

ACCEPTED AUTHOR VERSION OF THE MANUSCRIPT
80(1), 1st April 2021

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Please cite this article as: Rat M, Bogdanović S: *Ornithogalum sibthorpii* Greuter (Asparagaceae), an overlooked species in Croatia. *Acta Botanica Croatica*, DOI: 10.37427/botcro-2021-003.

This is a PDF file of a manuscript that has been accepted for publication. The manuscript will undergo and language and technical editing, formatting and author proofing before it is published in its final form.

***Ornithogalum sibthorpii* Greuter (Asparagaceae), an overlooked species in Croatia**

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Running title: *Ornithogalum sibthorpii* in Croatia

Abstract – *Ornithogalum sibthorpii* (Asparagaceae) is one of the early-flowering species, with scattered populations, distributed across the Balkan Peninsula and Turkey. It inhabits rocky places and clearings, open habitats, parks and marginal parts of wetlands. Based on known distribution, habitat preferences and literature records for the Balkan Peninsula, assumption was that this species might be distributed in Croatia as well. To confirm this, herbarium material was revised, and field investigations were organized. First report confirmed that *O. sibthorpii* is widespread along the eastern Adriatic coast, reaching inland Dinaric region too. To present nowadays known localities in Croatia, a distribution map is provided. A detailed morphological and leaf anatomical descriptions are given. Morphological affinities with similar species, *O. excapum* and *O. refractum*, are also briefly discussed, and identification key is given. All Croatian populations of *O. sibthorpii* resulted as diploids with chromosome number $2n=18$.

Keywords: anatomy, geophytes, karyology, morphology, *Ornithogalum*

Introduction

The genus *Ornithogalum* L. is characterized with wide ecological tolerance and pronounced morphological variability. According to Stevens (2017) it is one of the largest genera in the Asparagaceae family, with approximately 160 species (300 taxa). It is distributed in Africa, Europe and Southwest Asia (Speta 1998, Martínez-Azorín et al. 2013). Global databases records nearly 120 species with distribution in the Mediterranean region and rest of Europe (Govaerts 2019, Euro+Med 2006-2019). According to Nikolić (2019a, b) the Croatian flora comprises 19 *Ornithogalum* species, of which three are stenoendemic, and 10 species belong to the subgenus *Ornithogalum*.

Several discrete European *Ornithogalum* groups are differentiated based on flowering time; respectively species flowering in late winter/early spring, spring, and in late spring/early summer season. One of the species that flower in late winter/early spring season is *Ornithogalum sibthorpii* Greuter. It is rare and scattered species, distributed in western part of Turkey and in the Balkan Peninsula, reaching in north Dobrugea in Romania (Sibthorp and Smith 1809, Baker 1873a, b, Markgraf 1932, Radenkova 1964, Zahariadi 1966, 1977, 1980, Diklić 1975, Landström 1989, Speta 1990, Rat and Barina 2017, Rat 2019). It prefers karst,

rocky grounds, clearings and open habitats (Zahariadi 1980), and belongs to the group of *Ornithogalum* species that have underground scape, bearing from one to many flowers on the shortened inflorescence. Taxonomically significant characters compared with morphologically similar species in investigated region (*O. exscapum* Ten. and *O. refractum* Kit. ex Schltdl.) are more or less pronounced pulvinus, refracted pedicels at anthesis and bulbs without bulbils (Zahariadi 1980, Landström 1989).

There is one literature data which indicate that *O. sibthorpii* is distributed in Croatia. Ascherson and Graebner (1905-1907: 249) stated that *Ornithogalum nanum* Smith in Sibth. et Smith (today synonym of *O. sibthorpii*) is recorded in Ljubuški and Stolac in Bosnia and Herzegovina, near to the Croatian border. Furthermore, for general distribution they cited the Adriatic region which covers important part of the Croatian coast. For that reason, herbarium revisions and field trips were organized, with the aim to provide new data about species potential distribution in the western part of the Balkan Peninsula i.e. Croatia, and to complement it with biological and ecological descriptions.

Materials and methods

Field trips were organized during season in 2015 and 2016. Plant material was collected for morphological, anatomical and karyological analysis (Tab. 1). Voucher specimens are kept in the herbarium collections ZAGR and BUNS. Herbarium materials of *O. sibthorpii* were revised in BEO, BEOU, BP, BUNS, K, MKNH, SOM, SARA, W, WU, ZA, ZAGR and ZAHO. Virtual collections G, GZU and OXF were accessed as well. Herbaria acronyms follow Thiers (2019). Distribution map of *O. sibthorpii* in Croatia was produced in QGIS software ver. 3.10.

Observations and morphological analyses included qualitative and quantitative description of bulbs, leaves, scape and inflorescence with flowers. Ten fresh plants were collected and pressed for analysis. All observations were performed on fresh material using binocular stereomicroscope Leica M205C, while measurements were carried out using Digimizer image analysis software ver.4.2.6.0.

Anatomical investigations included description of leaf structure. Cross sections were cut from the middle part of leaf blade, using Leica CM 1850 cryostat, at -20 °C with cutting interval of 60 nm. Observations and measurements were performed using ZEISS light microscope AxioVision A2, equipped with a ProgRes Speed XT^{Core5} camera and CapturePro v.2.8.8 image analysis software.

For the chromosome counting and analyses, 10 bulbs were planted in pots, and young root tips were collected, treated with 0.5% colchicine solution for 1 h at room temperature, and then fixed with fresh solution of ethanol and glacial acid (3:1) for storage. To visualize metaphase chromosomes, root tips were hydrolysed with 1M HCl at room temperature, and then stained with Schiff's reagent. Standard squash technique was used for preparation of slides (Jong 1997). Image analysis and measurements of chromosomes were completed using KaryoType 2 software (Altinordu et al. 2016).

Results

Ornithogalum sibthorpii Greuter, Boissiera 13: 160 (1967) (Fig. 1)

Type – “In Arcadia, et prope Abydum, Martio florens”, Smith loc. cit. (OXF!; file name: Sib-0793).

Synonyms – *Ornithogalum nanum* Smith in Sibth. et Smith, Fl. Graec. Prodr. 1: 230 (1809); Fl. Graec. (Sibthorp). 4: 28, t. 333 (1823); non *O. nanum* (Burm. f.) Thunb., Prodr. Fl. Cap. 62 (1794).

Morphological description (based on Croatian material, Fig. 1) – Bulb hypogeal, ovoid, 12 – 15 × 0.8 – 20 mm, without bulbils; outer tunics light brown to brown. Leaves numerous, glabrous, longer than inflorescence, up to 3.5 mm wide, canaliculate, with median white stripe on the adaxial side. Scape short, mostly hypogeal, 1 – 5 cm long. Inflorescence up to 20 mm long, corymbose, with 1 – 8 (12) flowers. The overall length (scape + inflorescence) is ca. 5 cm. The lower pedicels refracted, 10 – 25 mm long, with the pulvinus at the base. Bracts shorter or equal to pedicels. Outer tepals 16 – 20 × 4 – 7 mm, with abaxial green stripe 4 – 6 mm wide. Inner tepals 15 – 21 × 5 – 8 mm, with abaxial green stripe 3 – 6 mm wide. Filaments 7 – 11 mm long; anthers 2.5 – 5 mm long. Ovary elongated to rounded, 5 – 6 × 3.5 – 5.5 mm, with 6 prominent ribs; style 4 – 6 mm long, longer or equal to ovary. Seeds globose, 1 – 2 mm in diameter, black, luminous, with reticulate testa.

Phenology – Flowering and fruiting time from March to June.

Leaf anatomy – Leaf has typical “*umbellatum*-type”, that is “U” shape on transverse cross section. Leaf blade is canaliculate, on abaxial side with 3 – 6 ribs. Longitudinal white stripe visible on the adaxial side is a consequence of non-continuous palisade tissue in the central part. On the cross section are visible 1-layered epidermis, 1-layered palisade tissue and mesophyll. Vascular bundles are arranged in two lines: larger bundles are in the centre of mesophyll, and smaller ones along palisade tissue of abaxial side. Other than vascular bundles, mesophyll contains large and small cavities, later usually with raphid crystals (Fig. 2).

Karyology – For karyological studies bulbs were available from localities Brgat, Donji Lapac and Nin. All investigated individuals were diploid, $2n=2x=18$ (Fig. 3a). Combined chromosome formula is $2n=2x=6m + 12sm$, and Stebbins karyotype asymmetry degree is 2A. Two chromosome pairs are long, three of medium length and four pairs are small chromosomes. Karyotype is characterized with chromosomes that have more or less gradual transition in size (Fig. 3b). Two pairs are metacentric, while others are submetacentric, with total haploid chromosome length $59.01 \pm 0.89 \mu\text{m}$ (Tab. 2). Satellited chromosomes were not detected.

Distribution and habitat – First herbarium specimens that confirmed presence of *O. sibthorpii* in Croatia were found in the collection of Ivo Horvat (ZAHO), and data back to the first mid of the 20th century. Since then this species was omitted in collections, most probably due to short vegetation period and early spring flowering time. However, herbarium revision of collections in GZU, W and WU, revealed that species was recorded earlier, but sparse data led to the neglecting of the species in Croatia. Recent field trips expanded known distribution range of *O. sibthorpii* in Croatia. It is spread from the southeast of the eastern Adriatic coast to the central Dalmatian region in west, and in north the central Dinaric region, which is only continental location in Croatia. According to all gathered data distribution map was created (Fig. 4). *O. sibthorpii* inhabits dry, clear, open habitats, dry hillsides as well as anthropogenic areas and bare surfaces. Estimated altitude in Croatia ranges from sea level up to 1000 m. In other regions of the Balkan Peninsula habitats and altitude are similar (Radenkova 1966, Rat et al. 2014, Rat and Barina 2017, Rat 2019).

Examined specimens (*specimina visa*) – **Croatia:** Lika, Krbava – Donji Lapac, Bare, u nižem vlažnijem dijelu polja, 07.06.1958, I. Horvat s.n. (ZAHO-41129, ZAHO-41130); Makarska (Dalmat.), 17.04.1931, M. Salzmann s.n. (GZU-057466); Orebić (Dalmatien), 16.04.; 19.04.1930, M. Salzmann s.n. (GZU 057466); Süddalmatien, Halbinsel Pelješac: zw. Orebić u. Sattel östl. des. Mte. Vipera, 16.04.1930, J. Eggler s.n. (GZU 103693); Süddalmatien, Halbinsel Pelješac: Kräuterfluren u. Macchie westl. von Orebić., 14.04.1930, J. Eggler s.n. (GZU 103694); Dalmatien, Gravosa [Dubrovnik], 10.04.1933, K. Ronniger s.n. [sub *Ornithogalum exscapum* Ten.] (W, Herbarium Karl Ronniger 5580); Dalmatia, in vineis

pr. Spalato [Split], 04.1870, Pichler s.n. [sub *Ornithogalum umbellatum* L. (Vis.)] (WU, Herbarium Kerner).

Identification key for *O. sibthorpii* and morphologically similar species in Croatia

1. Bulb with numerous bulbils (>10) outside of tunics; bracts equal or longer than pedicels..... *O. refractum*
- Bulb without bulbils; bracts equal or shorter than pedicels 2
2. Scape hypogeous, 20–50 mm long; inflorescence 1–8 (15)-flowered; lower pedicels refracted at anthesis, up to 30 mm long; pulvinus prominent; bracts subequal the pedicels *O. sibthorpii*
- Scape only partly hypogeous, 10–30 mm long; inflorescence 5–10-flowered; pedicels erect to divergent (basal pedicel), 35–42 mm long; pulvinus absent; bracts shorter than pedicels *O. exscapum*

Discussion

This species is known since the pre-Linnean period. Buxbaum (1728) described it for the first time from the region around Istanbul (Constantinopolium) in Turkey and he also provided first iconography of species. Species was overlooked by Linne, but later botanists who investigated Greece and eastern Mediterranean, Sibthorp and Smith, described *Ornithogalum nanum* as new species. *Ornithogalum nanum* Smith in Sibth. and Smith (1809) was described as: „*O. nanum*, corymbo simplici paucifloro glabro scapo longiore, bracteis ventricosis scariosis, foliis linearibus numerosis“. The locality is cited as „In Arcadia, et prope Abydum, Martio florens“, with reference to Buxbaum (1728). Abydum is ancient name for the region close to nowadays Cannakale in Turkey, and Arcadia is ancient region in Peloponnese, but as well in Antalya (Turkey) (Stearn 1967). Because of it, the precise *locus classicus* is not known. Detailed description of the species was published later (Sibthorp and Smith 1823). Herbarium specimen marked as type material is deposited in OXF herbarium, under the file number Sib-0793. Since *O. nanum* is later homonym of *O. nanum* (Burm. f.) Thunb., replacement name *O. sibthorpii* was published by Greuter (Greuter and Rechinger 1967), in honour to John Sibthorp (1758-1796) who collected plant material (Stearn 1984).

Although in most of the relevant databases (e.g. ThePlantList, Euro+Med Checklist) *O. sibthorpii* is synonymised with *O. sigmoideum* Freyn et Sint. we retain outlook given by Landström (1989), and later supported by results of Speta (1990), that *O. sibthorpii* is a good species which should not be underestimated without systematic investigations.

Results of the field trips organized in 2015 and 2016 confirmed our assumptions that *O. sibthorpii* is distributed in Croatia, and those data are presented by Rat and Bogdanović (2016) on the 5th Croatian Botanical Symposium. Based on this report and deposited herbarium specimens in ZAGR herbarium (ZAGR-55520, 55521), Nikolić (2019a, b) included it in the national list of the vascular flora. Historical data about distribution in Italy (Parlatore 1857) however have to be revised, since this record was included in distribution data for the species *O. mutabile* De Not., which is in meantime synonymized with *O. exscapum* by Peruzzi and Passalacqua (2002) and Garbari et al. (2003). Landström (1989) summarized differential morphological characters of *O. sibthorpii* and *O. exscapum*, describing *O. sibthorpii* with shorter pedicels, larger flowers, longer styles and anthers. Analysing material from Croatia, additional differential qualitative morphological characters for these two species are: pedicel shape (in *O. sibthorpii* refracted at anthesis, in *O. exscapum* ascending to deflexed), visible pulvinus in *O. sibthorpii*, and absence of pulvinus in *O.*

exscapum. Other informative parameter is bract/pedice length ratio; in *O. sibthorpii* bract and pedice are almost of the same length, while in *O. exscapum* bract is of the ca. half-length of pedice length.

Comparative morpho-anatomical and cytotaxonomical studies of *Ornithogalum* species that belong to the group of small plants (overall length up to 10 cm) with hypogee scape, including *O. sibthorpii*, have been done to clearly describe morphologically similar species in the area of Turkey and the Balkan Peninsula (Zahariadi 1962, 1965, 1977, Speta 1990, 2000). Both authors recognized three separated species: *O. sintenisii* Freyn, *O. sigmoideum* and *O. sibthorpii*. In addition, Speta (1990) described two new species, *O. saginatum* Speta and *O. plutulum* Speta, first from Romania and Moldavia, and later from the Balkan Peninsula.

Moreover, Landström (1989) recognized two ecotypes (montane and lowland) of *O. sibthorpii* and compare them to intermediate type, localised for the region of İstanbul (Turkey). These ecotypes are confirmed in Croatia as well, since for plants collected on higher altitudes in continental region (Donji Lapac, Lika, Dinaric area, 900 m a.s.l.) flowering time is in June. In Croatia, two distinct morphotypes of *O. sibthorpii* are observed: ascapose and scapose. Ascapose form (Fig. 1a) is characterized with short scape, and the inflorescence is more or less sessile on the ground, with numerous flowers; pulvinus is strong and prominent. Scapose form (Fig. 1b) is differentiate with scape up to 5 cm long, and inflorescence with only few (1 to 2) flowers (Fig. 1b). Comparing to results of Speta (1990) who investigated *O. sibthorpii* and related species in eastern part of the Balkan Peninsula, we can confirm that both morphotypes are mostly present at the same population. Going furthermore, he indicated that it is not unusual that two or more species from this group, that are morphologically invisible, coexist in one locality.

In the Balkan Peninsula, *O. sibthorpii* could be easily misidentified with *O. refractum*. Most informative discriminate characteristic is bulb without bulbils in *O. sibthorpii* (Landström 1989, Rat et al. 2014). It is not unusual to find both species in close localities, when in addition to bulb parameters flower characteristics can contribute to species identification (Rat et al. 2014). Furthermore, according to Rat et al. (2017) seed testa micromorphology can be used as taxonomically important character for recognition of *O. sibthorpii* from other *Ornithogalum* species. Globose seeds are typical only for *O. sibthorpii* (Fig. 1e) and *O. refractum*, while, former is differentiated as species with rather small seeds.

Cytological review by Cullen and Ratter (1967), with doubtful discussion on plant material, reported three main cytotypes for *O. sibthorpii* – *O. sigmoideum* group, in the region from Caucasus to Italy, with numerous aloploid series reported from different sources ($2n = 12, 16, 17, 18, 19, 20, 28$). They did not undertake any morphological measurements, and it was impossible for them to compare morphotypes and cytotypes, as well to discuss in more detail obtained results. Nonetheless, they recognized only two species *O. sigmoideum* and *O. sintenisii*, while *O. sibthorpii* is synonym of *O. sigmoideum*. Opposite review was presented by Speta (2000), who included both morpho-anatomical and cytological reports for 18 species that belong to this group. He recognized all species based on taxonomical characters, including described karyotypes. Derived from his results, *O. sibthorpii* is evidently separated from all other species, with $2n = 14, 28$ chromosome complements, while in *O. sigmoideum* they are $2n = 20, 24$. Chromosome arrangement $2n = 18$ was documented also by Speta (1990) in unresolved discussion in short notes. In addition to these species, for *O. sintenisii* chromosome number $2n = 12$ is recorded. Diverse chromosome numbers ($2n = 14, 16, 18, 24, 28$) for *O. sibthorpii* were recorded and documented from the Balkan Peninsula, by other authors as well. For Bulgaria, Markova (1972) reported three different chromosome numbers ($2n = 14, 16, 28$), while Lungeanu (1971) reported $2n = 18, 24$ for Romania.

Following evolutionary patterns in different *Ornithogalum* groups, it can be recognized that in almost all subgenera there are several groups or complexes that are characterized with “high morphological variability” and extensive chromosome numbers among samples. By now, for most of them that systematic studies have been undertaken it has been confirmed that every complex (i.e. *O. tenuifolium* in Africa and *O. umbellatum* in Europe) includes „good species“ and „transitional forms“ (cytotypes and morphotypes) that exists in nature thanks to vegetative reproduction (Stedje and Nordal 1984, 1987, Raamsdonk 1985, Raamsdonk and Heringa 1987, Andrić et al. 2015). Stedje and Nordal (1984) concluded that rapid evolution processes in *Ornithogalum*, visible as cytotypes differentiation, does not express its changes on morphological characters, but does indicate on evolutionary progress in the taxon. This statement evidently can be accepted for the *O. sibthorpii* related group that was studied in this research, taking into consideration that many similarities were observed.

Acknowledgements

We would like to thank Zrinka Ljubešić ex-president of the Croatian Botanical Society (HBoD) for financial support during the field trips to Nin and to Donji Lapac. We are grateful to our colleagues Milenko Milović and Marija Pandža who provide a new locality of *O. sibthorpii* from Sukošan. Research work of Milica Rat, as a part of PhD Thesis, was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 451-03-68/2020-14/ 200125).

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Fig. 1. *Ornithogalum sibthorpii* (Croatia, material from Brgat locality): a – ascapose form with prominent pulvinus (bulb without outer tunics), b – scapose form with small pulvinus, c – flower, d – gynoecium, e – seed micromorphology. Scale bar: a-d – 1 mm, e – 200 μ m.

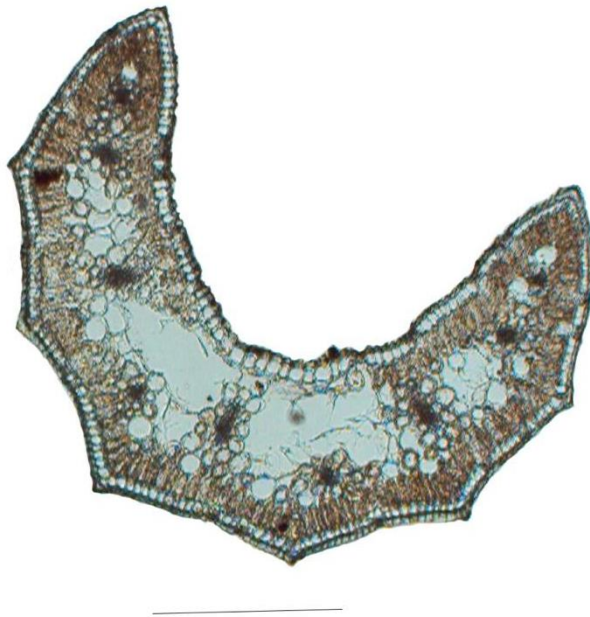


Fig. 2. Leaf “U” cross section of *Ornithogalum sibthorpii* (Croatia, material from Brgat locality). Scale bar: 50 μm .

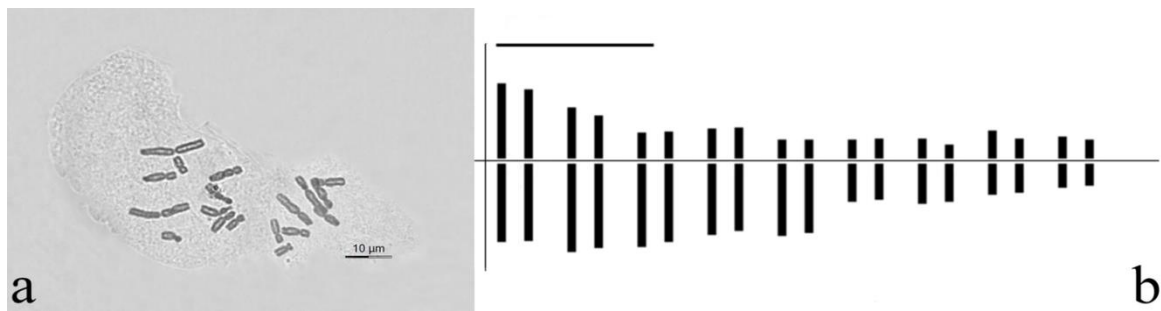


Fig. 3. Mitotic metaphase chromosome plate of *Ornithogalum sibthorpii* (Croatia, from Brgat locality): a – metaphase plate; b – karyogram ($2n=2x=18$). Scale bar: 10 μm .



Fig. 4. Distribution map of *Ornithogalum sibthorpii* in Croatia.

Tab. 1. List of examined plant materials of *Ornithogalum sibthorpii* in Croatia.

Locality	Latitude / Longitude	Collecting date	Collector(s)	Herbarium voucher number
Croatia, Brgat (South Dalmatia)	42°38'45.06" 18°09'30.63"	28.03.2015	M. Rat	BUNS-2-1151
Croatia, Nin (North Dalmatia)	44°14'48.58" 15°10'38.10"	23.04.2016	S. Bogdanović, V. Šegota, Z. Ljubešić	ZAGR-41135
Croatia, Donji Lapac (eastern part of the region of Lika)	44°31'36.82" 15°58'44.48"	06.05.2016	S. Bogdanović	ZAGR-55520 ZAGR-55521
Croatia, Sukošan (North Dalmatia)	44°02'59.10" 15°24'17.05"	05.04.2018	M. Pandža, M. Milović	ZAGR-46301

Tab. 2. Chromosome parameters for *Ornithogalum sibthorpii* in Croatia. Ten individuals are analyzed ($2n=2x=18$): four from Donji Brgat, three each from Nin and Donji Lapac. Number of analyzed metaphase plates is 40 in total (four per individual). Abbreviations: L – long arm length, S – short arm length, TAL – total absolute length, TRL – total relative length, m – metacentric, sm – submetacentric.

Chromosome pairs	L (μm)	S (μm)	TAL (μm)	TRL (%)	Type
I	6.20±0.72	5.13±1.03	11.32±1.39	10.51+8.68=19.19	m
II	5.96±0.48	3.12±0.65	9.08±0.84	10.10+5.29=15.38	m
III	5.41±0.55	2.08±0.51	7.49±0.97	9.17+3.52=12.69	sm
IV	4.52±0.99	2.18±0.50	6.71±1.30	7.66+3.70=11.36	sm
V	4.17±0.71	2.01±0.54	6.18±0.98	7.07+3.41=10.47	sm
VI	3.80±1.25	1.67±0.41	5.47±1.27	6.44+2.83=9.27	sm
VII	3.04±0.51	1.87±0.58	4.92±0.99	5.15+3.18=8.33	sm
VIII	2.82±0.58	1.54±0.29	4.36±0.62	4.78+2.61=7.39	sm
IX	2.10±0.82	1.39±0.51	3.49±1.18	3.55+2.36=5.91	sm