* and *Dicranum polysetum* Sw. This population was small: it included less than 200 *T. europaea* plants and occupied a territory about merely 100 m^2. In our previous studies (Tikhodeyev 2012), this population displayed extremely high rate of Ir (about 60%); that is unique to *T. europaea*.

III) Population K-3. This population was located in the *P. sylvestris* forest at the precipitous southwest coast of the island, approximately 8 m above the lake level. The tree, shrub and moss covers were similar to those of K-2. This population was as large as K-1. In our previous studies, it displayed medium rate of Ir (about 40%; unpublished).

Analysis of *T. europaea* flower structure. In the first decade of June 2015, we analyzed randomly chosen plants with undamaged first flowers in each population of *T. europaea*. We counted the number of S, P and St in each flower, thus getting the formula $S_xP_ySt_z$. If a certain P was cloven even slightly, we counted the number of its central veins, and each of them was considered as a separate P (Fig. 2). Based on the obtained formula, we reconstructed the origin of the flower, i.e. M and the arisen LDs. To analyze the second flower, if it opened, we labeled each studied plant and repeatedly screened it for several times till June 25. Similar analysis was carried out in June 2016.

Plant material harvesting and DNA isolation. From each *T. europaea* plant studied in 2016, we harvested 1–3 leaves for DNA isolation. Harvesting was accomplished in the end of June, when the flower structure analysis had been completed. We fixed the leaves in 1 ml of RNA*later*™ (a liquid for RNA stabilization; see https://www.thermofisher.com/order/catalog/product/AM7021). For DNA isolation, we used the CTAB protocol for plant tissues (Cullings 1992).

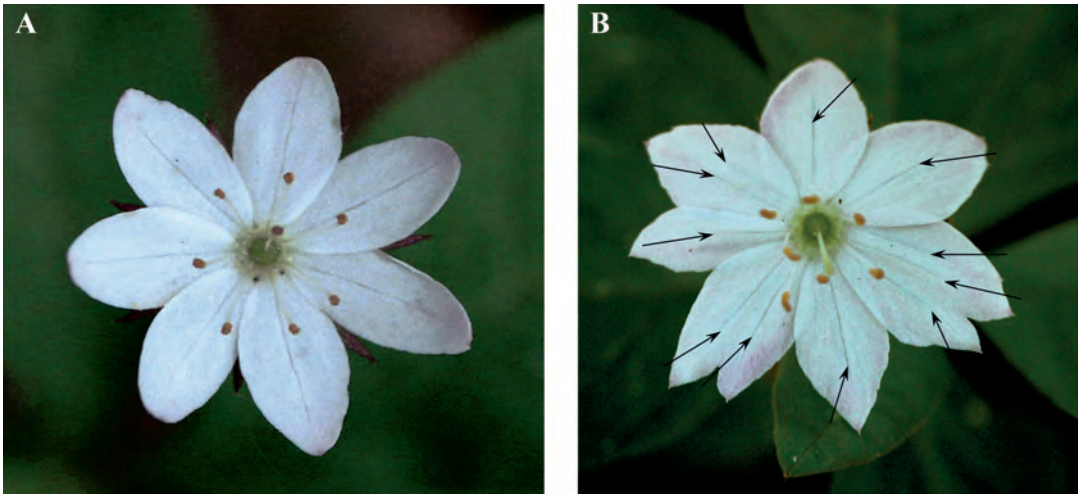

Figure 2. P count in *T. europaea*. A – the normal flower, in each P a single central vein is seen; B – the flower with 4 normal and 3 cloven P, the arrows indicate 11 central veins.

Table 1. The semi-specific primers used in RAPD analysis (Matveeva et al. 2008).

Primer	Sequence	Primer	Sequence
SR1	5'-AGCAGGTCAGGC-3'	SR11	5'-ACTTACCTGCCCTTC-3'
SR2	5'-AGCAGGTTGCCG-3'	SR12	5'-ACTTACCTGGAGCTG-3'
SR3	5'-AGCAGGTAGTCA-3'	SR13	5'-ACTTACCTGAGCCAC-3'
SR4	5'-AGCAGGTAATCG-3'	SR14	5'-ACTTACCTGCGCCGT-3'
SR5	5'-AGCAGGTAGGTC-3'	SR15	5'-ACTTACCTGGTCTTG-3'
SR6	5'-AGCAGGTGGTCC-3'	SR16	5'-ACTTACCTGCCTGAC-3'
SR7	5'-AGCAGGTGAACG-3'	SR17	5'-ACTTACCTGCGGGTG-3'
SR8	5'-AGCAGGTGTGAC-3'	SR18	5'-ACTTACCTGCGTAGG-3'
SR9	5'-AGCAGGTGGGTA-3'	SR19	5'-ACTTACCTGAACGCC-3'
SR10	5'-AGCAGGTGTGAT-3'	SR20	5'-ACTTACCTGTCGCAG-3'

RAPD analysis. PCR for RAPD analysis was performed with Taq polymerase (Evrogen, Russia) or SynTaq polymerase (Syntol, Russia) using the following program: 5 min at 95°C, then 35 cycles of 30 sec at 95°C, 30 sec at 45°C and 1 min 20 sec at 72°C and finally 5 min at 72°C. For each reaction, we used 40–80 ng of DNA.

We used a set of 20 semi-specific primers complementary to plants exon-intron boundaries (Table 1). 20 pairwise combinations of these primers (SR1+SR11, SR2+SR12, SR3+SR13, SR4+SR14, SR5+SR15, SR6+SR16, SR7+SR17, SR8+SR18, SR9+SR19, SR10+SR20, SR6+SR7, SR6+SR9, SR6+SR10, SR6+SR13, SR7+SR9, SR7+SR10, SR7+SR13, SR9+SR10, SR9+SR13 and SR10+SR13) were also used.

Statistical analysis. We used Fisher's exact test to compare the ratios between different types of plants or flowers.

Results

In 2015 and 2016, we analyzed the structure of *T. europaea* flowers in three natural populations on Konevitsa island and compared the obtained results with our previously published data. Despite dramatic differences in the weather (spring 2016 in Ladoga Lake region was atypically warm and caused a half month shift in vegetation time) each population stably reproduced the rate of Ir and the total frequency of LDs (Table 2). Thus, both parameters were quite stable with low dependence on the environmental influences. The ratio between the flowers with different M was more variable. In particular, the number of hexamerous flowers significantly increased in all three populations ($P_{Ho} < 0.001$) in 2016.

We labeled each studied plant and analyzed the structure of the second flower, if it opened. In different years, the number of the two-flowered plants significantly varied and appeared to be especially low in 2016 (Table 3). So, this parameter, like the ratio between the flowers with different M, was also strongly dependent on environmental influences. The amount of the obtained two-flowered plants was enough for statistical analysis in 2015 only. That year as well as in our previous study (Tikhodeyev 2012), we found no significant differences between the first

Table 2. Variation in flower structure in the studied populations of *T. europaea.*

Population	Year of study	Studied flowers									P_{Ho}[a]	f[b]	P_{Ho}[c]
		Σ	M						among them				
			5	6	7	8	9	10[d]	R	Ir			
K-1	2010[e]	82	0	17	61	4	0	0	75 (91%)	7 (9%)	0.79	0.09	0.84
	2015	83	1	18	64	0	0	0	78 (94%)	5 (6%)		0.06	
	2016	151	0	60	91	0	0	0	141 (93%)	10 (7%)		0.07	
K-2	2010[e]	220	1	2	97	106	12	2	84 (38%)	136 (62%)	0.92	0.83	0.78
	2015	205	0	7	135	58	5	0	82 (40%)	123 (60%)		0.75	
	2016	37	0	7	23	6	1	0	14 (38%)	23 (62%)		0.78	
K-3	2015	59	0	32	23	4	0	0	34 (58%)	25 (42%)	0.35	0.44	0.94
	2016	309	0	229	72	7	1	0	198 (54%)	111 (36%)		0.45	

[a] probability that the differences in the rate of Ir were random
[b] total frequency of LDs (all –1/+1 LDs were summarized)
[c] probability that the differences in the total frequency of LDs were random
[d] up to now, only irregular flowers with this M have been described
[e] Tikhodeyev (2012)

and the second flowers in M (Table 4) and the total frequency of LDs (Table 5). Thus, the sequence of flower opening is not the key factor underlying structural variation of *T. europaea* flowers.

From each *T. europaea* plant studied in 2016, we harvested several leaves for DNA analysis. That year, due to atypically warm spring, the majority of the plants in K-2 had completed their flowering before our work on Konevitsa island started. As a result, we could study only 37 single-flowered plants in this population. Since this amount was rather small, we also used 63 already deflorated plants from K-2. DNA from some plants appeared to be inappropriate for further analyses due to its low quality. Totally, we involved 297 DNA samples in RAPD analysis (66 from K-1, 92 from K-2, and 139 from K-3).

Using 20 semi-specific primers and their pairwise combinations (see Materials and Methods) we found 9 molecular markers suitable for RAPD analysis in *T. europaea*. 8 of these markers were obtained with the SR9+SR10 combination and were designated as #1–#8 (Fig. 3). One more suitable molecular marker was obtained with SR9 alone and was designated as #9 (Fig. 4). All

Table 3. The ratio between the single-flowered and two-flowered plants in the studied *T. europaea* populations.

Population	Year of study	Studied plants	among them		P_{Ho}[a]
			single-flowered	two-flowered	
K-1	2015	45	7	38	< 0.0001
	2016	150	149	1	
K-2	2015	179	153	26	0.013
	2016	37	37	0	
K-3	2015	32	5	27	< 0.0001
	2016	301	293	8	
Combined data for 2015		256	165	91	< 0.0001
Combined data for 2016		488	479	9	

[a] the probability that the differences in the ratio between the single-flowered and two-flowered plants were random.

Table 4. M differences between the first and second flowers of the two-flowered *T. europaea* plants (2015).

Population	Plants with				
	$\Delta = 0$	$\Delta = 1$		$\Delta = 2$	
		$M_I > M_{II}$	$M_I < M_{II}$	$M_I > M_{II}$	$M_I < M_{II}$
K-1 (n = 38)	28	4	5	0	1
K-2 (n = 26)	14	7	5	0	0
K-3 (n = 27)	15	2	6	4	0
P_{Ho}		> 0.60		–	

Notes: M_I – M of the first flower; M_{II} – M of the second flower in the same plant; Δ – difference between M_I and M_{II}; P_{Ho} – probability that the ratio between the $M_I > M_{II}$ and $M_I < M_{II}$ plants corresponds to 1 : 1; the amount of the two-flowered plants with $\Delta = 2$ was too small for statistical analysis.

other primers or their combinations gave similar sets of the PCR products in different *T. europaea* plants (data not shown).

In the studied natural populations of *T. europaea*, we found 53 polymorphic variants differing in at least one out of 9 molecular markers used (Table 6). The DNA samples from K-2 displayed low polymorphism (Fig. 5). Among these samples, we found only 5 polymorphic variants (No. 17, 27, 44, 45 and 52) and 3 of them (No. 17, 27 and 45) were represented by single plants. So, this small population was mostly clonal with very few founders. Both polymorphic variants abundant in K-2 (No. 44 and 52) showed similar high variation in flower structure (Table 7). Thus, there were no considerable relations between certain genotypes and flower structure in this population.

The DNA samples from K-1 and K-3 were highly polymorphic (Fig. 3; Table 6). We suppose that each of these populations originated from multiple genetically different founders whose clones have eventually overlapped and mixed. The fact that both K-1 and K-3 are significantly larger than K-2 is in good agreement with our suggestion. Notably, the polymorphic variants most abundant in K-2 (No. 44 and 52) were quite rare in both K-1 and K-3.

Discussion

Analysis of natural fluctuations in the flower organ numbers is a quickly growing branch of modern botany (Ellstrand 1983; Tikhodeyev et al. 2003; Choob & Yurtseva 2007; Ren

Table 5. LDs in the first and the second flowers of the two-flowered *T. europaea* plants (2015).

Population	Flowers	Total number of LDs						P_{Ho}
		–S	+S	–P	+P	–St	+St	
K-1	I (n = 38)	0	0	0	2	0	1	–
	II (n = 38)	0	0	0	1	1	0	
K-2	I (n = 26)	1	1	0	4	10	0	0.94
	II (n = 26)	1	1	0	2	9	0	
K-3	I (n = 27)	1	1	0	5	6	1	0.53
	II (n = 27)	0	0	0	6	3	0	
Combined data	I (n = 91)	2	2	0	11	16	2	0.77
	II (n = 91)	1	1	0	9	13	0	

Notes: I – first flowers; II – second flowers; P_{Ho} – probability that the differences in the frequencies of each type of –1 or +1 LDs were random; the amount of the two-flowered plants with LDs from K-1 was too small for statistical analysis.

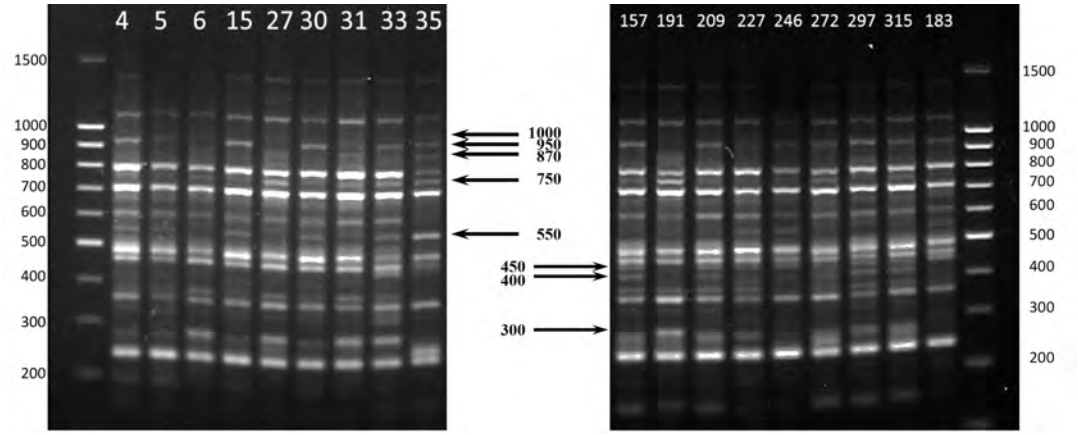

Figure 3. The PCR products obtained with the SR9+SR10 combination (SynTaq polymerase was used). Different plants from K-1 and K-3 are analyzed. The arrows indicate 8 molecular markers suitable for RAPD analysis in *T. europaea*; we designated these markers as #1–#8 in accordance with their length decrease. The approximate length (bp) of each molecular marker is shown near the corresponding arrow. Other PCR products were either present in all DNA samples, or poorly reproducible. Molecular weight standards and their lengths (bp) are shown at the left and right sides of the figure.

et al. 2010; Kitazawa & Fujimoto 2014; Ronse De Craene 2015). In many species, especially in Ranunculaceae, such fluctuations are believed to be stochastic since they fit statistics like the Poisson or beta distribution (for a review see Kitazawa & Fujimoto 2014). However, only one type of flower organs is usually analyzed; therefore, the origin of a certain fluctuation (M alteration, LD or both) remains unclear. As a result, different developmental events may be erroneously considered as identical, thus leading to wrong conclusions (Fig. 6).

Trientalis europaea is a suitable model plant in which the origin of a certain fluctuation can be reconstructed based on the flower formula $S_xP_ySt_z$ (Tikhodeyev & Tikhodeyeva 2001). Using this approach, we have previously demonstrated that all abovementioned mechanisms could be involved in flower structure variation even in the same *T. europaea* plant (Tikhodeyev 2012).

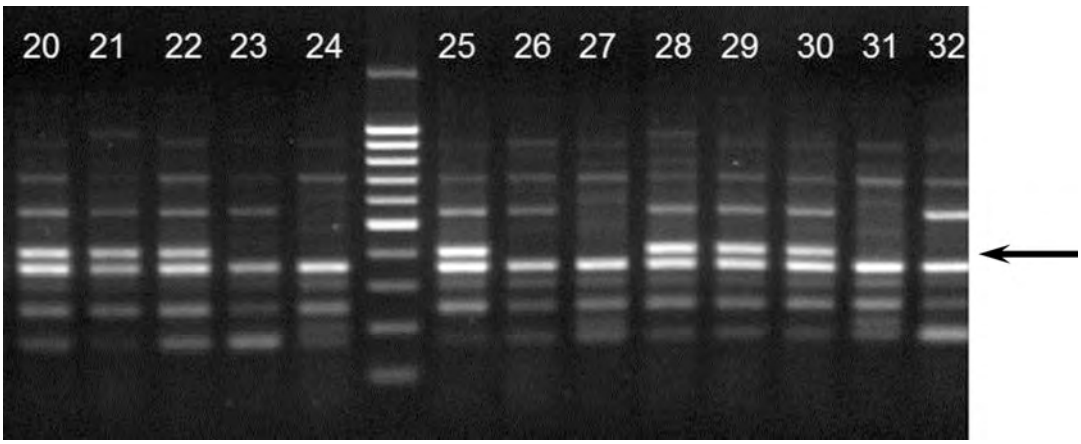

Figure 4. The PCR products obtained with SR9 alone (Taq polymerase was used). Different plants from K-1, K-2 and K-3 are analyzed. The arrow indicates one molecular marker (approximately 400 bp) suitable for RAPD analysis in *T. europaea* and designated as #9. Other PCR products were either present in all DNA samples or poorly reproducible. Molecular weight standards are the same as on Fig. 3.

Table 6. The ratios between different polymorphic variants in the studied *T. europaea* populations.

Polymorphic variants	Molecular markers									Populations		
	#1	#2	#3	#4	#5	#6	#7	#8	#9	K-1	K-2	K-3
1	-	-	-	-	-	-	-	-	+	0	0	1
2	-	-	-	-	+	+	+	-	+	9	0	2
3	-	-	-	+	-	-	-	+	-	2	0	0
4	-	-	-	+	-	-	+	+	+	1	0	0
5	-	-	-	+	+	-	+	+	-	2	0	0
6	-	-	+	-	-	-	-	+	-	1	0	0
7	-	-	+	-	+	-	-	-	+	1	0	0
8	-	-	+	-	+	+	+	-	+	0	0	1
9	-	-	+	+	+	-	+	+	-	2	0	0
10	-	-	+	+	-	-	+	+	+	1	0	0
11	-	+	-	-	-	-	-	-	+	5	0	7
12	-	+	-	-	-	-	-	-	-	0	0	2
13	-	+	-	-	-	+	+	-	+	0	0	13
14	-	+	-	-	-	+	+	+	-	0	0	7
15	-	+	-	-	+	-	-	-	+	2	0	7
16	-	+	-	-	+	-	-	-	-	2	0	0
17	**-**	**+**	**-**	**-**	**+**	**-**	**+**	**-**	**+**	**1**	**1**	**0**
18	-	+	-	-	+	-	+	-	-	1	0	0
19	-	+	-	-	+	+	+	-	+	1	0	14
20	-	+	-	+	-	-	-	-	+	0	0	13
21	-	+	-	+	-	-	+	+	+	0	0	1
22	-	+	-	+	-	+	+	-	+	2	0	18
23	-	+	-	+	+	-	-	-	+	3	0	4
24	-	+	-	+	+	-	-	-	-	2	0	0
25	-	+	-	+	+	-	+	-	+	0	0	2
26	-	+	-	+	+	-	+	+	+	1	0	0
27	**-**	**+**	**-**	**+**	**+**	**+**	**+**	**-**	**+**	**0**	**2**	**9**
28	-	+	+	-	-	-	-	-	+	1	0	0
29	-	+	+	-	-	+	+	-	+	0	0	2
30	-	+	+	-	-	+	+	-	+	0	0	1
31	-	+	+	-	-	+	+	+	+	0	0	2
32	-	+	+	-	+	-	-	-	+	7	0	0
33	-	+	+	-	+	+	+	-	+	0	0	4
34	-	+	+	+	-	-	-	-	+	0	0	2
35	-	+	+	+	-	+	+	-	+	0	0	4
36	-	+	+	+	-	+	+	+	+	0	0	6
37	-	+	+	+	-	-	-	-	+	0	0	2
38	-	+	+	+	-	+	+	-	+	0	0	2
39	-	+	+	+	+	-	-	-	+	6	0	5
40	-	+	+	+	+	-	-	-	-	1	0	0
41	-	+	+	+	+	-	-	+	+	2	0	0
42	-	+	+	+	+	-	+	-	+	1	0	3
43	-	+	+	+	+	-	+	-	-	1	0	0
44	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**+**	**0**	**59**	**5**
45	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0**	**1**	**0**
46	+	-	-	-	+	-	-	-	+	1	0	0
47	+	+	-	-	+	-	-	-	+	0	0	1
48	+	+	-	+	+	+	+	-	+	0	0	2
49	+	+	+	-	-	+	+	-	+	0	0	1
50	+	+	+	-	+	-	-	-	+	2	0	1
51	+	+	+	-	+	-	-	-	-	1	0	0
52	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**+**	**3**	**29**	**1**
53	+	+	+	+	+	+	+	+	+	2	0	0
The total number of the found polymorphic variants										30	5	32
Studied plants										66	92	139

Note: The polymorphic variants found in K-2 are marked in bold font.

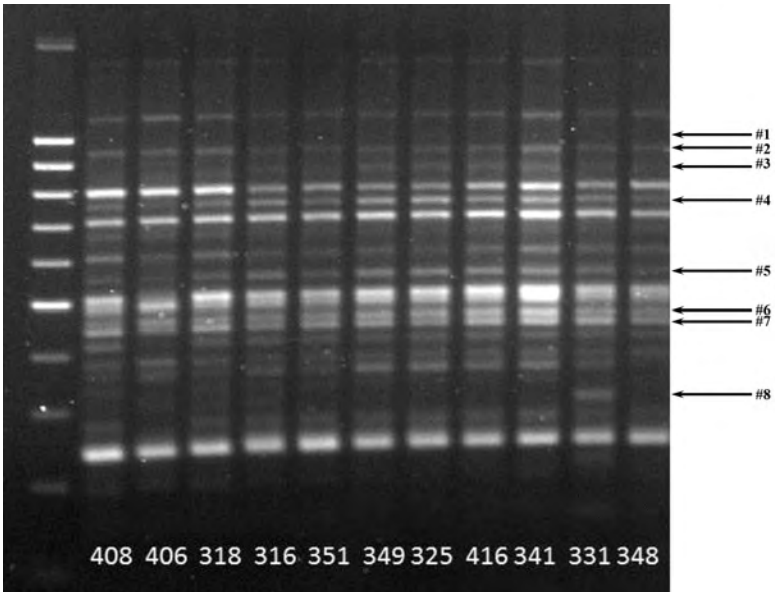

Figure 5. Low DNA polymorphism in K-2. The DNA samples of 11 plants are demonstrated. The PCR products were obtained with the SR9+SR10 combination (SynTaq polymerase was used). The arrows indicate molecular markers #1–#8. #1 is missing in all shown DNA samples except 325. #2, #5, #6 and #7 are characteristic of all DNA samples from K-2. #3 is characteristic of all shown DNA samples except 408 and 348. #4 is characteristic of all shown DNA samples except 406. #8 is missing in all shown DNA samples except 331. Molecular weight standards are the same as in Fig. 3.

Moreover, we have shown that each type of LDs fit the Poisson distribution (Tikhodeyev et al. 2003; Tikhodeyev 2012). But this fact itself did not prove that the studied events were exactly stochastic. To make an adequate conclusion, the impacts of the genotypic diversity, environmental influences and plant ontogeny in *T. europaea* flower structure should be estimated.

In the present article, we demonstrate that the rate of Ir and the total frequency of LDs were stably reproduced during several years in three natural populations of *T. europaea* despite significant differences in the weather (atypically warm spring 2016). Moreover, our results show that the studied variation is not dependent on the flower opening sequence. Thus, the role of environmental influences and plant ontogeny on variation of *T. europaea* flower structure is not crucial.

Table 7. Variation of flower structure in the most abundant polymorphic variants from K-2.

Polymorphic variants	Flower types								Rate of Ir	Total frequency of LDs
	R_6	I_{r6+1P}	I_{r6+1S}	R_7	I_{r7-1S}	I_{r8-1S}	I_{r8-2S}	Σ^a		
No. 44	2	1	1	4	7	2	1	18	67%	0.72
	M_6: 4^b			M_7: 11^b		M_8: 3^b				
No. 52	0	1	0	2	1	1	0	5	60%	0.60
	M_6: 1^b			M_7: 3^b		M_8: 1^b				
$P_{Ho} = 0.79^c$									$P_{Ho} = 0.89$	$P_{Ho} = 0.82$

[a] total number of the flowering plants studied by RAPD analysis
[b] total number of the flowers with certain M
[c] the probability that the differences in the ratios between 7 obtained types of flowers were random

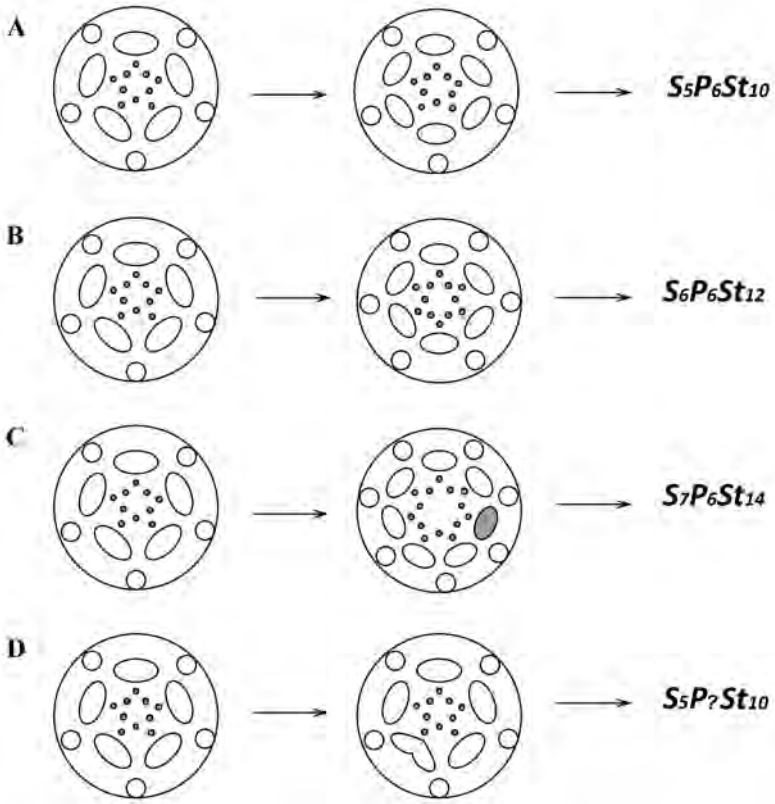

Figure 6. Schematic diagram of several possible ways underlying P hexamery in a hypothetical angiosperm with normal flower formula $S_5P_5St_{10}$. Potential sites for S, P and St formation are shown as circles, ovals and spots, respectively. A – the +1 LD in the P whorl; B – alteration of M resulting in 20% increase in the numbers of S, P and St; C – alteration of M resulting in 40% increase in the numbers of S, P and St, with the –1 LD in the P whorl; the affected site is shown in gray; D – one of the produced P is cloven. In A, B and C, the number of P is equal, but the underlying events occur with different frequencies and thus should be considered separately, otherwise statistical conclusions might be wrong. In D, subjective decisions in P count (5 or 6) are usually made and statistical analysis becomes inadequate; to avoid such subjectivity, additional criteria for P count are strongly required (see Materials and Methods and Fig. 2).

Up to now, only one study of molecular polymorphism in natural populations of *T. europaea* has been published (Patsias & Bruelheide 2011). However, variation of flower structure was not analyzed there. So, the present study is the first one, where the role of the genotype of *T. europaea* flower structure is investigated. Using RAPD analysis, we could demonstrate that the unique *T. europaea* population with extremely high rate of Ir was mostly clonal and included just several polymorphic variants. Moreover, we found no relations between flower structure and certain polymorphic variants in this population. So, high variation of *T. europaea* flower structure was not conditioned by the genotypic diversity, environmental influences or flower opening sequence. These data significantly support our suggestion that the key developmental events underlying the studied variation are exactly stochastic.

Extremely high variation of flower structure in K-2 can be explained in two ways. First, the founder(s) of this mostly clonal population might be genetically predisposed for high frequency of stochastic alterations in flower structure (the genotype does not determine the structure of a certain flower, but either provokes extra high sensitivity of a floral meristem to molecular stochasticity or strongly increases the level of molecular stochasticity). Since the polymorphic

variants No. 44 and 52 (most abundant in K-2) are rare both in K-1 and K-3, such suggestion seems to be quite realistic. Second, some specific environmental influences in K-2 might induce high frequency of various fluctuations in *T. europaea* floral meristems. This idea is less appealing because plant communities and abiotic factors in K-2 and K-3 are outwardly very similar, and the unique features of K-2 have been stably reproduced at least for 6 years.

Stochastic alterations of the phenotype under the same genotype, environmental influences, and developmental stage have been known for almost a century (Timofeev-Ressovski 1925; Astauroff 1930). However, they became included in the general concept of variability just recently (for a review see Tikhodeyev 2013). Such alterations represent a separate type of variability called autonomous (Astauroff 1930), random (Gartner 1990), realizational (Strunnikov & Vyshinski 1991) or fluctuational (Tikhodeyev & Zhurina 2004). This is an important step towards upgrading the basic biological concepts in correspondence with multiple 'non-canonical' data obtained since the end of the 20th century.

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