Cardamine × rhodopaea (Brassicaceae), a triploid hybrid from the West Rhodope Mts: Morphology, distribution, relationships and origin

Minčo Ančev[1], Petya Yurukova-Grancharova[1], Pepa Ignatova[1], Valentina Goranova[1], Stoyan Stoyanov[1], Elina Yankova-Tsvetkova[1] & Neyko Neykov[2]

[1] Department of Plant and Fungal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Science, Acad. G. Bonchev St., Bl. 23, 1113 Sofia, Bulgaria; e-mail: botmanch@bio.bas.bg (corresponding author)

[2] National Institute of Meteorology and Hydrology, Bulgarian Academy of Sciences, 66 Tsarigradsko chaussee, 1784 Sofia, Bulgaria; e-mail: neyko.neykov@meteo.bg

Abstract.   In the Bulgarian flora, the *Cardamine pratensis* group is represented by *C. penzesii, C. matthioli* and *C. rivularis.* In the West Rhodope Mts, in localities where *C. matthioli*, and *C. rivularis* occur sympatrically or parapatrically, populations of the triploid *C. × rhodopaea* occur. The morphological, karyological and embryological studies confirm its intermediate position between *C. matthioli* and *C. rivularis* in support of our hypothesis for its hybrid origin. This is the latest case of interspecific hybridization at diploid level and speciation with origin of triploid hybrids in the genus *Cardamine*, two of them being in the South Balkans, a region known as a glacial refuge.

Introduction

In the course of biosystematic studies of the family *Brassicaceae* in Bulgarian flora (Ančev 2001), a few specimens morphologically close to *Cardamine rivularis*, and a sample of live plants from a collection kindly submitted by Dr Antonina Vitkova, turned to be triploid and gave rise to the start of a project of more detailed investigation of the *Cardamine pratensis* group in the West Rhodope Mts. In Bulgarian flora, the European polyploid complex of *Cardamine pratensis*, known for its taxonomic comlexity (Lövkvist 1956; Marhold 1994, 1996; Franzke & Hurka 2000), is represented by the diploids *C. penzesii* Ančev & Marhold, *C. matthioli* Moretti and *C. rivularis* Schur, and a recently reported triploid with the provisonal name *C. × rhodopaea* (Ančev 2006, 2007). The area of dis-

tribution of *C. penzesii* is situated along the southern part of the Bulgarian western Black Sea Coast, and the coastal area of Northwest Turkey (Marhold & Ančev 1999). In a review summarizing the recent results from the investigations of representatives of the *Cardamine pratensis* complex in Europe (morphology, molecular systematics and biogeography), Franzke & Hurka (2000) classified *C. penzesii,* together with *C. rivularis,* in a "basal group" restricted to the Southeast Balkans. The geographical distribution of *C. rivularis* includes the South Carpathians and the high Bulgarian mountains: W. & C. Stara Planina, Mt Vitosha, Belasitza, Pirin, Rila, and W. & C. Rhodope Mts, at altitudes from (1000) 1400 m up to 2500 m. *C. matthioli* is distributed in C. and S. Europe (Pannonia and Carpathians, and southwards on the Balkan Peninsula in Albania, Serbia, Bulgaria, and Mainland Greece)

(Marhold & Tan 1999). In the Bulgarian flora, the species frequents the mountains in the central and southwestern part of the country. In the West Rhodope Mts, where *C. matthioli* and *C. rivularis* occur sympatrically or parapatrically, populations of triploid plants occur (Ančev 2006; Ančev & al. 2009). Furthermore, in this paper the athors have validated the earlier reported triploid with the provisional name *C. × rhodopaea* on the basis of its morphology, chromosome number, and results from tentative embryological studies (Ančev 2006). Presently, we have summarized the results from the recently provided morphological, karyological and embryological investigations on *C. × rhodopaea* and its putative parental species, as well as their distribution in the West Rhodope Mts., and have checked the hypothesis for its hybrid origin from morphologically closely related diploids *C. matthioli* and *C. rivularis*.

Material and methods

The investigations into *Cardamine × rhodopaea*, *Cardamine matthioli* and *Cardamine rivularis* – morhology, ecology, distribution, chromosome numbers and karyotypes, embryology and pollen quality – are based on field studies and field plant samples, as well as on herbarium specimens deposited in SO, SOA, SOM (Table 1; Appendix 1).

For evaluation of morphological variation in *C. × rhodopaea,* 10 quantitative characters (131 plants from five populations) were included in the morphometric analyses. Carnoy software (Schols & al. 2002) was used to measure up dried and then scanned floral parts, attached to paper by adhesive tape. The following characters, which proved to be useful in earlier studies of the *Cardamine pratensis* group (cf. Marhold 1996, Lihova & al. 2003, 2007), were measured: number of cauline leaves**,** number of leaflets on the stem leaf nearest to midpoint of the leafy part of the stem, number of leaflets on the middle cauline leaf, number of leaflets of rosette leaves**,** number of lateral inflorescences (longer than 1 cm), average length of sepals, average length of petals, average width of petals, average length of filaments of longer stamens, and average length of filaments of shorter stamens. In the description of species, 5 % and 95 % percentiles were set for the character value ranges, with 1 % and 99 % pecentiles in brack-

ets. Multiple boxplots and Wilks' Lambda statistics (Johnson & Wichern 2007) were used, in order to assess the *Cardamine* species distinction.

The chromosome counts and karyotype analysis were based on metaphase plates of root-tip mitosis fixed in the field or in the laboratory from individuals transplanted and cultivated in a greenhouse. Plants from 10 populations (30 individuals) of *C. × rhodopaea* were karyologically studied. In *C. matthioli* were studied 15 individuals from five populations, and in *C. rivularis* 22 individuals from seven populations.

The root tips were pretreated with 0.01 % colchicine for 30 min and fixed in ethanol/glacial acetic acid (3:1) for 24 hr in a refrigerator. Hydrolyzation was conducted in 1N HCl at 60 °C for 15 min. The root tips were transferred in HCl/ethyl ether (1:1) for 8 min, stained with haematoxylin after Gomori (Melander & Wingstrand 1953) for 30 min at 60 °C, squashed in 45 % acetic acid and mounted in Euparal. The chromosome lengths were measured with the programme Universal Desktop Ruler, v. 3.5 (AVPSoft, 2002–2010).

The material for embryological study (flower buds and open flowers) was collected periodically from individuals of 12 native populations (two from *C. mathioli*; three from *C. rivularis* and seven from *C × rhodopaea*) and fixed in a mixture of FAA (formalin: glacial acetic acid: 70 % ethanol in correlation 5:5:90 parts). Consecutively, the fixed plant material was treated according to the classical paraffin methods (Romeis 1948): embedded in paraffin; cut into 6–12 μm sections with a rotary microtome; and the sections stained with Heidenhain's haematoxylin and embedded in Enthelan, in order to get permanent slides. Differential staining revealed the features of the embryological structures and processes in male and female generative spheres during the ontogenesis, and made possible evaluation of the mature pollen quality.

Regarding pollen viability (potential fertility), the pollen grains in 40 anthers in each of the studied populations were numbered in a visual field at 100× magnification. According to the intensity of differential staining, the pollen was classified into two groups: 1. Viable pollen grains, potentially fertile and effective in the pollination process (stained pollen grains with reticulated sculpture and clearly distinguished vegetative and generative cells or sperms); 2. Non-viable pollen grains, sterile and inefficient (colourless or darkly stained degenerating pollen grains in which the vege-

Table 1. Population codes and origin of the plant samples and plant material (cultivated plants, root-tips, flower buds and flowers) from *Cardamine × rhodopaea*, *C. matthioli* and *C. rivularis*, used for the analyses: Morphometric analysis (Mma); Mitotic chromosome numbers, and karyotype drawings (2n, fig.); Embryological studies (Ems). Studied area: Bulgaria, W. Rhodope Mts. Fs – Forestry station; Nr – Natural reserve; Ps – protected site.

	Origin of the plant samples and plant material	Mma	2n	Ems
C. matthioli				
A0043	Fs Selishte, wet meadow, 1300 m, 05.06.2005		16	
A00563	Ps Meandri at river Ribna, Kara-tepe, wet meadows and brooks, 1450 m, 05.06.2005		16 Pl. I - B	+
A00579	Fs Beglika, east of Rakovo dere, 1600 m, wet meadows, 08.06.2005		16 Pl. I - B	+
A00631	Sout-east of Yundola, meadows, 1250 m, 05.06.2006		16 Pl. I - B	
A00617	South-east of Yundola, meadows, 1250 - 1300 m 05.06.2006		16	
C. rivularis				
A00571	West of vill. of Pobit kamak, 1300 m, wet meadows and brooks. 06.06.2005		16 Pl. I - C	+
A00577	Nr Beglika, wet meadows and brooks, 1600 m, 07.07.2005		16 Pl. I - C	+
A00649	Kartala, wet meadows and peat-bogs,1620-1650 m, 19.07.2006		16 Pl. I- C	
A00657	Dospatski pass, in the surroundings of Djenevra art. lake, 1550 m, wet meadows and peat-bogs along pine forests. 20.07.2006		16 Pl. III - C	
A00851	Komitite, south-west of dam Goljam Beglik, 1570 m, 23.06.2008.		16	
A00855	Nr Beglika,wet glades west of the Forestry Station, 1650 m, 24.06.2008.		16 Pl. I - C	
A00637	Rila Mt., brooks and peat-bogs above dam Belmeken, 1830 m, 18.07.2006		16	+
C. × rhodopaea				
A00561	Chepinska river valley, Ps Meandrite, 1240 m, 05.06.2005		24 Pl. I - A	+
A00565	Fs Selishte, 1300 m, wet glades along coniferous forest outskirts. 05.06.2005.	+	24 Pl. I - A	
A00567	Wet meadows along Dospat reiver, sout-east of Medeni poljani, 1300 m, 06.06.2005	+	24 Pl. I - A	+
A00569	Wet meadows between Pobit kamak and Medeni poljany, 1300 m, 06.06.2005	+	24	+
A00573	About 2 km west of Pobit kamak, in wet meadows and along brooks, 1300 m, 06.06.2005	+	24 Pl. I - A	
A00575	Kartala, wet meadows and peat-bogs,1620-1650 m 19.07.2006		24 Pl. I - A	+
A00615	Glades along brooks sout-east of Yundola, 1250 m 05.06.2006		24	
A00623	Soth of Fs Besslet, wet meadows, 1800 m, 06.06. 2006		24	+
A00625	Fs Besslet, along brooks in wet meadows,1800-1850 m, 06.06.2006		24 Pl. I - A	+
A00627	Ps Meandri at river Ribna, Kara-tepe, wet meadows and brooks, 1400 – 1450 m, 07.06.2006	+	24	+

tative and generative cell or sperms were not discernable and also empty, transparent, shrunken, without clear sculpture). The chromosome number, karyotypes, main embryological structure and processes in the male and female generative sphere, as well as pollen fertility were established after observations with LM "Olympus" CX2. Microphotographs were taken by high-performance NICON Camera and LM Olympus-UCMAA3. Voucher specimens from all analyses performed in this study were deposited in SOM.

Results

Cardamine × *rhodopaea* Ančev, sp. nov. (Fig. 1)

Diagnosis. Perennial herb, (20) 35–50 (55) cm tall. Stem erect, simple. Rosette leaves pinnate, with (5) 7–25 shortly petiolulate, or almost sessile, broadly-oblanceolate to subrotundate leaflets; terminal leaflet conspicuously larger than the lateral ones, at the base slightly reniform. Cauline leaves (3) 6–12 (14), lower pinnate with 8–17 oblong-ovate, oblanceolate or elliptic, shortly petiolulate or almost sessile leaflets, terminal leaflet oblanceolate, crenate, cuneate at base; uppermost leaves pinnatisect. Inflorescence racemose. Flowers balmy fragrant, petals pale-violet to pale-white-violet, seldom violet, 5.0–10.8 mm long, 2.5–5.5 mm wide. Stamens 6, anthers pale-yellow, occasionally slightly pale-purplish before dehiscence. Pollen grains 3–6-colpate, prolate, rather different in size. Chromosome number 2n = 3x = 24.
Illustration: Fig. 1

Type. Bulgaria, West Rhodope Mts. About 2 km westwards of village of Pobit Kamak, population of numerous plants in flower, growing in wet meadows bordering on springs and brooks, 1300 m, 06.06.2005, M. Ančev, A0573 (**holotype** SOM 168553 !; **isotypes** SOM !).

Description. Perennial herb, under moist conditions viviparous – at the base of the leaflets of the lower cauline leaves young plants with short rootlets develop. Rhizome short, ascending, without stolons. Stem erect, simple, very seldom branched near the base, (20) 35–50 (55) cm tall, glabrous or occasionally with sparce to dense appresed hairs at the base. Rosette leaves pinnate, with (5) 7–25 shortly petiolulate, or almost sessile, 9–14 mm long, 9–16 mm wide, broadly-oblanceolate to subrotundate leaflets, glabrous, or with very short, erect hairs on the margins; terminal leaflet 12–15 mm long, 9–15 mm wide, conspicuously larger than the lateral ones, more or less crenate, slightly reniform at the base; occasionally immature leaves on the rachis with short apressed hairs. Cauline leaves (3) 6–12 (14), glabrous, lower pinnate with 8–17 oblong-ovate, oblanceolate or eliptic, 6–16 mm long, 4–14 mm wide, shortly petiolulate or almost sessile leaflets, the number of leaflets diminishing gradually up the stem, slightly ascending, crenate; terminal leaflet (8) 15–25 mm long,

(7) 10–12 mm wide, oblanceolate, cuneate at base; uppermost leaves pinnatisect. Inflorescence racemose with 1–9 lateral branches. Flowers with well expressed balmy fragrance, functionally, seldom morphologically protogynous (the style protruding from the still closed flower bud); sepals 2.6–4.9 mm long, with membranaceus 0.3–0.5 mm wide margin**;** petals pale-violet, pale-white-violet, seldom violet, obovate, 5.0–10.8 × 2.5–5.5 mm; stamens 6, anthers pale-yellow, occasionally slightly pale-purplish before dehiscence, occasionally some of them sterile, non-dehiscent; filaments of longer stamens 1.95–6.15 mm, these of shorter stamens 0.79–4.4; short stamens with nectar ring at the base, the two pairs of longer stamens with narrow conical nectar gland at the base. Pollen grains 3–5 (6)-colpate, prolate, rather different in size. P = (23.75) 26.25–32.50 (36.25) μm, E = (22.50) 25.00–30.00 (35.00) μm..

Chromosome number 2n = 3x = 24.

Cardamine × *rhodopaea* differs from the putative parental species *C. matthioli* and *C. rivularis* by 3–14 cauline leaves (2–18 in *C. matthioli*, 3–12 in *C. rivularis*); the pale-violet, pale-white-violet, seldom violet, balmy fragrant flowers with petals 5.0–10.8 × 2.5–5.5 mm (in *C. matthioli* white, without fragrance, with petals 5.0–9.0 × 2.5–5.5 mm; in *C. rivularis* purple, without fragrance, with petals 6.1–10.5 × 3.0–6.5 mm); pollen grains 3–6-colpate, prolate, rather different in size (in *C. matthioli* and *C. rivularis* 3–4-colpate, prolate, not very different in size); and the triploid karyotype with 2n = 24 (in *C. matthioli* and *C. rivularis* diploid with 2n = 16). (Fig. 1; Table 2).

The multiple boxplots presented in Fig. 3 give a summary of the empirical distribution of the Cardamine species based on the metric LSpl, Lptl, LLFlm, LSFlm, and Wptl measurements (For the abbreviations see in Table 3). The box indicates approximately the lower and upper quartiles, i.e. the 25 % and 75 % quantiles and the median (50 % quantile), whereas the whiskers specify the smallest and largest observations. Overall, the discrimination between *Cardamine* species is significant (Wilks' Lambda = 0.33945, approximately Fisher F distributed F(10,360) = 25.789 with tail probability p-tail < 0.000).

Distribution and ecology.
Cardamine × *rhodopaea* occurs in the West Rhodope Mts. in a region more or less confined by the riv-
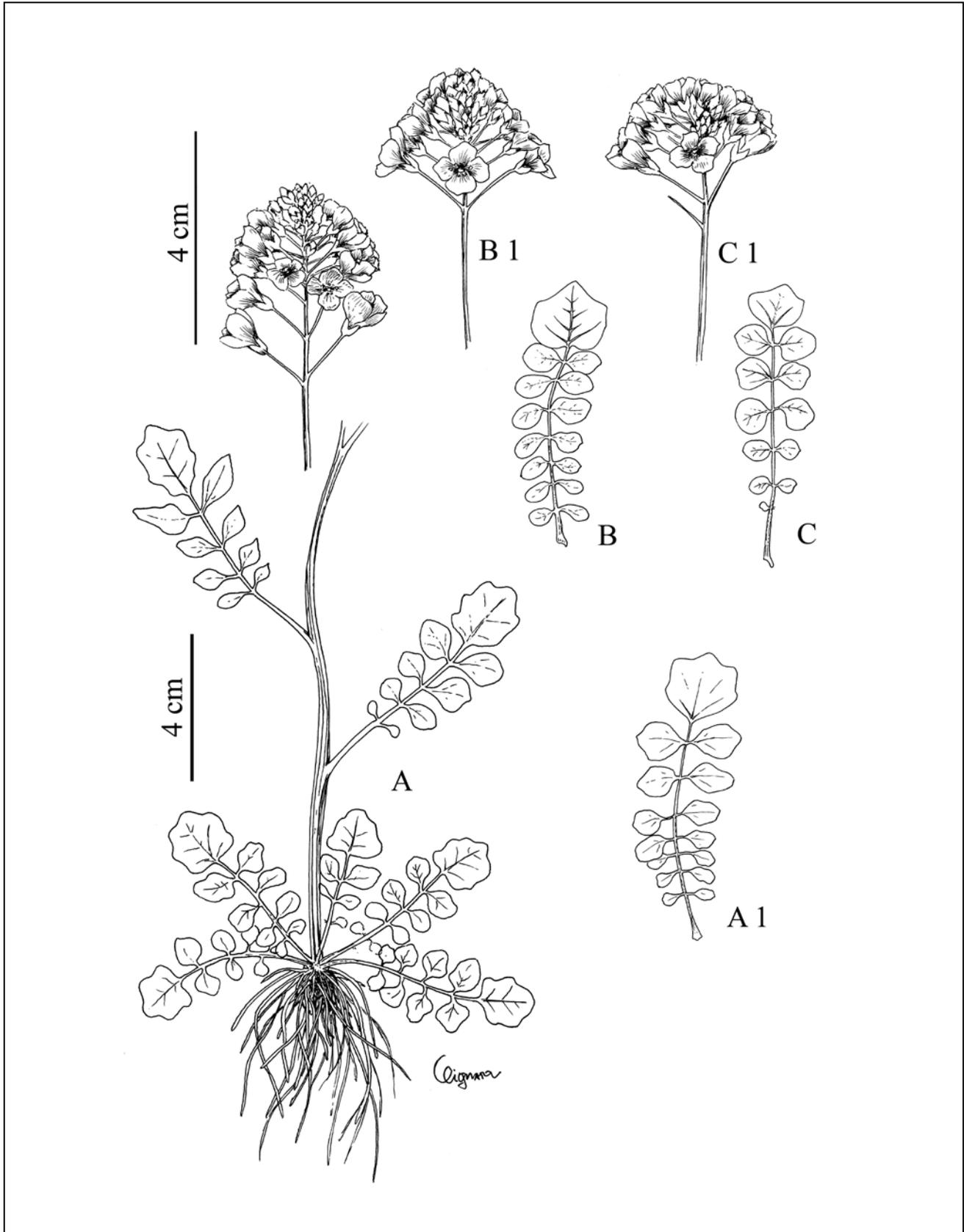
Fig. 1. Plant and plant fragments of: **A.** *Cardamine × rhodopaea,* habitus with short rhizome, stem with rosette leaves, two lower cauline leaves and inflorescence; **A1** – rosette leaf. **B.** *C. matthioli* – rosette leaf; **B1** – fragment of inflorescence; **C.** *C. rivularis* – rosette leaf; **C1** – fragment of inflorescence. (Drawing by Assen Ignatov).

Table 2. Characters distingushing *C. × rhodopaea* from *C. matthioli* and *C. rivularis.*

Characters	Cardamine × rhodopaea	C. matthioli	C. rivularis
Stem	(20) 35–50 (55) cm	11–50 cm	12–45 cm
Rosette leaves			
Terminal leaflet	distinctly larger than lateral ones	distinctly larger than lateral ones	approximately the same size as lateral ones
Lateral leaflets	(2) 3–12 pairs	2 5–8 pairs	5–11 pairs
Cauline leaves	(3) 6–14	2–18	3–12
Flowers			
development	dichogamous, functionally or morphologically protogynous	dichogamous, functionally or morphologically protogynous	dichogamous, functionally or morphologically protogynous
Scent	balmy fragrant	without fragrance	without fragrance
Sepals length:	2.6–4.9 mm, average 3.6 mm	1.9–4.3 mm, average 2.6 mm	3.2–4 mm, average 3.5 mm
Petals	Pale violet, white-violet or violet 5.0–10.8 × 2.5–5.5 mm	white 5.0–9.0 × 2.5–5.5 mm	Purple 6.1–10.5 × 3.0–6.5 mm
Anthers before anthesis	pale-yellow, occasionally slightly pale purplish	yellow	purplish
Pollen grains	3–6 colpate, rather different in size P = 23.75–36.25 μm E = 22.50–35.00 μm	3 (4) colpate, similar in size P = 22.50–28.75 μm E = 22.50–27.50 μm	3 (4) colpate, similar in size P = 25.00–30.00 μm E = 23.75–28.75 μm
Chromosome number	24 (3x)	16 (2x)	16 (2x)
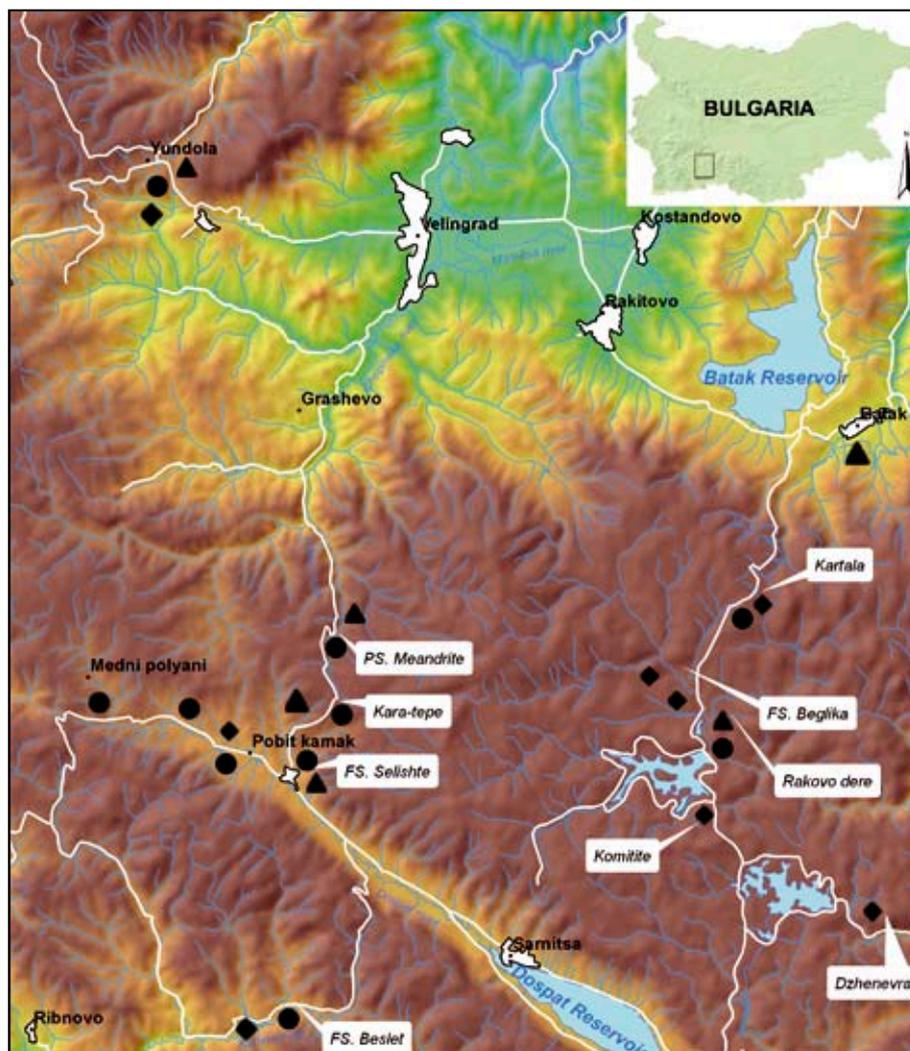
Fig. 2. Distribution of *Cardamine × rhodopaea* (●), *C. matthioli* (▲), and *C. rivularis* (■) in the West Rhodope Mts.

ers Chepinska to the west and northwest, Matnitza and Stara Reka to the north, Dospat and Kanina to the south and southwest, and Vacha to the east. In this area the species localities are concentrated mostly in wet mountain meadows bordering on springs and brooks, in catchments of artificial lakes, frequently in boggy glades, from the zone of beech forests upwards to the coniferous forest belts, at altitude 1200–1300 m and up to about 1900 m. (Fig. 2).
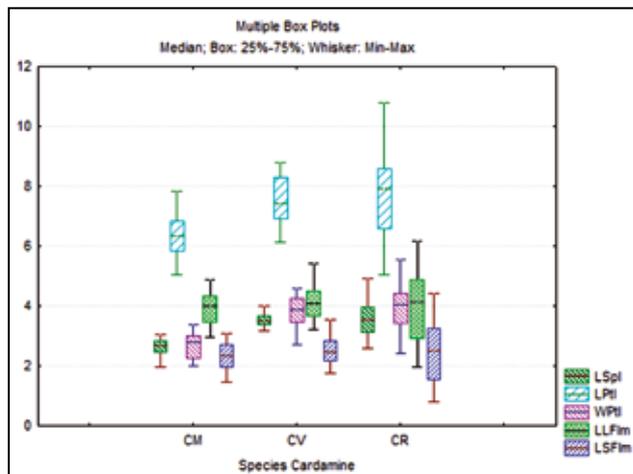

Fig. 3. Variation in flower characters in *Cardamine matthioli* (CM), *C. rivularis* (CV), and *C. × rhodopaea* (CR). For character abbreviations see Table 3.

Table 3. Flower characters included in the morphometric analysis.

LSpl	average length of sepals mm
LPtl	average length of petals mm
WPtl	average width of petals mm
LLFlm	average length of long filaments mm
LSFlm	average length of short filaments mm

Reproductive biology. Flowering in late May to late July (early August). Cross-pollinating, supposedly allogamous plant. Reproduces by seed, at least part of them result of agamospermy; and vegetatively, under moist conditions on the midrib of lower cauline leaves young plantlets are produced.

Ecological and phytosociological characteristics

Cardamine × rhodopaea, *C. matthioli* and *C. rivularis* are ecologically related to fresh-water spring, marsh and fen habitats. Syntaxonomically, the vegetation in which they take part belongs to the classes of *Montio-Cardaminetea* Br.-Bl. & Tüxen ex Br.-Bl. 1948 and *Scheuchzerio-Caricetea fuscae* Tüxen 1937 (Mucina 1997). *C. rivularis* prefers permanently damp habi-

tats, often bordering on water, where it forms populations with few to numerous individuals. *Cardamine × rhodopaea* and *C. matthioli* predominately participate in the vegetation of mountain transitional mires and quaking bogs, related to the class *Scheuchzerio-Caricetea fuscae*. Syntaxonomical studies of that class in Bulgaria have been carried out by Hájková & al. (2008). Their phytosociological data and descriptions of plant associations from Bulgarian mires from the lake areas of Shiroka Polyana, Batak and Beglika in the Rhodope Mts. have pointed out to a floristic content similar with that of the plant communities in which *C. × rhodopaea* and *C. matthioli* participate in the mires in the area of Yundola, Beglika, Beslet, Selishte, and the villages of Pobit Kamak and Medeni Polyani. *Cardamine × rhodopaea* occasionally also demonstrates affinity to spring and brook vegetation, where possibly replaces *C. rivularis* or both species grow together.

Cardamine × rhodopaea and *Cardamine matthioli* are elements of the vegetation of the mountain fens in the coniferous forest belt, along with *Carex nigra* (L.) Reichard, *C. canescens* L., *C. echinata* Murray, *C. rostrata* Stokes, *C. vesicaria* L., *C. leporina* L., *Scirpus sylvaticus* L., *Eleocharis palustris* (L.) R. Br., *Blysmus compressus* (L.) Panz. ex Link, *Alopecurus aequalis* Sobol., *Cynosurus cristatus* L., *Glyceria fluitans* (L.) R. Br., *Dactylorhiza majalis* (Rchb. f.) P.F. Hunt & Summerh., *Geum rivale* L., *G. coccineum* Sibth. & Sm., *G. rhodopeum* Stoj. & Stef., *Galium palustre* L., *Lychnis flos-cuculi* L., *Sagina saginoides* (L.) H. Karst., *Stellaria alsine* Grimm, *Caltha palustris* L., *Lathyrus pratensis* L., *Trifolium hybridum* L., *Trifolium aureum* Pollich, *Chaerophyllum hirsutum* L., *Oenanthe banatica* Heuff., *Juncus articulatus* L., *J. thomasii* Ten., *J. bufonius* L., *Epilobium palustre* L., *E. tetragonum* L., *Myosotis scorpioides* L., *Ranunculus acris* L., *R. repens* L., *R. flammula* L., *Filipendula ulmaria* (L.) Maxim., *Potentilla palustris* (L.) Scop., *P. erecta* (L.) Raeusch., *Parnassia palustris* L., *Lysimachia nummularia* L., *Primula farinosa* L., *Veronica serpyllifolia* L., *V. scutellata* L.

Cardamine rivularis occurs with high frequency in communities of the class *Montio-Cardaminetea*, setting up rich fens (alliance *Cratoneurion commutati*) along springs and edges of fast-running high mountain rapids (Hájková & al. 2006). Other characteristic species occupying brook habitats and growing together with *C. rivularis* are: *Viola biflora* L., *Geum rivale* L., *Geum coccineum* Sibth. & Sm., *Primula farinosa*

L., *Soldanella rhodopaea* F. K. Mayer, *Pinguicula balcanica* Casper, and *Dactylorhiza majalis* (Rchb. f.) P.F. Hunt & Summerh.

Chromosome numbers and karyotypes

Ten populations of *C.* × *rhodopaea* were karyologically investigated. The karyotypes in all studied individuals were triploid, with 2n = 3x = 24 (Plate I-A, Table 1). They occured in a hybride zone, sympatrically or parapatrically with the putative parental species. The chromosomes in the investigated karyotypes were of Sm-type, and in four of the studied populations (A0561, A 00573, A00575, A00627) 1–2 pairs of almost M-type chromosomes persisted. The karyotypes demonstrated some asymmetry in the length of chromosomes, varying within the limits of 0.82–2.54 µm. A group of 4–6 pairs of long chromosomes with 2.00–2.54 µm length could be traced, as well as a group of 3–4 pairs of short chromosomes of 0.82–1.14 µm .

The studied karyotypes of *C. matthioli* from the W. Rhodope Mts were diploid, with 2n = 16 (Plate I-B, Table 1). The counting was the same as reported earlier from Mt Vitosha (Ančev & al. 1997). The chromosomes were of the Sm-type, close in length, varying from 0.83 to 1.47 µm, as in most of the studied populations, a pair of SAT-chromosomes with micro-satellites were observed.

The studied karyotypes of *C. rivularis* from the W. Rhodope Mts were also diploid, with 2n = 16 (Plate I-C, Table 1). The chromosome number coincides with earlier counts in populations from the Vitosha and Rila mountains (Kuzmanov & Kozhuharov 1969; Ančev & al. 1997). The chromosomes were 1.10–2.50 µm long, in 2–4 pairs were longer, and in 4–6 pairs shorter, all but one of them of the Sm-type. In population A 00855 (Plate I-C), a pair of SAT-chromosomes was observed.

Embryological study of *C. matthioli*, *C. rivularis* and *C.* × *rhodopaea*

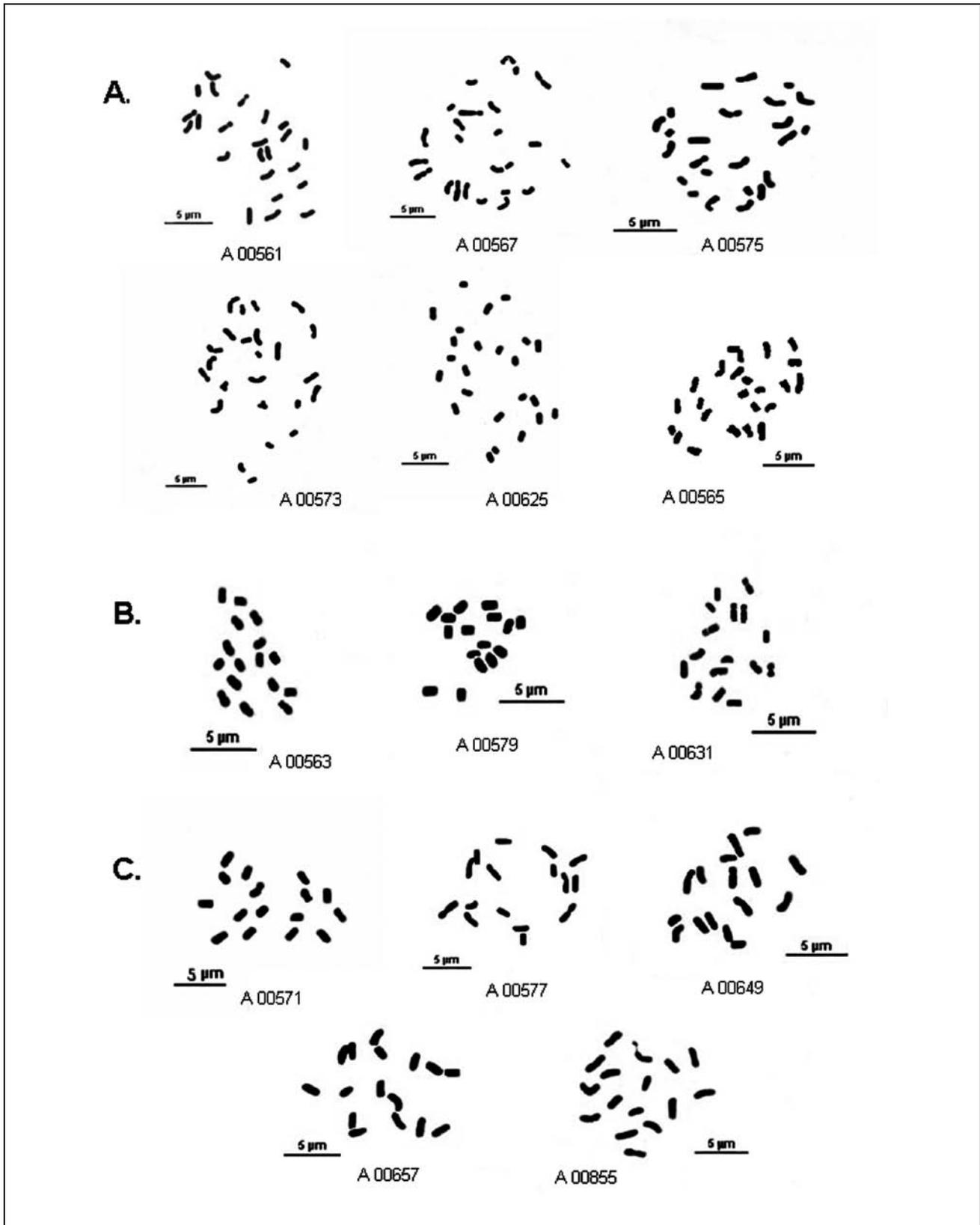### Anther and development of the male gametophyte (Plate II, Figs 1–6)

The anthers of the three studied taxa were tetrasporangiate. Furthermore, in the hybrid, a reduction of the anther locules from four to two as a result of degeneration or lysis of the connective tissue was observed. The anther wall was four-layered (Plate II,

Fig. 1). Some morphological characteristics of the epidermal cells were observed. Thus, in *C. rivularis* and *C. matthioli* the cells were smaller in size, the cell walls were thiner, and not so strongly inflated, as compared to the epidermal cells in the anther wall of the triploid hybrid. Regarding the endothecium, in *C.* × *rhodopaea* the fibrous thickenings of its consisting cells were poorly expressed (apomorphic feature), as compared to those in the endothecium of anther walls in *C. matthioli* and *C. rivularis.* In *C.* × *rhodopaea,* the anther wall comprised usually one middle layer (seldom two), while in *C. rivularis* and *C. matthioli* two middle layers were more often observed. That layer is most ephemeral in *C.* × *rhodopaea,* degenerating until the beginning of homeotypic division of the meiosis in the microspore mother cells (MMCs), while in the two diploid species that process took palce later on. The one-row glandular tapetum towards the epidermis and the connective tissue usually consisted of cells that are different in size and shape. Into the initially uninuclear tapetum cells, a multiplication from one up to two, or seldom four nuclei ran as a result of mitotic division. Within the three taxa, there were found differences between the populations in relation to the morphological characteristics of the anther tapetuma, such as the shape of its cells, size and thickness of the cell walls (thinner and smaller in *C. rivularis* and the thickest in *C.* × *rhodopaea*). The tapetum remained cellular until the end of the anther ontogenesis.

The sporogenous tissue in the anthers was multilayered (Plate II, Fig. 1). Meiosis in the MMCs ran normally in the diploids, while in the triploid were observed more deviations and degenerations leading to formation of different type of microspore tetrads, degenerating and sterile pollen. The microsporogenesis was simultaneous and as a result of that tetrahedral, seldom isobilateral (Plate II, Figs 2, 3) and even more seldom T-shaped microspore tetrads formed. More varied types of the microspore tetrads formed in *C.* × *rhodopaea* resulted from the deviations registered during the meiosis in MMCs and microsporogenesis. Usually in the anthers an earlier degeneration of the tapetum layer was observed, namely at the stage of the microspore tetrads (Plate II, Fig. 3).

The mature pollen was observed as bi-, tricellular and usually tricolpate. In the diploid taxa, morphologically uniform (Plate II, Figs 4, 5) and to a comparatively high degree fertile (viable) pollen formed

Plate I.

Microphotographs of karyotypes of: A. *Cardamine × rhodopaea,* 2n = 24; **B.** *Cardamine matthioli*, 2n = 16; **C.** *Cardamine rivularis,* 2n = 16 (for population codes and origin of the plant material see Table 1).

Plate II.
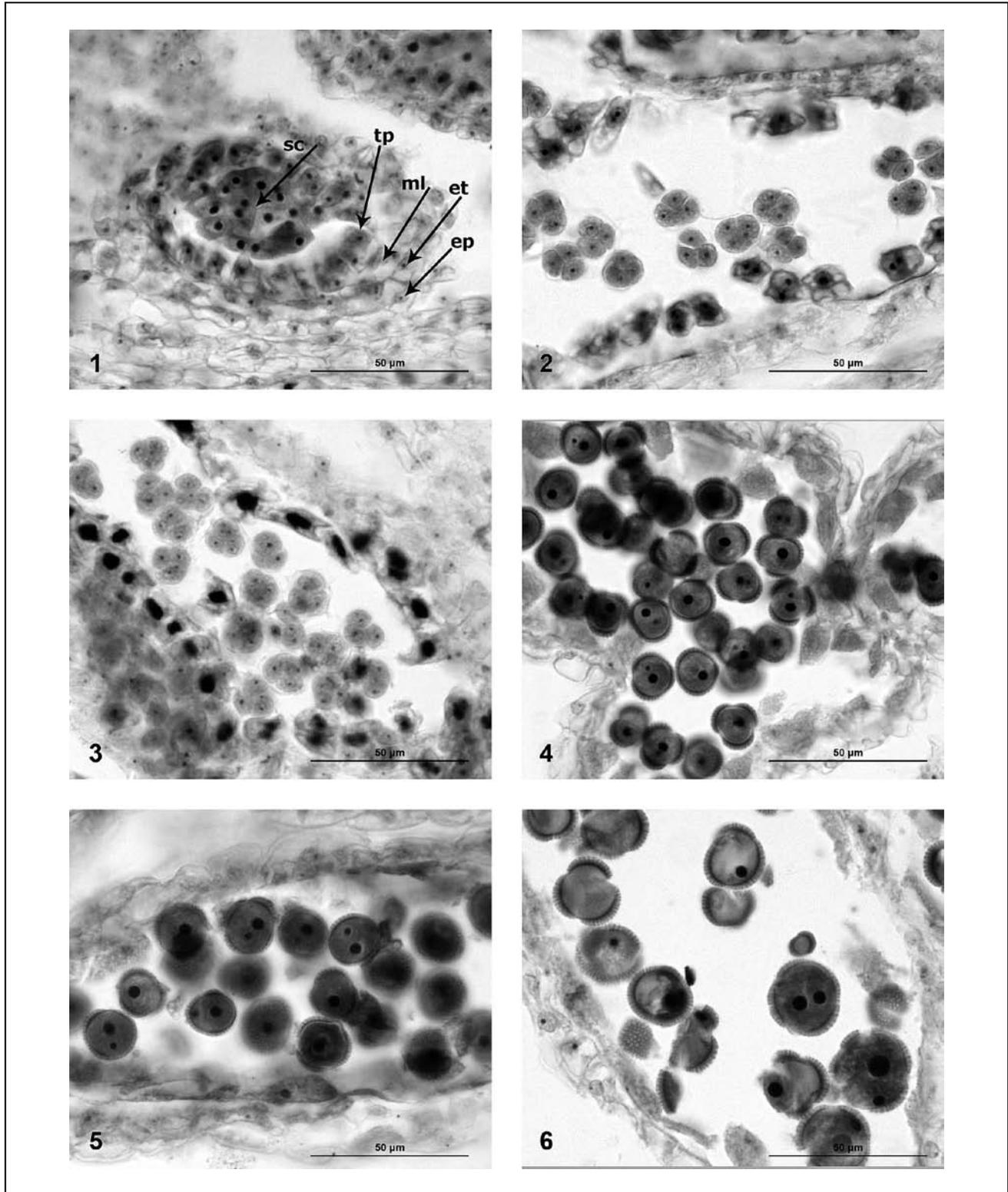
Anther and development of the male gametophyte in the studied *Cardamine* taxa: 1 – sporogenous tissue (**sc**) and four-layered anther wall in *C.* × *rhodopaea* (**ep** –epidermis, **et** – endothecium, **ml** – middle layer, **tp** – tapetum). **2** – microspore tetrads in an anther locule of *C. rivularis*. **3** – microspore tetrads in an anther locule of *C.* × *rhodopaea* and degenerating tapetum layer. **4** – morphologically uniform mature pollen grains (fertile) in *C. rivularis*. **5** – morphologically uniform mature pollen grains (fertile) in *C. matthioli*; **6** – different in size and shape fertile and degenerating pollen grains in *C.* × *rhodopaea*.

(from 62.44 % in population A00577 to 77.16 % in population A00571 in *C. rivularis* – Fig. 5, and from 58.47 % in population A00579 to 76.48 % in population A00563 in *C. matthioli* – Fig. 4). In most anthers of the triploid *C. × rhodopaea*, tricolpate mature pollen grains were often observed, different in size and shape, as well as tetracolpate, penta- and hexacolpate pollen grains, or such with two vegetative cells (Plate II, Fig. 6) instead of one. Furthermore, in many anthers of *C. × rhodopaea*, degenerative processes were found which were more strongly expressed and to a higher degree, as compared to the diploid species. That is why, in the triploid many pollen grains were often found to be sterile (non-viable) and ineffective (Plate II, Fig. 6). As a result of all deviations and degeneration processes observed during the meiosis in MMCs and microsporogenesis, the quantity of sterile pollen in many anthers of *C. × rhodopaea* varied within a wide range (from 24.49 % in population A00627, up to 79.85 % in population A00567 (Plate II, Fig. 3).

The linear discriminant analysis of the data for the fertile (viable) and sterile (nonviable) pollen grains in *C. matthioli, C. rivularis* and *C. × rhodopaea* did not demonstrate statistically proved differences between the putative parental species and the triploid hybrid.

Ovule and development of the female gametophyte
(Plate III, Figs 1–6)

The false septum into superior ovary was poorly developed in the hybrid *C. × rhodopaea,* as compared to the studied diploid species. In each ovary of the hybrid, over 10 (up to 14–16) ovules formed, while in *C. matthioli* and *C. rivularis* their number was smaller (8–10). In *C. rivularis*, a large number of archesporium cells (3–5) in an ovule was found (Plate III, Fig. 1), while in *C. × rhodopaea,* in some ovules a reduction of multicellular archesporium to bi- and even unicellular one (apomorphic feature) was observed. In the three taxa, usually one archesporial cell underwent a further development, functioning directly as a megaspore mother cell and, after meiosis, a linear megaspore tetrad formed in the ovule (Plate III, Fig. 2). The embryo sac (ES) in many ovules of *C. × rhodopaea*, a dyad instead of a megaspore tetrad, formed in the ovule as result of a destroyed meiosis and then the ES began to develop apomictically from the lower megaspore of the dyad, following the *Taraxacum*-type meiotic diplospory (Plate III, Fig. 3). The mature ES consisted of an egg cell and two synergids, two polar nuclei and

three antipodals in the chalazal part (Plate III, Fig. 4). Sporadically, development of two ESs was observed in the hybrid, in parallel in an ovule (Plate III, Fig. 5) – a proof of potential polyembryony.

In the diploids *C. rivularis* and C. *matthioli*, the legitimate embryo and endosperm developed after porogamous double fertilization. The embryogenesis began before the endospermogenesis. The embryo development followed the Onagrad-type, with a long suspensor, the apical cell of which was usually clearly vacualized (Plate III, FIg. 6).

In some ovules of the triploid hybrid, the embryo developed from unfertilized egg cell (parthenogenesis), while in other ovules it developed after fertilization (like in the diploids). Initially, the endosperm was nuclear (Plate III, Fig. 6) and subsequently transformed to cellular, when the embryo was already multinucleate (usually after the globular embryo stage). The presence of a different amount of fertile pollen in the anthers of the hybrid (sometimes in sufficiency) supposedly meant that in the ovules with an apomictic diplosporous ES, the endosperm development was not autonomous and ran after a pseudogamy.

In general, interpopulation differences regarding the features of the male and female generative spheres concerned the time of origin, existence and degeneration of some main embryological structures and their morphological characteristics were not so considerable, in order to change the embryological characterization established for the three studied taxa.

Discussion

Morphological variability of *Cardamine × rhodopaea,* the triploid karyotype, characteristics of the embryological processes, heteromorphic pollens, species distribution and ecology suggest that *C. × rhodopaea* has a hybrid origin. The putative parental species, *C. matthioli* and *C. rivularis*, occur sympatrically or parapatrically with the triploid. They are diploid amphimictic species with embryologically balanced reproductive systems that reproduce by seeds and vegetatively. The studies into the microsporogenesis and pollen quality show that both form morphologically more or less uniform tricolpate (seldom tetracolpate), mostly fertile pollen grains.

Our investigations of the morphological variability, chromosome numbers, ploidy level, and embry-

Plate III.
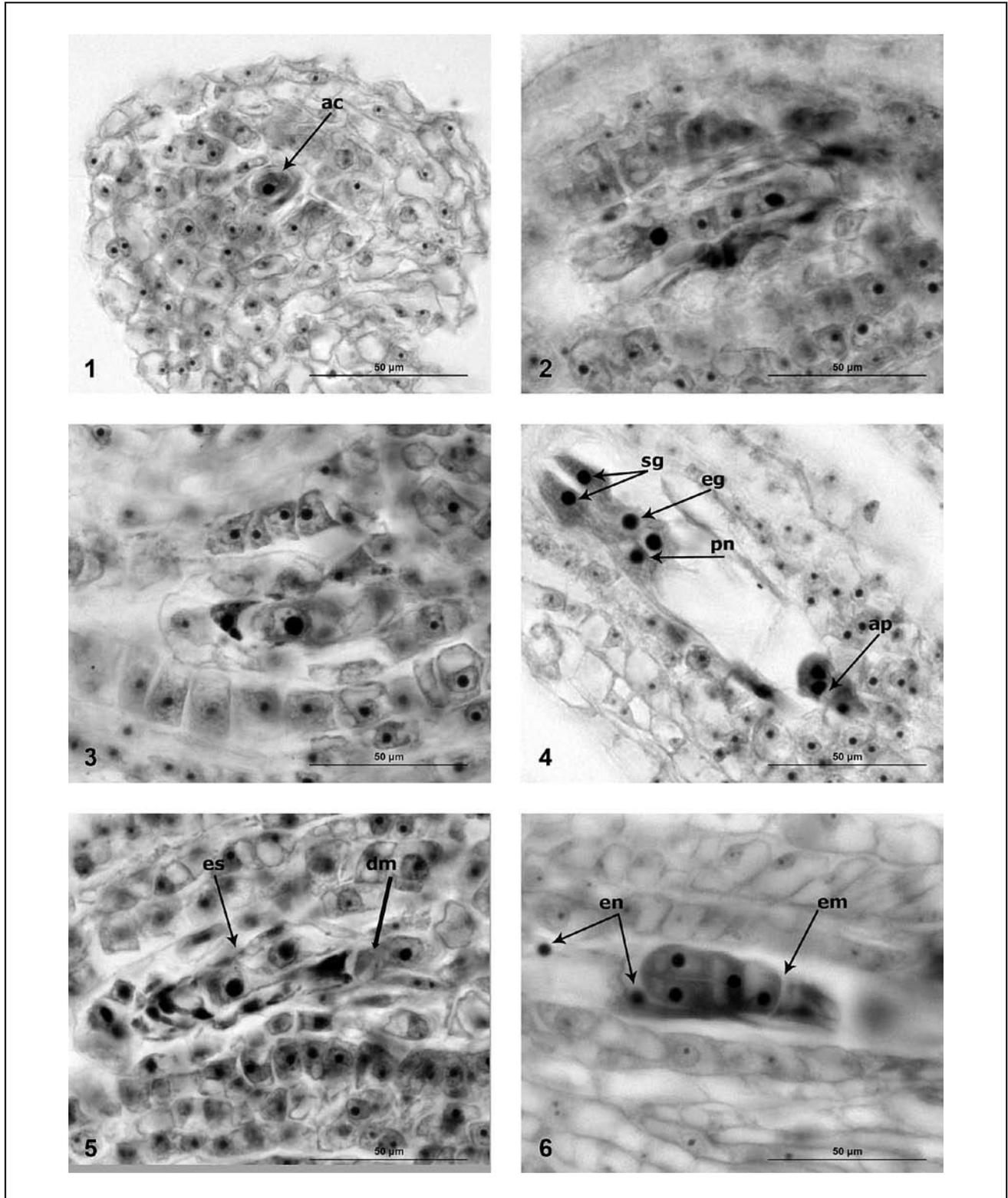
Ovule and development of the female gametophyte in the studied *Cardamine* taxa: 1 – ovule with unicellular archesporium (**ac**) in *C. × rhodopaea*. **2** – linear megaspore tetrad in an ovule of *C. rivularis*. **3** – megaspore dyad in an ovule of *C. × rhodopaea*. **4** – Mature ES in *C. rivularis* with an egg cell (**eg**), two synergids (**sg**), two polar nuclei (**pn**) and three antipodals (**ap**). **5** – two nuclear ES (**es**) and initiation of a diplosporous *Taraxacum*-type ES development from the chalazal megaspore of a dyad (**md**) in an ovule of *C. × rhodopaea*. **6** – Onagrad-type young embryo (**em**) and two endosperm nuclei (**en**) in *C. matthioli*.

ological characteristics suggest that *C. × rhodopaea* is an amphiapomictic triploid, with unbalanced embryological processes and high plasticity of the embryological structures, found also in other polyploids of the family *Brassicaceae,* and especially the *Taraxacum*-type diplosporous apomixis (Naumova & al. 2001; Taskin & al. 2004; Yurukova-Grancharova & al. 2004). *Cardamine × rhodopaea* produces rather heteromorphous, 3–6 colpate polen grains. The pollens are medium-sized or large, stained (effective), with well distinguished vegetative and generative cells (or sperms), or small and giant, with lacerated walls, empty (ineffective).

We believe that *C. × rhodopaea* is a recent polyploid, most probably arising from interspecific hybridization and "fusion of an unreduced gamete with a reduced gamete to produce a triploid" (cf. Levin 2002).

Because of the higher morphological similarity of triploid plants with *C. rivularis,* we suppose that most of the genome of the triploid comes from this species. Analyzing the karyotypes of the triploid *C. × rhodopaea* and the putative parental species *C. matthioli* and *C. rivularis,* it could be speculated that in the karyotype of *C. × rhodopaea,* which constitutes of two parental genomes, a set of 16 longer chromosomes (8 pairs) came from the unreduced gamete of *C. rivularis*, and 8 chromosomes (4 pairs) of the reduced gamete came from the genome of *C. matthioli.*

The triploid *C. × rhodopaea* originated as a result of spontaneous interspecific hybridization in zones of sympatric distribution of the putative diploid parental species *C. matthioli* and *C. rivularis.* In view of the observed pattern of geographic and vertical distribution of the triploid populations in environmental diversity of mountainous topography with high summits, deep river valleys, gorges and ravines, we suggest that *C. × rhodopaea* emerged as a result of several hybridization events in different parts of the West Rhodope Mts, in the catchment area of the rivers Chepinska, Dospat, Kanina, Devinska, and Stara Reka, and formed a mosaic hybrid zone. The hybrid populations irradiated along the valleys of mountain rivers and their feeders, presently grow in wet meadows bordering on springs and brooks, in bogy glades, in catchments of artificial lakes, starting from the beech forests at 1200–1400 m a.s.l., and upwards to the coniferous belt at 1800–1900 m a.s.l., possibly in the region of the summits Syutkite and Batashki Snezhnik, up to about 2000 m a.s.l. In southwestern direction,

the hybrid zone extends to Dabrash in the upper flow of river Kanina, northwestwards to the Avramovi Kolibi saddle and Yundola, in the north to Mt Batashka, and in the east along river Vacha (Fig. 2). Such a hypothesis seems quite probable under the conditions of strongly rugged topography of the West Rhodope Mts and the observed characteristics of distribution of the triploid *C. × rhodopaea* and the diploids *C. matthioli* and *C. rivularis.* It is most unlikely that *C. × rhodopaea* has emerged as a result of a single-time interspecies hybridization in a sympatric zone of the putative parental species, and that later on the hybrid generations have produced the presently distributed populations in the West Rhodope Mts.

The restricted distribution of *C. × rhodopaea* supposes a young hybrid origin. Most probably, hybridization was stimulated by the changes in structure and composition of the mountain flora and vegetation in the Rhodope Mts, as a result of climatic oscillations and human interference (deforestation, stockbreeding and agriculture), since the Neolitic, traced out in the pollen diagrams and palaeoethnobotanical finds (Bozilova & al. 2012).

The pattern of hybridization in the hybrid zone in the West Rhodope Mts resembles the case of *C. × insueta* (3x) found at Urnerboden, Central Switzerland (Urbanska-Woritkiewicz & Landolt 1972; Urbanska & al. 1997). Mention deserves the fact that in the area of distribution of *C. × insueta,* a neopolyploid species (*C. schulzii,* 6x) was also found, supposedly at a young phylogenetic age (Urbanska & al. 1997). In the area of distribution of *C. × rhodopaea* in the West Rhodope Mts, no other theoretically expected polyploid cytotypes were found, except hybrid triploid plants. This is the latest find of a triploid hybrid in the genus *Cardamine,* after *C. × insueta* (*C. pratensis* (*C. rivularis* auct.) × *C. amara*) (Urbanska & al.1997; cf. Marhold & Lihova 2006), and *C. matthioli × C. acris* subsp. *pindicola* reported from NW Greece (Perny & Marhold 2006). *Cardamine matthioli × C. acris* subsp. *pindicola* and *C. × rhodopaea* emerged in the South Balkans, both with participation of *C. matthioli* in a region known as a glacial refuge, characteristic with the dynamic processes of plant successions in the Late Glacial and the Holocene (Bozilova 1996; Bozilova & al. 2000; Palamarev 2003; Bozilova & al. 2012).

The results of our biosystematic studies into the *Cardamine pratensis* group in W. Rhodope Mts provide some convincing evidence of active hy-

bridization processes within the group, with poly-ploidization, speciation and distribution of the here described triploid *Cardamine × rhodopaea*. Nevertheless, the preliminary investigation of the genetic variability of *Cardamine × rhodopaea* and its putative parental species *C. matthioli* and *C. rivularis* based on ITS sequences of nrDNA did not yield straightforward evidence of a hybrid origin. Direct sequencing indicated the presence of multiple ITS repeat types not only in the assumed hybrid but also in the putative parents. Some of the intraindividual single-site polymorphisms observed in *C. × rhodopaea* showed additive patterns, but the entire ITS sequence variation need to be explored properly in all three species, prior to drawing final conclusions. Alternatively, other markers (single-copy nuclear genes, DNA-fingerprinting methods) could also be employed. Thus, molecular evidence will be needed in future so as to reveal the precise identity of the parental species, directionality and frequency of hybridization events, assessment of the hybrids' age and their evolutionary history (Zozomová-Lihová, pers. communication).

Appendix.
List of examined herbarium specimens of *Cardamine matthioli, C. rivularis* and *C. × rhodopaea*.

Cardamine matthioli

Bulgaria. W. Stara Planina: Ribni Vir locality near Vitinya pass, 02.05.1973, J. Koeva (SO); **Sofia region**: in pratis humidis inter pagum Kamartzi et Mirkovo, 10.05.1904, I. Urumov, sub *C. hayneana* (SOM); Kazichene. B. Stafanov (SOA); Chepinzi, 21.05.1921, D. Jordanov sub *C. pratensis* L. Rev. K. Marhold as *C. matthioli*; marshland in the area of Kazichensko Blato, 30.03.1930, D. Jordanov sub *C. pratensis* var. *matthioli* (SO); marshland in the area of former Kazichensko blato, 50 m, 21.04.1968, N. Vihodcevsky sub *C. pratensis* var. *matthioli* (SO); **Mt.Vitosha region**: Dragalevski Livadi, 25.05.1927, N. Stojanov (SOA); Dragalevski Livadi, 900 m, 16.04.1894, St. Georgiev sub *C. pratensis* (SO); Knyazhevo, meadows, V. Stribrny sub *C. hayneana,* rev. K. Marhold as *C. matthioli* (SOM);**West Frontier Mountains:** Mt Osogovska**,** on the banks of river Bistrishka, 05.05.2000, B. Sidjimova (SO); **Struma Valley:** wet places with *Typha* sp. near Rila town, 15.04.2007, D. Dimitrov (SOM); marshes at Strumski Chiflik, Blagoevgrad Distr., 325 m, 21.04.1949, B. Kitanov sub *C. pratensis* (SOM); **Mt Slavyanka:** wet meadows near Paril, 900 m, 02.05.1991, I. Pashaliev (SOM); **Mesta Valley:** along a brook in meadows near Gotse Delchev, 02.10.2010, V. Goranova, & K. Vassilev (SOM); **Pirin Mts:** wet places southwestwards of Bansko, 1000 m, 22.06.2010, D. Dimitrov (SOM); **Ri-la Mts:** in pratis humidis agri Samokovensi ad urbem Samokov, 930 m, 21.05.1911, B. Davidov sub *C. hayneana* (SO, SOM); in silvaticis humidis agri Samokovensi, 1000 m, 05.05.1909, B. Davidov sub *C. hayneana* (SOM); Near sumit Damga, 2400 m, 29.07.1961, M. Simeonovski sub C. pratensis (SO); Borovetz, wet glades, 1350 m, 12.06.1997, M. Anchev (SOM); Predela pass, 16.05.1961, M. Simeonovski sub *C. pratensis* (SO); **W. Rhodope Mts:** Batashko Blato, 19.05.1920, B. Ahtarov sub *C. hayneana* (SOM); Alabak, in wet glades, 31.07.1953 (SO); wet meadows near artificial lake Batak, July, 2005, M. Anchev (SOM); Yundola, wet glades, 1250 m, 05.06.2006, M. Anchev (SOM); wet glades at artificial lake Shiroka Polyana, 15.06.2007, V. Goranova (SOM); Kara-Tepe locality at river Ribna, 1450 m , 05.06.2005, M. Anchev (SOM); wet meadows east of Rakovo Dere, 1600 m, 08.06.2005, Goranova & S. Stojanov (SOM); flooded glades, southeastwards of Rakovo Dere, 08.06.2005, M. Anchev (SOM); **Thracian Lowland:** wet meadows near Boshulya village, Pazardzhik distr., 26.04.1949, B. Kitanov sub *C. pratensis var. matthioli* (SOM).

Cardamine rivularis

W. Stara Planina: Berkovski Balkan, 05. 1898, I. Stamboliev (SOM); **C. Stara Planina:** above Gabrovo, 06.1907, B. Davidov (SOM); **Mt Vitosha:** Kupena,

17.07.1929, T. Georgiev (SOA); in pratis humidis alpinis, 17.07.1904, I. Urumov (SOM); in peatbogs in the Kamen Del area, 2000 m, 13.06.1920, B. Ahtarov (SOM); in peatbogs, 1400 m, 27.05.1951, N. Efremov (SO); Reznyovete, 1950 m, 06.1912, A. Drenovsky & B. Ahtarov sub *C. pratensis* var. *alpicola* (SOM); in pratis humidis alpinis, 06.1902, J. Mrkvicka & B. Ahtarov sub *C. pratensis* var. *alpicola* (SOM); Selimitza, 1950 m, 10.07.1932, B. Ahtarov (SOM); near Selimitsa, 24.06.2004, R. Tsonev (SO); Suhoto Ezero, 06.07.2004, R. Tsonev (SO); **Pirin Mts:** ad Papas Gyol, 16.VII.1929. S.G. sub *C. pratensis* var *rivularis* (SOA); near Banderitsa chalet, 1800 m, 14.07.1969, B. Kuzmanov (SOM); **Rila Mts:** Chernata Skala, 7.VI.1917, N. Stojanov (SO); supra fluv. Bistritza, distr. G. Dzumaja, 19.VI.1931. N. Stojanov & T. Georgiev (SO); on the slopes of Belmeken, along streams, 12.VII.1947, S.Valev & A. Janev, sub *C. pratensis* var. *alpicola* (SO); Bela Rila, sub lacum Dolna Leva Reka, 2350 m, 17.07.1911, B. Davidov (SOM;); in rivularibus, Cherni Iskar, 2310 m, 05.08.1920, B. Davidov (SOM); Tzarska Rila, Bistritsa, 1300 m, 05.06.1911, B. Davidov (SOM); in rivularibus agri Samokovensis ad riv. Bistritza, 1000 m, 03.051909; Bela Cherkva, 950 m, 20.04.1910, B. Davidov (SOM); Ambaro – Ribni Ezera, 25.06.1901, A. Toshev (SOM); B. Davidov (SOM); E. Rila, peak Slavov, 1900 m, 05.10.2007, D. Dimitrov (SOM); Cherna Rila, Sedemte Ezera, 2250 m, 21.07.1909, B. Davidov (SOM); ad rivularibus ad Bistrishki Ezera, 2450 m, 07.07.1909, B. Davidov (SOM); **W. Rhodope Mts:** Beglika, VII. 1935, D. Antonov sub *C. pratensis* var. *alpicola*. Rev. K. Marhold as *C. rivularis* (SOA); Beglika, wet meadows, 1600 m, 14.07.1976, B. Kuzmanov, (SOM); Beglika, along river Suisuza, west of Beglika Forestry Station, 1600 m, 07.07.2005, M. Anchev (SOM); supra pagum Batak, 1600 m, 20.06.1926, B. Davidov (SOM); west of Shiroka Polyana, 11.08.1967, N. Andreev (SOM); peatbogs in meadows southeastwards of Syutka, 25.07.1940, D. Jordanov (SO).

Cardamine × *rhodopaea*

W. Rhodope Mts. Ca. 2 km west of Pobit Kamak village, in wet meadows bordering on brooks and springs, 1300 m, 06.06.2005, M. Ančev (SOM); wet meadows along river Dospat, southeastwards of Medeni Polyani village, ca. 1300 m, 07.06.2005, M. Ančev (SOM); wet glades along coniferous forests, near Selishte Forestry Station, 05.06.2005, M. Ančev (SOM); wet glades

alnog river Ribna, together with *Cardamine matthioli* and *C. rivularis*, 05.06.2005, M. Anchev (SOM); wet meadows northeastwards of Karatepe locality, 1400 m, 07.06.2006, M. Anchev (SOM); Beglika Forestry Station, Rakovo Dere, wet meadows, 08.06.2005, M. Anchev, S. Stojanov & V. Goranova (SOM); wet meadows and peatbogs in the locality Kartala, 1620–1650 m, 19.07.2006, M. Ančev (SOM); in the surroundings of Beslet Forestry Station, 1850 m, 06.06.2006. M. Ančev (SOM); glades in the surroundings of peak Beslet, 06.06. 2006, M. Anchev (SOM); meadows west of Avramovo rail station, 05.06.2006, V. Goranova & S. Stojanov (SOM).

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