Karyological Analysis of *Cyclamen hederifolium* s.l. in Greece

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Received April 5, 2020; accepted May 12, 2020

Summary The current study aims to contribute to the further karyological knowledge of the very diverse species *Cyclamen hederifolium*. The species is represented in Greece by two subspecies, *C. hederifolium* subsp. *hederifolium* and *C. hederifolium* subsp. *crassifolium*, which are not widely accepted as taxonomically distinct taxa. The subspecies are analyzed karyologically and morphometry of the most important karyological features is used to compare them, attempting to reveal the taxonomic relationships between the two subspecies. However, the karyological analysis proved that all karyotypes are symmetrical and both subspecies have both diploid and tetraploid populations. The applied karyomorphometry and multivariate analysis provided no special pattern and it was unable to differentiate the subspecies from a karyological point of view.

Keywords *Cyclamen*, Greek flora, Karyomorphometry, Multivariate analysis, Polyploidy.

Cyclamen hederifolium Aiton (Primulaceae) is a species with a summer-autumn flowering period and one of the widest distributions from SE France to SW Turkey, including most of the Balkan countries and many Mediterranean Islands (Meikle and Sinnott 1972). *C. hederifolium* is characterized by its great morphological variability, especially concerning the shape, color, and pattern of its leaves, but also the color of the flowers, from pale to intense reddish-purple (Meikle and Sinnott 1972). Due to this variability, *C. hederifolium* is often confused with other species such as *C. africanum* Boiss. & Reut. (Clennett 2002), which does not occur in Greece and *C. confusum* (Grey-Wilson) Culham, Jope & P. Moore, which is an endemic species of Crete island. The latest was classified as *C. hederifolium* subsp. *confusum*, but it has been upgraded to species level, based on morphological, molecular and cytological data (Culham *et al.* 2009).

In Greece, *C. hederifolium* consists of two subspecies: the typical *C. hederifolium*, with a wide distribution throughout the whole country, and *C. hederifolium* subsp. *crassifolium* (Hildebr.) Culham, Denney & P. Moore, which is a Greek endemic taxon, restricted to Peloponnisos, Sterea Ellas, and some of the Ionian Islands (Moore and Jope 2011). The two subspecies are thought to differ in the chromosome number ($2n=34$ for the typical species and $2n=68$ for subsp. *crassifolium*) and the morphology (fleshiness of their leaves) (Culham

et al. 2009). The Checklist of Vascular Plants of Greece (Dimopoulos *et al.* 2013) reports the presence of both subspecies. However, according to the “Atlas of the Aegean Flora” (Strid 2016), subsp. *crassifolium* is not considered to fulfill the criteria for the taxonomic rank of subspecies.

Even though several populations from Greece have been studied karyologically, all available data refer to *C. hederifolium* s.l. making the comparison of the two subspecies impossible. The aim of the current study is to contribute to the subspecies' taxonomy by providing more chromosome counts and by comparing both taxa's karyomorphometry for the first time.

Materials and methods

Plant material of *C. hederifolium* s.l. was collected from 20 populations, covering the areas where both subspecies are distributed. Tubers were cultivated at the Laboratory of Systematic Botany in Agricultural University of Athens and herbarium specimens were deposited at the Herbarium of the University (ACA). Root tips were pretreated in 8-hydroxyquinoline (0.002 M w/v) for 6 h in room temperature, then fixed in 3:1 (v/v) absolute ethanol:glacial acetic acid for 24 h at 4°C and stored in 75% ethanol at –20°C. Hydrolysis in 1 M HCl at 60°C lasted for 15 min and staining was achieved after 2.5 h in Feulgen reagent (Darlington and La Cour 1969). The stained root tips were put on a slide with a drop of fresh 45% (v/v) acetic acid and then squashed according to Östergren and Heneen (1962), and Kamari (1976). At

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DOI: 10.1508/cytologia.85.219

Fig. 1. Photomicrographs of mitotic metaphase plates of *C. hederifolium* subsp. *hederifolium*: A. $2n=2x=34$ (voucher no K120); B. $2n=4x=68$ (voucher no C3); and *C. hederifolium* subsp. *crassifolium*: C. $2n=2x=34$ (voucher no K98); D. $2n=4x=68$ (voucher no C5). Scale bars=10 μ m.

Table 1. Populations of *C. hederifolium* subsp. *hederifolium* and *C. hederifolium* subsp. *crassifolium* studied along with their voucher numbers and level of ploidy.

Taxon	Location	Voucher no	Ploidy level
<i>C. hederifolium</i> subsp. <i>hederifolium</i>	Aegean Isl. Lesvos: Petra—Kalloni	K67	2x
	Ionian Isl. Kerkira: Argirades	K114	2x
	Ionian Isl. Kerkira: Temploni	K120	2x
	Peloponnisos. Ilia: Krestena	K69	2x
	Epirus. Preveza: Kanali	K121	4x
	Peloponnisos. Achaia: Mega Spilaio—Kalavrita	C4	4x
	Peloponnisos. Achaia: Patra	K78	4x
	Peloponnisos. Korinthos: Egialos—Xilokastro	C3	4x
<i>C. hederifolium</i> subsp. <i>crassifolium</i>	Ionian Isl. Zakynthos: Agios Leon	K84	2x
	Peloponnisos. Arkadia: Moni Kaltezon	K98	2x
	Peloponnisos. Messinia: Kampos	K76	2x
	Peloponnisos. Messinia: Raftopoulo	K75	2x
	Stereia Ellas. Attiki: Penteli—Dionisos	C8	2x
	Aegean Isl. Poros: Russian Bay	C9	4x
	Ionian Isl. Kefalonia: Koutavos	C5	4x
	Ionian Isl. Kefalonia: Faraklata	C6	4x
	Ionian Isl. Lefkada: Melissa Gorge	C2	4x
	Ionian Isl. Zakynthos: Kiliomenos	K83	4x
	Ionian Isl. Zakynthos: Korithi	K79	4x
	Peloponnisos. Messinia: Milea	C13	4x

least five plates per population were observed using an AXIOLAB Zeiss microscope. Chromosome terminology follows Levan *et al.* (1965), Stebbins (1971), and

Kamari (1976), taking into consideration comments and suggestions by Sybenga (1959), Bentzer *et al.* (1971) and Favarger (1978).

Table 2. Karyomorphometric indices of studied populations of *C. hederifolium* subsp. *hederifolium* and *C. hederifolium* subsp. *crassifolium* along with their voucher number.

Taxon	Voucher no	2n	x	max l+s (μ m)	min l+s (μ m)	TCL (μ m)	ACL (μ m)	THL (μ m)	CV _{CL}	M _{CA}	CV _{CI}
<i>C. hederifolium</i> subsp. <i>hederifolium</i>	K67	34	2	2.65	1.47	70	2.06	35	14.69	25.06	17.18
	K114	34	2	3.41	1.59	84.77	2.49	42.39	14.75	21.36	14.5
	K120	34	2	1.82	1.14	49.09	1.44	24.55	10.02	18.82	16.59
	K69	34	2	2.33	1	52.67	1.55	26.33	22.07	16.3	17.9
	K121	68	4	2.67	1	118.33	1.74	29.58	20.99	21.58	19.27
	C4	68	4	2.5	1.25	123.96	1.82	30.99	15.36	27.66	18.22
	K78	68	4	4.37	2.19	206.56	3.04	51.17	16.35	30.02	22.17
	C3	68	4	2.5	0.83	104.17	1.53	26.04	18.48	29.74	21.45
<i>C. hederifolium</i> subsp. <i>crassifolium</i>	K98	34	2	3.23	1.76	82.06	2.41	41.03	12.81	25.81	14.79
	K76	34	2	1.67	0.67	36.33	1.07	18.17	23.66	19.8	19.8
	K75	34	2	2.67	0.67	64	1.88	32	23.08	22.14	17.34
	C8	34	2	2.5	1.25	62.71	1.84	31.35	14.53	28.47	19.08
	C9	68	4	3.5	1.5	135.5	1.99	33.87	25.64	29.31	17.66
	C5	68	4	1.87	1.05	96.88	1.42	24.22	16.37	27.99	15.75
	C6	68	4	3.12	1.56	148.13	2.18	37.03	16.49	25.36	14.71
	C2	68	4	1.87	0.83	99.38	1.46	24.84	36.55	26.52	24.34
	C13	68	4	2.5	1.04	112.92	1.66	28.23	18.25	25.97	17.53

Fig. 2. PCA analysis based on six karyomorphometric parameters ($2n$, x , THL, CV_{CL} , M_{CA} , CV_{CI}). (●) stands for diploid *C. hederifolium* subsp. *hederifolium*; (○) tetraploid *C. hederifolium* subsp. *hederifolium*; (▲) diploid *C. hederifolium* subsp. *crassifolium*; (△) tetraploid *C. hederifolium* subsp. *crassifolium*.

The karyomorphometrical study provides, apart from chromosome counts, a presentation of the ploidy level, total (TCL), average (ACL), and total haploid (THL) chromosome length. Concerning the asymmetry of the karyotype, the interchromosomal asymmetry (CV_{CL} , Peruzzi and Altinordu 2014) and the intrachromosomal asymmetry (M_{CA} , Peruzzi and Eroğlu 2013) were calculated. Furthermore, the coefficient of variation of the centromeric index (CV_{CI} , Paszko 2006), measuring the centromere position heterogeneity was estimated. A multivariate analysis (PCA) concerning six karyological parameters ($2n$, x , THL, CV_{CL} , CV_{CI} , and M_{CA}) was also applied.

Results and discussion

Twenty populations were studied karyologically. All karyotypes observed are symmetrical, consisting of metacentric and submetacentric chromosomes (Fig. 1).

Both taxa have diploid ($2n=2x=34$) and tetraploid ($2n=4x=68$) populations, showing that ploidy level does not match with a specific subspecies (Table 1). More specifically, *C. hederifolium* subsp. *hederifolium* is found diploid in material from Lesvos (Aegean Isl.), Kerkira (Ionian Isl.) and Krestena (Peloponnisos), while tetraploid in material from Preveza (Epirus), Achaia and Korinthos (Peloponnisos). Regarding *C. hederifolium* subsp. *crassifolium* diploid karyotypes are observed in Arkadia (Peloponnisos), as well as Attiki (Sterea Ellas), which is the *locus classicus* of the taxon. On the other hand, tetraploid populations are found in Poros (Aegean Isl.), Kefalonia and Lefkada (Ionian Isl.). Also, Messinia (Peloponnisos) and Zakynthos (Ionian Isl.) have both diploid and tetraploid populations of *C. hederifolium* subsp. *crassifolium*.

Our findings on chromosome counts are in accordance with existing literature. *C. hederifolium* s.l. has been found diploid (Strid and Franzen 1981, Bennett and

Grimshaw 1991, Samaropoulou *et al.* 2010, Strid 2015) in populations from Andros, Ikaria, Naxos (Aegean Isl.), Evangelistria (Peloponnisos), Mt. Olimpos (Thessalia) and Mt. Parnitha (Sterea Ellas), as well as tetraploid (Bennett and Grimshaw 1991, Kriemadi *et al.* 2002) in populations from Poros (Aegean Isl.), Lefkada and Zakynthos (Ionian Isl.). Even though triploidy ($2n=3x=51$) has been reported in material from Italy (Bennett and Grimshaw 1991), to our knowledge, it has never been confirmed for material from Greece.

Karyomorphometric parameters were analyzed in 17 populations (Table 2). The largest chromosome length ($4.37\mu\text{m}$) is observed in a tetraploid population of *C. hederifolium* subsp. *hederifolium* from Achaia (Peloponnisos) and the smallest chromosome length ($0.67\mu\text{m}$) in two populations of *C. hederifolium* subsp. *crassifolium* from Messinia (Peloponnisos). However, the TCL and ACL are variable, even between populations of the same subspecies and same ploidy level. The indices CV_{CL} , M_{CA} , and CV_{CI} prove that all karyotypes are symmetrical concerning both the size and the morphology of the chromosomes. When the PCA is applied, no special pattern can be provided either in order to distinguish the two subspecies. On the contrary, the only differentiation present is caused by the level of ploidy (Fig. 2).

As already mentioned, one of the criteria for the taxa's current taxonomy is the number of chromosomes and levels of ploidy ($2n=2x=34$ for subsp. *hederifolium* and $2n=4x=68$ for subsp. *crassifolium*). However, the hereby karyological analysis proves that both subspecies have diploid and tetraploid populations and their geographical distribution seems to be random. Moreover, the applied karyomorphometry is also unable to differentiate the subspecies. Concluding, the current taxonomy of *C. hederifolium* s.l. remains intriguing. Thus, further studies are needed to clarify whether *C. hederifolium* subsp. *hederifolium* and *C. hederifolium* subsp. *crassifolium* are distinct subspecies, and if their distinction based on the ploidy level is an important taxonomical character.

Acknowledgements

We are grateful to The Cyclamen Society for the award that funded the current study. Moreover, we would like to thank Prof. Emerita G. Kamari and Prof. Emeritus D. Phitos and our colleagues Ch. Kyriakopoulos and E. Katopodi for collecting live material.

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