The elusive *Juncus minutulus*: a failure to separate tetra- and hexaploid individuals of the *Juncus bufonius* complex in a morphometric comparison of cytometrically defined groups

Tajemný *Juncus minutulus* – neúspěšný pokus odlišit tetra- a hexaploidní jedince komplexu *Juncus bufonius* při morfometrickém srovnání cytometricky definovaných skupin

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Screening of nuclear genome size was carried out on ca 2400 plants from over 120 mainly Central-European localities of the *Juncus bufonius* group. Besides the diploid level, corresponding to known diploid species (in this case *J. ranarius*, *J. hybridus* and *J. sorrentini*), two polyploid cytotypes were detected, conforming with the tetraploid and hexaploid levels treated by some authors as separate species: *J. minutulus* and *J. bufonius* s. str. The relationship between nuclear DNA content and the number of chromosomes was verified by chromosome counting. Polyploidy, as opposed to agmatoploidy can, therefore, account for the karyological variation. The 2C values of diploid, tetraploid and hexaploid individuals were ca 0.65, 1.18±2.8% and 1.84±1.6% pg 2C DNA, respectively. No other cytotype or statistically significant variation in nuclear genome size was found. To asses the utility of hitherto published morphological characters distinguishing *J. minutulus* from *J. bufonius* s. str., measurements of seven floral and three vegetative quantitative characters were obtained (no less than 10 measurements per flower, 30 per plant) for 358 mature plants of known ploidy level from 47 localities. Diverse ordination and clustering techniques did not indicate the presence of any grouping in the dataset. Canonical discriminant analysis and stepwise variable selection indicated that inner tepal length followed by mean capsule width and mean capsule length were the most useful characters for identifying the two ploidy levels; however, the estimated 10-fold cross-validation error rate of a simple k nearest neighbour classification analysis was 0.45. Other analyses corroborated this result. No new morphological character that would allow successful separation of tetraploids from hexaploids was discovered. This provides independent support for the opinion of some previous authors that *J. bufonius* L. is best treated as a single variable species comprising two cytotypes that are inseparable using hitherto suggested diagnostic characters until convincing proof to the contrary is available.

K e y w o r d s: chromosomes, discriminant analysis, flow cytometry, *Juncus bufonius*, *J. minutulus*, morphometrics, polyploidy

Introduction

Annual rushes of the *Juncus bufonius* L. (*Juncus*, subgen. *Agathryon*, sect. *Tenageia*) kinship, commonly known as toad rushes, comprise at least six currently accepted species: *J. bufonius* s. str., *J. hybridus*, *J. minutulus*, *J. ranarius*, *J. sorrentini* and *J. turkestanicus* (Kirschner 2002c, see therein for authors of plant names). The centre of diversity of *Juncus* subgen. *Agathryon* lies in the western Mediterranean. This group is highly variable in overall appearance and both quantitative and, to a limited extent, qualitative traits, yet notoriously poor in the number of diagnostic characters. It is therefore considered taxonomically intricate (Holub 1976, Kirschner 2002c and others). The most widely distributed of the species, with cosmopolitan or almost cosmopolitan ranges, represent three chromosome ploidy levels: (i) *J. ranarius*, a diploid with 2n = ca 34 chromosomes; (ii) *J. minutulus*, a tetraploid with 2n = ca 72 chromosomes; and (iii) *J. bufonius* s. str., a hexaploid with 2n = ca 108 chromosomes (van Loenhoud & Sterk 1976, Mičieta & Mucina 1983, Cope & Stace 1985, Kirschner 2002c). All members of the group occur most frequently in open wet habitats. Bernhardt (1993) studied the dynamics of *J. bufonius* during succession and found that it is a rapid colonizer and reaches its highest population densities in the first two years. Toad rushes have few recorded uses for mankind. For example, some native Americans use a toad rush decoction as an emetic agent (Moerman 1998). The current distribution of these widely distributed species owes much to human-facilitated dispersal (Snogerup 1980, Kirschner 2002c). Information on the distribution of *J. minutulus* is the least complete. Only a very few localities are based on karyological data and most rely on morphological identification. No independent marker other than chromosome counts, such as DNA polymorphisms or nuclear DNA content, was ever employed to discriminate between tetraploids and hexaploids. The utility of flow cytometry for this task has not been tested.

The relationship between *J. bufonius* s. str. and *J. minutulus*, and especially the status of the latter taxon, is debatable. Different authors have published opposing opinions and data to support them. One group of authors favours distinguishing narrowly defined species (e.g. Holub 1976, van Loenhoud & Sterk 1976, Mičieta & Mucina 1983) and another is more inclined towards accepting wider species delimitations (e.g. Foerster 1969, Cope & Stace 1973, Novikov 1978, Stasiak 1978b). This lack of consensus is reflected in different floras, in which *J. minutulus* either is (e.g. Snogerup 1971, 1980, Kirschner 2002a, c) or is not (e.g. Stace & Thompson 1997, Sell & Murrell 2006) accepted as a separate species depending on the author(s).

Holub (1976), in a short article on the presence of *J. minutulus* in Czechoslovakia, maintains that by measuring quantitative traits it is possible to differentiate between individuals of *J. minutulus* and *J. bufonius* s. str. even when they grow sympatrically and form mixed clumps. He is of the opinion that the smaller size of some flowering and fruiting organs in *J. minutulus* "seems completely constant" and that, with experience, the two species can be distinguished relatively easily.

Dutch authors van Loenhoud & Sterk (1976) record three discrete ploidy levels (which they called "cytodemes") within this group in the Netherlands: (i) 2n = 27–37; (ii) 2n = 58–78 and (iii) 2n = 81–115. To compare the morphology of the three ploidy levels, they sampled two flowers from a comparable part of the inflorescence of 10 plants per ploidy level (i.e. altogether 60 flowers of 30 plants of three ploidy levels). They then carried out

a series of Wilcoxon tests to assess the differences among the three groups and used their results to choose a set of supposedly differentiating characters. They then used these characters to identify 584 herbarium sheets of the *J. bufonius* group in order to ascertain the geographic distribution and ecological preferences of the three cytotypes. Merely mentioning the existence of the name *J. minutulus*, Novikov (1978) cites Holub (1976) but dismisses the justification for accepting *J. minutulus* (and also "*J. erythropodus*") as species, stating that these "minute taxa" are conditioned by soil properties.

Stasiak (1978b) investigated the morphology of *J. bufonius* and *J. ranarius* (under the name *J. ambiguus* Guss.) in Poland employing biometric methods described in Stasiak (1978a). She sampled 50 or 30 individuals from 21 Polish localities and collected data on 22 characters. Interestingly, although she cites Novikov (1978), Stasiak does not cite van Loenhoud & Sterk (1976) and Holub (1976). Stasiak uses the name *J. minutulus,* as described by Kreczetovich & Gontscharov (1935), without mentioning the earlier homonym *J. minutulus* (Albert et Jahand.) Prain, which is the valid name according to Holub (1976) and Kirschner (2002c). Regarding *J. minutulus* (i.e. *J. minutulus* Krecz. et Gontsch.), she completely rejects the taxon, saying that it is a form which is entirely conditioned by soil properties and that both *J. bufonius* and *J. ranarius* produce such phenotypes. She does not address the karyological difference between *J. minutulus* and other species.

Cope & Stace (1983) support the recognition of five species in this group, four of them being diploid (*J. foliosus, J. ranarius, J. hybridus* and *J. sorrentini*) and the fifth comprising known polyploids (i.e. *J. minutulus* and *J. bufonius* s. str.). These authors hold the view that polyploids represent either "…a single, highly polymorphic species" or "several taxa whose distinctions are partially obscured by intermediates." Cope & Stace (1983) acknowledge that Snogerup (1971), Snogerup (1980) and van Loenhoud & Sterk (1976) accept *J. minutulus* on account of cytological, morphological and ecological differences but claim that these differences are "far from clear, at least at the level of the individual." Cope & Stace (1983) also suggest that the variation in polyploids, great as it is, is even greater in areas where they co-occur with diploids.

Mičieta & Mucina (1983) criticized Stasiak (1978b) for rejecting *J. minutulus* as a taxon without considering karyological data and because her claim that plants ascribed to this species are in fact mere "rachitic" forms is based on a single sample. They also direct some criticism towards Cope & Stace (1983) for not accepting *J. minutulus* as a species, saying that Cope and Stace base their assertions on an erroneous understanding of the species' description. Mičieta & Mucina (1983) carried out their own numerical taxonomic study of 34 Slovak populations (15–25 specimens from each) of the *J. bufonius* complex, measuring 11 characters (50 measurements per character). The statistical approaches they used include principal component analysis, reciprocal averaging, single linkage clustering, average linking clustering, McQuitty's similarity analysis, Ward's method, Lance-Williams beta-flexible method, one-way ANOVA and F-tests. They conclude, in general agreement with van Loenhoud & Sterk (1976), that the following characters are statistically significant for the separation of *J. ranarius* (2n = 34), *J. minutulus* (2n = ca 72) and *J. bufonius* (2n = ca 100–110): length of capsules, length of seeds, length of inner tepals, length of anthers and the anther to filament length ratio. All clustering techniques they used, with the sole exception of single linkage clustering, always separated *J. minutulus* in

their data. Still, this was not enough to convince authors such as Cope & Stace (1973, 1983, 1985) to accept *J. minutulus* as a species.

Cope & Stace (1985) studied stomatal length in the *J. bufonius* polyploid complex and conclude that there are three groups of species that seem to correlate more with ecological specialization than with the level of ploidy. Anyhow, Snogerup (1971) used stomatal length to successfully predict the chromosome count of *J. turkestanicus*. Cope & Stace (1985) present a hypothesis that polyploids within the *J. bufonius* complex have possibly arisen through hybridization of diploids, allotetraploidization and subsequent backcrossing with diploids and tetraploids, which produce hexaploids, possibly in a polytopic manner, a process that may be still continuing. They view the *J. bufonius* aggregate as an example of the "pillar complex" as conceived by Babcock & Stebbins (1938; see also Stace 1992).

Previous studies never thoroughly address the question whether chromosome counts in the group correlate with nuclear genome size. The sole previously published value of nuclear DNA content for *J. bufonius* (ploidy level not indicated) is that of 2.60 pg 2C DNA, which was determined by Feulgen staining (Leitch et al. 2005, citing Band 1984 pers. comm.). Kirschner (2002c) suggests that agmatoploidy might account for at least part of the observed differences in chromosome counts, as is the case in the allied genus *Luzula* (see Nordenskiöld 1961, Kirschner 2002b, Kuta et al. 2004). In a self-published website, Harmaja (2003) discusses the supposed differences between *J. minutulus* and *J. bufonius* and proposes a few additional distinctions (see Table 1).

Unless stated otherwise, the name "*Juncus minutulus*" is used here to refer to *J. minutulus* (Albert et Jahand.) Prain., that is, the taxon described by Albert & Jahandiez (1908) and promoted to species rank by Prain (1921), not the later homonym *J. minutulus* Krecz. et Gontsch., which was independently chosen by Kreczetovich & Gontscharov (1935) for a similar, even though more narrowly circumscribed, group of plants. Snogerup (1985) accepts the species *J. minutulus* Krecz. et Gontsch. in the Flora of Turkey and not *J. minutulus* Albert et Jahand., unlike before in Snogerup (1971). He also states that, among the records of the occurrence of *J. bufonius* he cites, some "probably include forms resulting from hybridization with *J. minutulus* and *J. turkestanicus*." Cherepanov (1973) viewed *J. minutulus* Albert et Jahand. and *J. minutulus* Krecz. et Gontsch. as distinct taxa, but he is the only author to hold this opinion. *Juncus bufonius* excluding *J. minutulus* is referred to here as *J. bufonius* s. str. and the term *J. bufonius* s.l. is used when including *J. minutulus* in *J. bufonius*.

A modern study comparing *J. minutulus* and *J. bufonius* requires an independent marker other than chromosome numbers. Flow cytometry seems to be the best choice (see Suda & Pyšek 2010), provided that observed karyological differences are not caused by agmatoploidy. The objectives of the present study were to ascertain whether agmatoploidy or polyploidy is behind the observed differences in chromosome numbers and, if cytometry shows the existence of different DNA-cytotypes, to undertake a new morphometric comparison of cytometrically defined groups to verify the purported morphological differences between tetra- and hexaploids and improve our knowledge of the group.

Table 1. – Non-comprehensive list of published diagnostic characters of *Juncus minutulus* and *J. bufonius* s. str.

Character	*Juncus minutulus*	*J. bufonius* s.str.	Reference
Plant height	1–2 cm	—	Albert & Jahandiez 1908
	0.8–5.0 cm	10–50 cm	Kreczetovich & Gontscharov 1935
	0.5–5.0 cm	5–50 cm	Snogerup 1971
	3–15 (–25) cm	1–5 (–50) cm	Snogerup 1985
	< 10 cm	—	Chicouène 1996
	ca 1.5–6.0 (–15) cm	(2–) 5–40 (–45) cm	Kirschner 2002c
Number of flowers	2–15 (–20)	few to > 100	Snogerup 1971
	2 or 3	—	Chicouène 1996
	ca 2–15 (–30)	—	Kirschner 2002c
Capsule length	1.5–2.5 mm	4–4.5 mm	Kreczetovich & Gontscharov 1935
	2.5–3.0 mm	(3.0–) 3.5–5.0 mm	Snogerup 1971, 1985
	2.5–3.0 mm	3–5 mm	Holub 1976
	2.5–3.0 mm	3.0–4.0 mm	van Loenhoud & Sterk 1976
	2–3 mm	—	Chicouène 1996
	2.5–3.0 mm	3.5–5.0 mm	Kirschner 2002c
	2.2–3.2 mm	3.5–4.5 mm	Harmaja 2003
Tepal to capsule length ratio	slightly longer	clearly longer	Harmaja 2003
Capsule shape	ovoid, obtuse	ovoid to subellipsoid	Snogerup 1971
	obtuse	ovoid to almost ellipsoid	Snogerup 1985
	oblong-ovoid to ellipsoid	capsule narrowly ovoid to subellipsoid	Kirschner 2002c
Outer tepal length	2.5–3.5 mm	6.5–7.5 mm	Kreczetovich & Gontscharov 1935
	4.0–6.5 mm	(4.5–) 6.0–8.0 mm	Snogerup 1971
	3.5–4.5 mm	4–8 mm	Holub 1976
	2.5–4.5 mm	4.5–6.5 mm	Harmaja 2003
	3.5–4.5 (–6.0) mm	4.0–7.5 mm	Kirschner 2002c
Inner tepal length	2–3 mm	5–6 mm	Kreczetovich & Gontscharov 1935
	3.0–4.5 mm	4.5–6.5 mm	Snogerup 1971
	ca 3.0–4.0 mm	ca 3.5–6.0 mm	Kirschner 2002c
Number of stamens	3, rarely 6	6	Snogerup 1971
	usually 3	usually 6	Holub 1976
	3 or 6	6	Snogerup 1985
	generally 3	6	Chicouène 1996
	2 or 3, rarely 6	6, rarely 3	Kirschner 2002c
Stamen length	1–1.2 mm	2 mm	Kreczetovich & Gontscharov 1935
Stamem to tepal length ratio	1/3–1/2	1/3–1/2	Snogerup 1971
	< 1/2	usually < 1/2	Kirschner 2002c
Anther length	0.2–0.6 mm	—	Snogerup 1971
	0.2–0.5 mm	0.5–1.7 mm	Holub 1976
	0.2–0.6	0.5–1.0 mm	Snogerup 1985
	0.2–0.6	0.5–1.0 mm or more	Chicouène 1996
	0.2–0.5 mm	(0.3–) 0.5–1.0 (–1.2) mm	Kirschner 2002c

Character	*Juncus minutulus*	*J. bufonius* s.str.	Reference
Anther to filament length ratio	1/5–1/1	2/5–1/1 (sometimes longer)	Holub 1976
	1/4–1/3	1/3–1	Snogerup 1985
	1/4–1/3	2/5	Chicouène 1996
Style length	0.1–0.4 mm	ca 0.5 mm	Snogerup 1971
	0.1–0.4 mm	0.4–0.5 mm	Kirschner 2002c
Stigma length	1–1.5 mm	ca 1 mm	Snogerup 1971
Stigma shape	divergent or almost contorted	deflected	Snogerup 1971
	recurved	± patent	Kirschner 2002c
Seed length	0.35–0.40 (–0.50) mm	0.40–0.55 mm	Snogerup 1971
	0.30–0.45 mm	ca 0.5 mm	Holub 1976
	0.36–0.42 mm	0.41–0.49 mm	van Loenhoud & Sterk 1976
	0.3–0.4 (–0.5) mm	—	Chicouène 1996
	0.30–0.45 mm	0.40–0.55 mm	Kirschner 2002a
Seed width	0.23–0.27 mm	0.24–0.30 mm	van Loenhoud & Sterk 1976
Seed shape	ovate, with 0.3 mm long dark mucro	ovate-elliptical, 0.3 mm	Kreczetovich & Gontscharov 1935
	ovoid to ellipsoid	ellipsoid, sometimes suboblique	Snogerup 1971
Overall colour of plant	paler, partly yellowish green	fairly dark greyish green	Harmaja 2003
Colour of mature capsule	pale to dark red (at apex at least)	green or pale brown	Harmaja 2003
Colour of mature capsule	mostly present	mostly absent	Harmaja 2003
Leaf width	< 1 mm	0.5–2.0 mm	Snogerup 1985
Leaf shape	short, usually subterate	flat, but margins often convolute	Snogerup 1985
Bract length	< 1 mm	< 2.5 mm	Kreczetovich & Gontscharov 1935
Stem	very thin, with usually only one small leaf below inflorescence	—	Snogerup 1985
Mode of pollination	cleistogamy	chasmogamy and cleistogamy	Snogerup 1971, Kirschner 2002c
	usually cleistogamy	cleistogamy or chasmogamy	Snogerup 1985

Materials and methods

Plant material

Plants sampled for the purpose of this study were collected between 2005 and 2008 in Europe (see Appendix 1) at altitudes ranging from sea level (Corsica and Italy) to ca 1900 m a.s.l. (Mt Petros, Ukraine). In a small number of cases, seeds from older herbarium collections, including a few from other continents, were also used (see Appendix 1). The

sampling strategy, both at the geographic and microhabitat scale, was based on the cluster method of random sampling. No stratification was carried out, and plants were sampled without prior assessment of their morphology. In practice, many populations were sampled in the regions visited (from different habitats when possible). From these populations, several plants growing close to each other in different parts of the population and along any obvious ecological gradient (e.g. in water regime or vegetation cover) were collected. The sample size varied in different populations, ranging from tens of individuals to about a hundred, but around 20 individuals were sampled at most localities. Plants were kept moist in plastic bags, refrigerated and analysed cytometrically as soon as possible. After being analysed, they were kept as herbarium specimens. Material that could not be analysed fresh was dried and pressed, and seeds from it were later used to obtain fresh tissue. To eliminate the (very real) danger of seed cross-contamination, loose seeds were discarded and only seeds extracted directly from their capsules still attached to the dried plants were used. Seeds stored in paper envelopes germinated readily throughout the year in Petri dishes filled with water and placed inside a growth chamber at temperatures of 25 and 10 °C in a 12-hour day/night cycle. Plants intended for further cultivation in the greenhouse were transferred to pots containing wet soil. Nomenclature follows Kirschner (2002c).

Flow cytometry

Approximately 2400 plants were analysed by flow cytometry. Up to 10 plants were analysed simultaneously in some FCM runs. As the internal reference standard, *Glycine max* cv. 'Polanka' (2C = 2.50 pg DNA) was used in most analyses; when soybean was unavailable, a minority of analyses were carried out with *Solanum lycopersicum* cv. 'Stupické polní tyčkové rané' (2C = 1.96 pg DNA). Based on a direct comparison with *Glycine max* cv. 'Polanka' (2C = 2.50 pg DNA), the 2C value of the *Solanum lycopersicum* standard used was found to be 2.07 pg of DNA. Analyses carried out with *Solanum lycopersicum* were not included in calculations of 2C values of samples and were only used for ploidy estimation. Soybean was the internal standard of choice because, firstly, the peak of tomato is located too close to the peak of hexaploid *J. bufonius* (see Fig. 1D) and, secondly, the position of the tomato peak overlaps the G2 peak of hexaploid *J. bufonius*.

Nuclei were prepared in a two-step procedure described by Doležel et al. (2007). Intact leaf tissue of the plant(s) being analysed was chopped together with leaf tissue of the internal reference standard in 0.5 cm^3 of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990). The sample was filtered through a 42-µm nylon mesh and incubated for 10 min. at room temperature. The staining solution consisted of 1 cm^3 of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$) supplemented with propidium iodide and RNase IIA (both at final concentrations of 50 $\mu g \cdot cm^{-3}$) and ß-mercaptoethanol (2 $\mu l \cdot cm^{-3}$). The fluorescence intensity of isolated nuclei (5000 particles) was recorded using a Partec CyFlow SL cytometer equipped with a diode-pumped solid state laser 532 nm (Cobolt Samba, 100 mW output power). The gain of the cytometer was adjusted to position the peak of the internal standard on channel 200. The Partec Ploidy Analyser PA-II with mercury arc lamp (UV) illumination was used for analyses of DAPI-stained nuclei. Data were acquired using the device's default software Partec FloMax version 2.4d (Partec GmbH, Münster, Germany) and saved in Flow Cytometry Standard version 2.0 format (Dean et al. 1990) of the Society for Analytical Cytology. Peak detection was done using the binary proprietary software's inbuilt functions.

Although only CVs below 3% are usually considered acceptable (Marie & Brown 1993, Galbraith et al. 1998), this requirement may be relaxed somewhat, and CVs up to 5% can be considered acceptable if better precision cannot be achieved (Doležel & Bartoš 2005). A threshold value of 3.5% was arbitrarily chosen. Any analysis in which the CV of either the peak of the samples or the internal standard exceeds this limit was not included in nuclear genome size calculations.

Whole seedlings as young as a few days, leaves, stems, bracts and parts of the inflorescence, except the flowers, were analysed to test for the presence of ontogenetic variation and endopolyploidy. Same samples were analysed repeatedly to establish whether any temporal variation occurs.

Chromosome counting

Germinating seeds were pre-treated in saturated 1,4-dichlorobenzene for two hours, placed in freshly prepared Carnoy's I solution (3:1 95% ethanol to glacial acetic acid), left at room temperature for 24 hours and stored in a refrigerator for up to one month. Wet mounts were prepared for viewing. A sprout was dipped in a solution of 1 EtOH : 1 HCl (1:1 95% ethanol to 38% hydrochloric acid) and placed in water for a few minutes. The root tip was then cut off on the mount, water was drained and a drop of stain added. A cover slip was then placed over the specimen and squashed with a needle. The cover slip was lifted with a razor blade so that the stain reached the whole specimen and the squash was completed. Chromosomes were then counted under a light microscope with a 100× immersion lens.

Morphometry

A total of 1371 flowers of 358 plants (166 tetraploid and 192 hexaploid) from 47 localities were included in the morphometric analyses. Six floral and three vegetative quantitative characters (mostly no less than 10 measurements per flower, 30 per plant) were measured. Cytometrically identified vouchers are gradually being made available at the herbarium of Charles University in Prague (PRC).

The position of a flower in the inflorescence influences the size and shape of its organs in that the smallest flowers and least mature capsules are usually found at the ends of the stem or its branches. In the bottom and central part of the inflorescence, however, the spatial distribution of quantitative values appears to be random. Therefore, all or no less than three well developed flowers on each plant, excluding those at the extremities of the inflorescence, were measured. The following quantitative morphological characters were studied: plant height (PH), stem thickness at base (STB), capsule length (CL), capsule width (CW), outer tepal length (OTL), inner tepal length (ITL), filament length (FL) and anther length (AL); in addition, the number of stamens in each flower (NoS) and flowers on each plant (NoF) were counted. In addition, whether each plant had clustered flowers (CLUST) and whether each capsule was bilocular (BILOC) were recorded. The following additional characteristics were calculated for each plant: mean capsule length (CLAVG), mean capsule width (CWAVG), mean outer tepal length per flower ($OTLAVG_0$), mean outer tepal length (OTLAVG), mean inner tepal length per flower ($ITLAVG_0$), mean inner tepal length (ITLAVG), mean filament length per flower ($FLAVG_0$), median filament length per

flower ($FLMED_0$), maximum filament length per flower ($FLMAX_0$), mean filament length (FLAVG), median filament length (FLMED), maximum filament length (FLAVG), mean anther length per flower ($ALAVG_0$), mean anther length (ALAVG) and mode number of stamens (NoSMOD). Morphometric measurements were done on dry material under a stereo zoom microscope (models SZX12 and SZ51; Olympus Corp., Tokyo, Japan) equipped with an eyepiece micrometer. Tepal, capsule lobe, anther and filament length were measured using the eyepiece micrometer calibrated with an accuracy of 0.05 mm using a 10 mm gauge block at 20-fold magnification. Seed and capsule lobe dimensions and shape were obtained via image analysis (see below). Plant height (PH) was measured with an accuracy of 5 mm using a ruler.

Occasionally, when an anther adheres to the stigma during cleistogamous pollination it does not detach from its filament but comes away with part of the filament, leaving only a short stump. Markedly short filaments are therefore often present among longer ones. For this reason, maximum filament length (FLMAX) or median filament length (FLMED) were preferred to mean filament length (FLAVG). The mean is likely to be influenced by uneven tearing of filaments.

Seed dimensions were recorded using computer image analysis with the help of ImageJ (Image Processing and Analysis in Java; Rasband 1997–2009). Images were acquired using a flat-bed scanner. Prior to image analysis the scale of each image was set (Analyze > Set Scale), and each image was converted to an 8-bit bitmap (Image > Type > 8-bit) and then binarized by setting a threshold value that separated the objects from the background (Image > Adjust > Threshold). Particles touching the image edges were excluded (Exclude on Edges). Seeds occasionally touched each other in spite of attempts to prevent them from touching. This problem was ameliorated by using the watershed algorithm (Process > Binary > Watershed). Finally, particle analysis (Analyze > Analyze particles) extracted the needed properties of each particle. Particle length was determined as maximum calliper length, also called maximum Feret's diameter, which is the distance between theoretical parallel lines that are drawn tangent to the particle profile and perpendicular to the ocular scale.

Data were analysed in the GNU R statistical computation and graphics environment (R Development Core Team 2008). Univariate normality was tested by the 'shapiro.test' (Shapiro-Wilk normality test) procedure from the 'stats' library. The Kruskal-Wallis rank sum test (function 'kruskal.test' from 'stats') was used as a non-parametric method for comparing distributions of variables between two groups. Spearman's correlation coefficient was computed using the 'cor' function of the 'stats' library. Multivariate normality was tested using the function 'mshapiro.test' from the library 'mvnormtest'. Multivariate outliers were detected by use of the function 'aq.plot' from 'mvoutlier' package. Homogeneity of variances was tested using the non-parametric routine 'fligner.test' for the Fligner-Killeen test from the 'stats' library and the 'hov' procedure from the library 'HH'. Principal component analysis was done using 'prcomp' ('stats') with centering and scaling enabled (with scale=TRUE). Generalized canonical discriminant analysis was done with the 'candisc' procedure from the library of the same name. Classification discriminant analysis was then done with the non-parametric method 'sknn' (simple k nearest neighbours) from the 'klaR' package. Prediction error rates were estimated using 'errorest' from the 'ipred' library. Partition plots were produced using the 'partimat' function from the 'klaR' package.

Results

Plants material for flow cytometry was obtained from over 120 mainly central-European localities; of which 47 were included in the morphometric study. Some additional material, mostly older herbarium specimens or seeds, was also included (see Appendix 1).

Nuclear DNA content

Both PI and DAPI staining provided clearly defined G1/G0 peaks and G2 peaks, which were sometimes obscured by background noise. Background noise was negligible when fresh material was used and increased significantly as the material deteriorated over time. Approximately 400 flow cytometric analyses of over 2400 plants from 120 populations yielded altogether three peak positions relative to the peaks of one of the internal standards used (*Glycine max* and *Solanum lycopersicum*). The fluorescence of 2C nuclei of all samples was below that of both internal standards. The mean ratios of sample peak positions to the positions of peaks of *Glycine max* were 0.260:1, 0.472:1 and 0.736:1 (these values were calculated using analyses in which the CVs of both peaks did not exceed 3.5% using propidium iodide staining, i.e. 9, 26 and 62 FCM runs; all other analyses agree with these results). In absolute numbers (based on the 2C value of 2.5 pg DNA of *Glycine max*), three levels of nuclear DNA content were found: (i) with ca 0.65, (ii) 1.18±2.8% and (iii) 1.84±1.6% pg 2C DNA. The three levels of nuclear DNA content are well-defined and there are no intermediate values. The lowest level was found only in plants morphologically ascribable to one of the diploid species (see Fig. 1C). Assuming that the lowest ploidy level is represented by diploid plants, the two higher DNA contents correspond to tetraploid and hexaploid levels, since one is slightly less than double the diploid value and the other is just below triple the DNA content of diploids. There is no indication that the nuclear genome size varies within or among the diploid species analysed (*J. hybridus, J. ranarius* and *J. sorrentini*), but this was not firmly established, as this study concentrated on polyploids within the *J. bufonius* group. At the tetra- and hexaploid level, there is remarkable uniformity (see Fig. 1B). No FCM analysis suggested any other cytotype or statistically significant variation in nuclear DNA content. The P-values of the Shapiro-Wilk normality test on values of tetraploid and hexaploid 2C values are 0.0261 and 0.6569, respectively. Therefore, the null hypothesis that the 2C values have a normal distribution is not rejected. In the tests for the occurrence of endopolyploidy, no FCM analysis of a single plant ever resulted in multiple peaks.

Mixed tetra- and hexaploid populations were found at many localities. In populations consisting of both tetraploid and hexaploid individuals, numerous FCM runs showed two sample peaks (see Fig. 1A), often even when analysing single tufts. In many cases, a single tetraploid stem grew attached by its roots to a cluster of hexaploid stems or vice versa. When reanalysing mixed samples, the source of the secondary peak could always be identified. In Corsica (locality number 222; Appendix 1), di-, tetra- and hexaploid plants were found growing together in a single puddle. The diploids were identified as *J. sorrentini* and *J. hybridus* and were easily separable from the two polyploid cytotypes using morphological characters considered diagnostic (Cope & Stace 1983, Kirschner 2002c). A similar situation was found in herbarium material from Cyprus (see Fig. 1D).

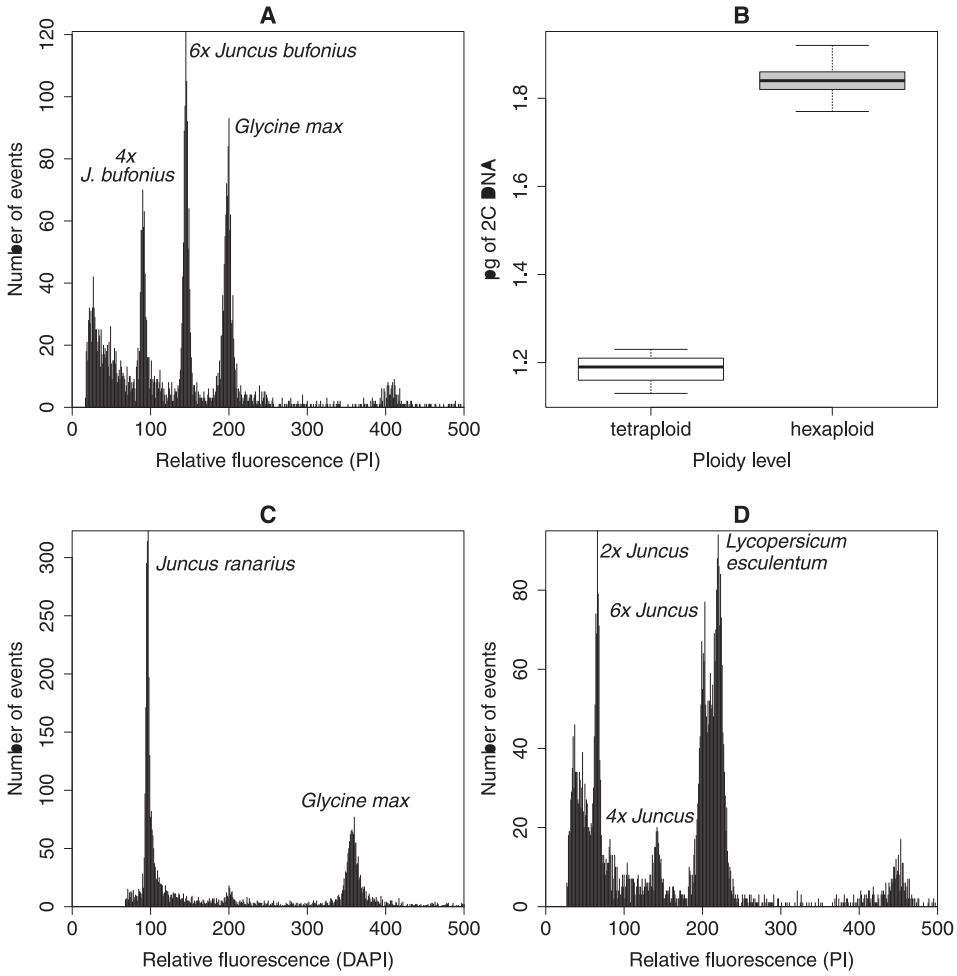

Fig. 1. – Results of flow cytometric screening of toad rush populations. (A) A simultaneous analysis using propidium iodide staining of tetraploid and hexaploid plants co-occurring at a single locality. The CVs of the tetra- and hexaploid peaks at channels 89.74 and 144.27 were 4.42% and 2.44%, respectively; the CV of the peak at channel 197.18 of the internal standard was 2.71%. (B) Boxplots of 2C values of tetraploid and hexaploid plants expressed as pg of DNA estimated based on analyses using propidium iodide staining in which the CV of the peaks was below 3.5%. (C) Flow cytometric analysis of the diploid species *J. ranarius* using DAPI staining. The CVs of the sample peak at channel 95.44 and the internal standard peak at channel 357.20 were 2.54% and 2.11%, respectively. (D) Result of a cytometric analysis using propidium iodide of seedlings germinated from herbarium material collected at one locality in Cyprus. The analysis revealed the presence of diploid, tetraploid and hexaploid plants on a single herbarium sheet.

Chromosome counts

To verify that the observed differences in nuclear DNA content correspond with differences in chromosome numbers, an attempt was made to obtain chromosome counts from seedlings previously analysed cytometrically and belonging to the three DNA cytotypes. Although the counts were imprecise, there were indications that the three levels of nuclear DNA content correspond to the three distinct albeit approximate chromosome counts: (i) ca 30 < 2n < ca 40; (ii) ca 60 < 2n < ca 80; and (iii) 2n > 90.

Morphometry

Using cytometrically determined plant material, a multivariate morphometric comparison was used to examine the purported morphological differences between tetraploids and hexaploids sometimes referred to as *J. minutulus* and *J. bufonius* s. str. A total of 1371 flowers on 358 plants (166 tetraploid and 192 hexaploid) collected from 47 localities were included in the morphometric study (see Table 1). One person measured seven floral (capsule length, capsule width, outer tepal length, inner tepal length, filament length, anther length and the number of stamens) and three vegetative (plant height, number of flowers and thickness of stem at base) quantitative characters of plants belonging to two ploidy levels occurring in mixed and remote single-cytotype populations (mostly no less than 10 measurements per flower and 30 per plant).

The distribution of plant height (PH) and number of flowers (NoF) in tetraploid and hexaploid plants appears to be very similar (see boxplots in Fig. 2A). In the number of flowers (NoF), both ploidy levels show a large number of outliers with high values of up to more than 80 flowers per stem. Note that the notches on the boxplots of the two ploidy levels overlap noticeably, which suggests that their medians do not differ. Plant height (PH) does not have a normal distribution in either tetraploids or hexaploids, even after transformation. The number of flowers (NoF) is a log-normal variable in tetraploids after a logarithmic transformation is applied.

Thickness of stem at base (STB) is a very variable character and was not statistically significantly different in the two cytotypes (data not shown). Therefore, it was omitted from further statistical analyses.

Capsule length (CLAVG) in tetraploids has a normal distribution with or without being log-transformed; however, in hexaploids the distribution is neither normal nor log-normal, probably because of the large number of outliers (see Fig. 2B). Capsule width (CWAVG), after log-transformation, is normally distributed in plants of both ploidy levels.

The boxplots of outer tepal length (OTL) shows an especially large number of outlying values (Fig. 2B). Mean outer tepal length (OTLAVG) has a log-normal distribution in both tetra- and hexaploids. The mean inner tepal lengths (ITLAVG) are normally distributed in tetra- and hexaploid plants.

Filament length (FL) presented a challenge, since there is considerable variation even within individual flowers, possibly caused by uneven shortening of the filament when an anther is torn off by a growing capsule. Occasionally it was noticed that anthers were attached to the stigma or to the wall of the fruit valve, which were still connected to a part of a filament. Therefore, it was decided to use the maximum filament length in each flower and not the mean (as in other variables). FLMAX does not have a normal distribution even after transformation (see Fig. 2C). Measurements of ALAVG were not obtained for many

plants because anthers were no longer present. This was the case for 69 plants. Even when log-transformed, ALAVG does have a normal distribution in tetraploids, but in hexaploids the P-value of its Shapiro-Wilk test marginally exceeds 0.05.

As expected, the number of stamens (NoS) is variable and has a bimodal distribution with peaks at three and six, and less frequently at two, four and five (see Fig. 2D). The number of stamens (or filaments) varies within ploidy levels, populations and even individual plants. Flowers with bilocular capsules tend to have two stamens. Only 53% of all the plants included in the morphometric study appeared to have a constant number of stamens (NoS of two, three or six). The true percentage is lower, since not every flower was examined.

In initial tests, image analyses of seed dimensions failed to reveal any statistically significant difference (using Welch two sample t-tests) in seed dimensions between tetraploid and hexaploid individuals (results now shown), so seed data was not included in further analyses. At both ploidy levels, seed dimensions approximately spanned the ranges of both species (not shown).

A scatter plot matrix of seven floral and three vegetative quantitative characters (not shown) does not seem to indicate a striking relationship between any pair of variables, possibly with the sole exception of OTL and ITL (see Table 2 for summary statistics of variables used in multivariate analyses).

The Shapiro-Wilk multivariate normality test led to the rejection of the null hypothesis that the dataset has a multivariate normal distribution (P-value = 5×10^{-19}). This P-value was returned when all variables except Ploidy and NoSMOD were included. Omitting variables with non-normal distributions (i.e. PH, FLMAX, ALAVG) did not make the dataset normal ($P = 2 \times 10^{-6}$). Omitting multivariate outliers also did not result in the data matrix having a multivariate normal distribution ($P = 3 \times 10^{-13}$). Because some of the variables used in the analyses do not have a normal distribution and the dataset does not have multivariate normal distribution in either group, the non-parametric Spearman's rank correlation coefficient was used as a measure of correlation among variables. Spearman's correlation coefficients among the variables in the dataset are not excessively high, the highest correlation coefficient being 0.81 (between OTLAVG and ITLAVG). It does not exceed 0.8 for any other pair of variables, so there is no need to omit any of the variables from the analyses. Note that Ploidy is correlated least of all the variables. Comparison of the correlation coefficients for tetraploid and hexaploid plants and the values in the two groups, seem to be similar. To test the null hypothesis that the variances in each of the two groups are the same, the Fligner-Killeen (median) test was used. The Fligner-Killeen test of homogeneity of variances (P-value of 0.516) did not lead to the rejection of the null hypothesis that the variances in the two groups differ. Therefore, the data matrix was considered to be homoscedastic.

Principal component analysis (PCA) was performed on the data set with individuals as OTUs after standardization. The resulting biplot (Fig. 3A) consists of one large cloud of plants with increasing density towards the centre, a few isolated outlying individuals and a small group of observations, and the PCA does not indicate that the tetraploid and hexaploid groups differ. The first principal component explained 46% of the variance, the second 15%. The component loadings of the variables were rather low, often negative and exceeded 0.5 in only a few cases (Table 3). Dropping variables did not help reveal any differences. Separate PCAs were done for tetraploid and hexaploid plants with similar

Table 2. – Summary statistics of morphometric variables used in multivariate analyses. PH – plant height, NoF – number of flowers on each plant, CLAVG – mean capsule length, CWAVG – mean capsule width, OTLAVG – mean outer tepal length, ITLAVG – mean inner tepal length, FLAVG – mean filament length, FLMAX – maximum filament length, ALAVG – mean anther length.

	Minimum		1st quartile		Median		Mean		3rd quartile		Maximum	
Ploidy level	4x	6x	4x	6x	4x	6x	4x	6x	4x	6x	4x	6x
PH	1.70	2.00	9.00	9.00	14.00	13.25	14.45	13.93	19.00	17.50	30.00	34.00
NoF	2.00	2.00	8.00	7.00	13.00	12.00	18.62	18.17	20.00	22.00	91.00	91.00
CLAVG	2.13	2.43	2.86	3.02	3.05	3.26	3.05	3.30	3.24	3.52	3.90	5.03
CWAVG	0.63	0.75	0.95	1.05	1.05	1.17	1.06	1.20	1.16	1.34	1.50	2.00
OTLAVG	3.11	3.34	4.36	4.46	4.69	4.83	4.73	4.93	5.15	5.29	7.08	7.83
ITLAVG	2.05	2.62	3.15	3.46	3.42	3.78	3.45	3.86	3.75	4.27	5.03	5.14
FLAVG	0.89	0.88	1.12	1.20	1.23	1.30	1.24	1.31	1.32	1.38	1.92	2.34
FLMAX	0.95	1.00	1.30	1.39	1.45	1.50	1.46	1.53	1.55	1.60	3.60	3.60
ALAVG	0.20	0.22	0.35	0.42	0.45	0.49	0.48	0.51	0.53	0.58	1.70	1.25

Table 3. – Principal component analysis loadings for the first six principal component axes (PC1 – PC6). Values greater than 0.5 are in bold. PH – plant height, NoF – number of flowers on each plant, CLAVG – mean capsule length, CWAVG – mean capsule width, OTLAVG – mean outer tepal length, ITLAVG – mean inner tepal length, FLMAX – maximum filament length, ALAVG – mean anther length, NoSMOD – mode number of stamens.

	PC1	PC2	PC3	PC4	PC5	PC6
PH	–0.325	**0.524**	–0.101	0.148	–0.230	0.059
NoF	–0.262	**0.625**	0.035	0.256	0.062	–0.387
CLAVG	–0.413	–0.268	0.060	0.004	–0.108	–0.047
CWAVG	–0.348	–0.385	–0.012	0.108	0.263	–0.677
OTLAVG	–0.400	–0.110	0.172	0.345	–0.032	**0.524**
ITLAVG	–0.430	–0.235	0.091	0.146	–0.066	0.180
FLMAX	–0.106	0.148	**0.855**	–0.470	0.051	–0.039
ALAVG	–0.303	–0.020	–0.337	–0.608	–0.558	–0.079
NoSMOD	–0.294	0.158	–0.319	–0.413	0.737	0.267

results. The first principal component explained 48% of the variance in tetraploids and 45% in hexaploids. PCA ordination therefore does not indicate any appreciable difference between tetraploid and hexaploid groups in this data set, which might indicate that tetraploids and hexaploids are morphologically indistinguishable. A detrended correspondence analysis (DCA) was performed on the same dataset with a similar result. The PCAs indicate the presence of multivariate outliers. A comparison of ordered squared robust Mahalanobis distances of the observations against the empirical distribution function makes it possible to identify the outliers. The multivariate outliers identified belong to both ploidy levels, come from various localities and are positioned in different parts of the ordination space.

Various different clustering algorithms (e.g. k-means clustering, hierarchical clustering, partitioning around medoids) were tested, but none of them managed to separate to any appreciable extent tetraploid from hexaploid individuals, be it within or between populations (results not shown).

The dataset does not fulfil all the assumptions of linear discriminant analysis (LDA) on account of it not having a multivariate normal distribution. Nevertheless, a parametric

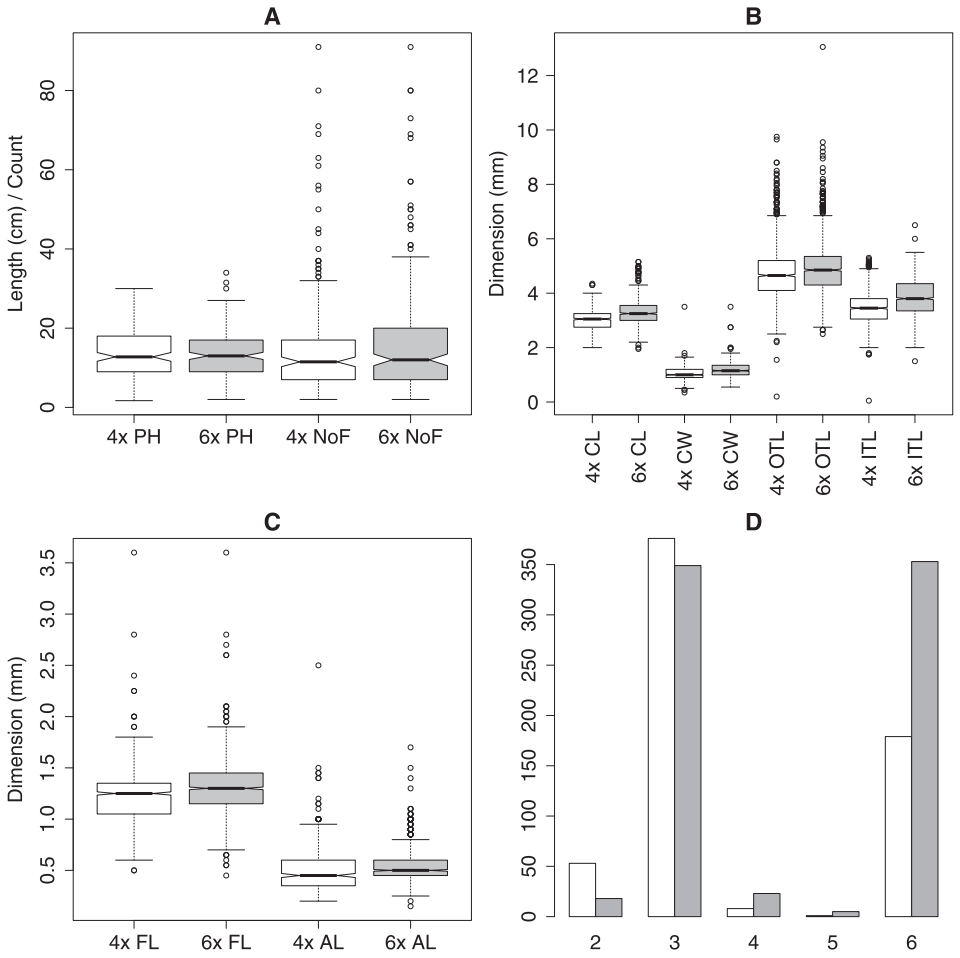

Fig. 2. – Comparison of values of quantitative morphological characters measured in tetra- and hexaploid individuals of *Juncus bufonius* s.l. (A) Boxplots of plant height (PH) and the number of flowers (NoF) of tetraploid and hexaploid plants. (B) Boxplots of capsule length (CL), capsule width (CW), outer tepal length (OTL), inner tepal length (ITL) of tetraploid and hexaploid plants; C – Boxplots of filament length (FL) and anther length (AL) of tetraploid and hexaploid plants. (D) Barplots of the number of stamens (NoS) recorded for tetraploid and hexaploid plants.

approach, such as generalized canonical discriminant analysis, was still useful for descriptive purposes. The data matrix is homoscedastic and there is not a large difference between the Spearmann correlation matrices of tetraploid and hexaploid observations. For classification analyses, however, a non-parametric method is preferred.

In the output of the generalized canonical discriminant analysis (Fig. 3B), the distributions of tetraploid and hexaploid observations along the canonical axis overlap considerably. Moreover, the means of the two groups are both positioned near the centre of the discriminant axis and the box of the hexaploid boxplot crosses the zero point on the first

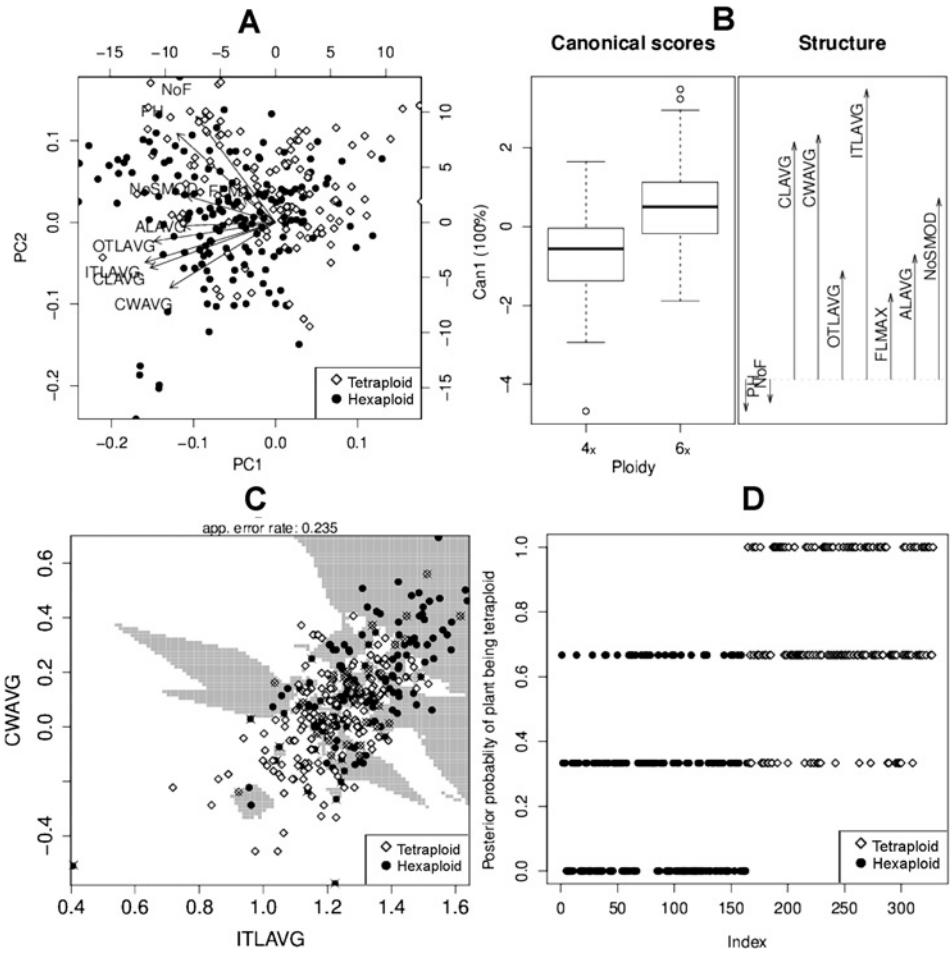

Fig. 3. – Results of multivariate analyses of morphometric data for tetra- and hexaploid individuals of *Juncus bufonius* s.l. (A) Principal component analysis for tetraploid and hexaploid plants. (B) Output of generalized linear canonical discriminant analysis. (C) Partition plot showing the output of stepwise variable selection based on SKNN classification. Tetraploid group: white background; hexaploid group: grey background. Incorrectly classified observations are crossed out. The apparent and 10-fold cross-validated error rate of this particular analysis was 0.23 and 0.36, respectively. (D) Prediction based on simple k nearest neighbours classification. The apparent error rate of this classification analysis is 0.16. 10-fold cross validation increases the error rate to 0.45.

canonical axis. Canonical structure values of PH and NoF are negative. The rest are positive, the largest being the value for ITLAVG followed by CLAVG and CWAVG, which are close to each other, FLMAX, and OTLAVG and ALAVG, which are similarly low. Many ALAVG measurements are missing from the data set. Because the structure coefficient of ALAVG in the canonical discriminant analysis is only 0.310, this variable was omitted from further analyses in order to increase the number of observations to 358. Since the data set violates the assumption of multivariate normality, a non-parametric method was chosen for the classification discriminant analysis, namely the simple k nearest neighbour

discriminant (SKNN) classifier. For this analysis, the number of tetraploid and hexaploid observations was the same. The SKNN procedure using three nearest neighbours misclassified 16% of the individuals in the training set. This apparent error rate has an optimistic bias. Using the 10-fold cross-validation estimator, the misclassification error of the analysis is estimated at 0.45 (see Fig. 3D). This analysis was also tested for individual mixed populations (and groups of populations) with similar results. Stepwise variable selection based on SKNN classification using the default performance measure (i.e. correctness rate or 1 – error rate) returned the following formula: 'Ploidy ~ CWAVG + ITLAVG'. The overall 10-fold cross-validated error rate, however, was 0.36 (the apparent error rate was 0.23; see Fig. 3C).

Diploid plants notwithstanding, plants with clustered flowers were rarely encountered. Most tetraploid or hexaploid individuals had remote flowers although plants with clustered flowers were abundant in a few populations. All these populations, however, also included plants with single, remote flowers as well as continuous transitions towards clusters of flowers. This applied to both tetraploids and hexaploids.

In some of the collections there were plants with stems that had a conspicuous red colouring – sometimes at the base but often of the entire plant. This was observed in plants of both ploidy levels at many localities. During repeated visits to the same locality in different seasons and different years, the red colouration was not constant.

Signs of cleistogamy could be observed in most of the plants studied. It appears to be a universal phenomenon in polyploids of the *J. bufonius* complex. On a few occasions, chasmogamous flowering in hexaploid plants, but never in tetraploids, were recorded.

Discussion

The presence of three previously reported ploidy levels within the *Juncus bufonius* complex is confirmed. Crucially, the FCM data together with the albeit limited chromosome counts showed that differences in nuclear DNA content reflect the number of chromosomes and that these plants are polyploid and not merely agmatoploid. That is, as suggested previously (van Loenhoud & Sterk 1976, Mičieta & Mucina 1983, Cope & Stace 1985) the *J. bufonius* group is a polyploid complex including diploids, tetraploid and hexaploid individuals, but no octoploids as suggested by Cope & Stace (1985) or any other ploidy level. Whether agmatoploidy, in addition to polyploidy, occurs in the *J. bufonius* complex remains an open question, as there are an insufficient number of chromosome counts to rule it out. As for aneuploidy or dysploidy, the sensitivity of even the best of cytometers is an order of magnitude lower than would be needed to detect differences involving one or a few chromosomes (Loureiro et al. 2010). This and the low precision, great laboriousness and low success rate of chromosome counting in toad rushes preclude any reliable assessment of aneuploidy and dysploidy in this group, at least in the near future. Nothing in the results indicates the occurrence of hybrids between a tetraploid and a hexaploid parent. In addition, no pentaploid or any other abnormality was encountered. Cleistogamy probably almost eliminates the possibility of any natural cross-pollination between the cytotypes, so hybridization probably occurs extremely rarely, if at all. On the other hand, the frequent physical contact between tetra- and hexaploid individuals increases the chances of cross-pollination.

The flow cytometric screening had to cope with a large number of mixed-ploidy samples. Purely hexaploid populations were by far the most numerous, but mixed populations composed of tetraploid and hexaploid individuals were common, especially in some regions. By contrast, exclusively tetraploid populations were rare. It is likely that a much higher proportion of populations include both ploidy levels because one or the other for some reason went undetected. In a few cases, populations appeared pure one year, but the other cytotype was found the next, e.g. when conditions were more favourable and the overall population size noticeably larger. The incidence of mixed samples was high even when attempting to analyse individual plants, as the two ploidy levels often grow in such proximity that they form mixed-ploidy pseudoclumps. This is because seeds from different individuals get randomly shuffled in water, mud or soil and germinate so close to each other that they form mixed-ploidy pseudotufts. It was always possible to localize the source of the contamination by dividing each mixed tuft into single stems. In regions where diploid species are present, they too were found in mixed samples. Holub (1976) estimated that 20% of Czechoslovak herbarium specimens of *J. bufonius* s.l. contain both tetraploid and hexaploid plants. Interestingly, in the present study, the percentage of mixed populations in Czech Republic also reached about 20%.

We have significantly broadened the knowledge on the distribution of the tetraploid cytotype of *J. bufonius* s.l. in Europe, but more data is still needed to make any general statements, which is why a map is not presented. One important conclusion that can be drawn from this data is that the tetraploid cytotype is not rare or endangered, at least in most of the parts of Europe sampled. Some European red lists (e.g. Kålås et al. 2006) list *J. minutulus* among endangered taxa, but this may well be due to a lack of data. The presence and distribution of tetraploids in areas where *J. minutulus* is considered rare or endangered needs to be verified using flow cytometry. For this purpose, seeds obtained from herbarium collections can be used, provided that they have retained their germination ability. Tetraploids were not found in some of the regions visited (notably the Ukrainian Carpathians, southern Carpathians and south-eastern Slovakia), but in areas where tetraploids were detected they appear to be fairly abundant. Of course, population numbers fluctuate depending on natural conditions, such as weather, succession and the extent of habitat disturbance, so the occurrence of *J. bufonius* of both cytotypes is largely unpredictable. In summer 2008 it was often hard to find toad rushes in some parts of Central Europe, but the next year they were abundant in suitable habitats, often found thriving in places that are usually too dry for them to grow. The absence or rarity of tetraploids in some regions might be a result of seasonal or year-to-year fluctuations.

Differences in the ecological requirements of *J. minutulus* and *J. bufonius* s. str. are frequently mentioned in the literature (e.g. Holub 1976, van Loenhoud & Sterk 1976, Mičieta & Mucina 1983 and Kirschner 2002c), but the frequency with which tetraploid and hexaploid plants co-occur in various different habitats indicates that, for the most part, the ecological preferences of both ploidy levels can be considered identical. An experimental study comparing the behaviour of the two ploidy levels under controlled conditions might yield interesting results. Despite not having collected any data on the phenology of tetraploids and hexaploids, it is likely, as previously cited by other authors, that tetraploids can be found somewhat earlier and often mature more rapidly than hexaploids. This, however, can be observed only in mixed populations that are not subjected to any noticeable ecological gradient, as microhabitat conditions easily overshadow any intrinsic difference

in phenology. Still, these opinions are based on subjective observations and a proper study should be conducted to ascertain whether there is any constant difference in phenology between the two cytotypes.

The prevalence of cleistogamy is another topic frequently dealt with by previous authors. Although cleistogamy was not specifically studied it was routinely recorded during this study. This was indicated by anthers attached to the stigma at the top of many capsules. These were found in both tetra- and hexaploid plants. In both cytotypes, there were rarely noticeably elongated anthers (up to 2.5 mm), which sometimes even exceed the length of their filaments. This finding is similar to that published by Shah (1963). The extremely long anthers recorded in this study are not a stable trait but rather an anomaly found in some flowers of some plants in some populations. Extremely long anthers tend to be black, which might suggest that they are a result of an infection.

It is stated by all previous authors (e.g. Holub 1976, van Loenhoud & Sterk 1976, Cope & Stace 1978, Mičieta & Mucina 1983, Cope & Stace 1985, Chicouène 1996, Kirschner 2002c, Harmaja 2003) that values of most quantitative traits of *J. minutulus* and *J. bufonius* s. str. overlap to a certain degree. The current results shed new light on the extent of the overlaps. For example, values of capsule length, the most frequently presented diagnostic character, overlap by 58%. Plant height and the number of flowers overlap even more (> 85% and 100%), which indicates that "minutulus" may not be a well chosen epithet after all. A similar situation was found in values of other characters that are used in identification keys, such as lengths of outer and inner tepals, filaments and anthers. The number of stamens, another purportedly diagnostic character, also turned out to be of no diagnostic value whatsoever. That tetraploid individuals with six stamens and tri-staminate hexaploid individuals occur has already been noted by previous authors (e.g. Kirschner 2002c). The current results clearly show that bi-, tri- and hexastaminate flowers occur frequently in plants of both ploidy levels, often even on the same individual.

The results of the ordination and discriminant analyses confirmed that it is impossible to reliably separate tetraploid plants using supposedly diagnostic morphological characters. This applies to both sympatric and allopatric occurrences of the two ploidy levels. This supports the view of Cope & Stace (1983) that the supposed morphological distinctions between *J. minutulus* and *J. bufonius* s. str. are fuzzy at the level of the individual and that tetraploids should be pooled together with hexaploids under the name *J. bufonius* L., at least until new evidence proves otherwise, relegating the name *J. minutulus* (Albert et Jahand.) Prain and its synonyms to the synonymy of *J. bufonius* L.

The results contradict those of van Loenhoud & Sterk (1976) and Mičieta & Mucina (1983). As mentioned in the introduction, van Loenhoud & Sterk (1976) conclude that certain quantitative characters are diagnostic based on a series of Wilcoxon tests. The Wilcoxon rank sum tests of each of the characters considered in this study lead to the rejection of the null hypothesis that tetraploid and hexaploid values have the same continuous distribution (with P-values < 0.0000). This null hypothesis could not be rejected for plant height, the number of flowers and, marginally, the average outer tepal length. Still, the ordination and discriminant analyses show that none of the characters allow the separation of tetraploids from hexaploids. This demonstrates that univariate statistics alone are not useful for selecting diagnostic characters.

Mičieta & Mucina include in their analyses only eight tetraploid accessions from six localities (two were sampled twice) and compared them to 17 accessions of hexaploid

plants. Only three of their localities harboured both tetraploids and hexaploids. On the other hand, in the current study both allopatric and sympatric occurrences of the two cytotypes in 120 populations (47 included in morphometric analysis), of which many were mixed ploidy, were recorded. It is likely that a broader part of the actual range of values of quantitative values in natural populations of the two cytotypes was sampled, better reflecting the variability of the species. Furthermore, chromosome counting demands a relatively large investment of effort compared to flow cytometry. Understandably, this could have lead previous authors to preferentially count chromosomes in populations that were observably morphologically different. By contrast, in this study indiscriminate FCM analyses were carried out, that is, there was no prior assessment of morphology. Although this approach resulted in a vast amount of mixed ploidy samples and thus "wasted" FCM runs, it allowed the assessment of the degree of morphological differentiation in the zone of morphological overlap of the two cytotypes. Of course, this sampling strategy is not perfect, either, but probably not to the extent that it strongly influences the results. A seriously flawed sampling design would be expected to bias the results in the opposite direction, i.e. towards a false positive (e.g. through under-representation of intermediates or over-representation of extremes). Intra-cluster correlation inherent in the cluster sampling method used probably somewhat increased the observed similarity between the two groups. In this case, however, this is not viewed as a problem, since anyone trying to compare toad rushes in the field is likely to end up at least partially switching to the clustering method due to mixed-ploidy pseudotufts, which must be divided and re-analysed in order to separate the two ploidy levels.

Because the two ploidy levels may be misidentified so easily, it is likely that many of the 26 localities cited by Holub (1976) are erroneous. Holub claims to be able to reliably tell apart *J. minutulus* and of *J. bufonius* s. str. even in mixed populations or clumps, but this is unlikely using the characters he used. It is also likely that many of the 584 herbarium sheets classified by van Loenhoud & Sterk (1976) using their "diagnostic" characters were identified erroneously. Flow cytometry, thanks to the high germination ability of toad rush seeds can be easily employed to identify mixed-ploidy herbarium specimens.

One obvious shortcoming of the present study is that only a small number of morphological characters were studied. At the beginning of the study, including a somewhat larger set of characters was considered, but the opportunity provided by flow cytometry to observe many individuals from numerous populations resulted in the discarding of some of them (e.g. capsule colour, capsule shape, plant colour or branching characteristics). The red colouring of plants is probably caused by environmental conditions and occasionally occurs in both tetraploids and hexaploids. The only characters that seemed to make sense in the large amount of cytometrically identified material are those included in the statistical analyses. Seed ornamentation, stomatal length and possibly dimensions of other types of cells (on the capsule surface, for example) should be included in any follow-up study.

Possibly of interest is the question as to why hexaploids are more widely distributed than tetraploids. In some parts of the world, *J. bufonius* s.l. is considered an invasive alien, but cytometric data or chromosome counts from most of the world are missing. Some studies (Kubešová et al. 2010) show that invasive species tend to have smaller genomes than their non-invading relatives. In *J. bufonius* s.l., however, it looks as though hexaploids have some competitive advantage. Since both cytotypes are potentially invasive, it would be interesting to know the cytotype composition of toad rush populations in places where the species is considered a neophyte.

More questions can be answered by a molecular study. For instance, are mixed tetraploid/hexaploid populations a result of independent colonizations by two unrelated cytotypes or have hexaploids arisen polytopically from tetraploid progenitors? *Juncus minutulus* may still be a justifiable taxon from a biosystematic perspective, though a cryptic one, if molecular phylogenies support it. It is possible that the border between *J. bufonius* and *J. minutulus* (and possibly other species) does not run along differences in ploidy level. Polyploids often originate polytopically over time (e.g. Soltis et al. 2007), so it may be the case that some hexaploid plants are phylogenetically closer to extant tetraploids than others. Independently arising hexaploids may have blurred the distinction between otherwise distinct species. Cope & Stace (1985) suggest that the birth of higher polyploids in the *J. bufonius* complex may be an ongoing process. The original morphology-based treatment of the two distinct species *J. minutulus* and *J. bufonius* could still be viable (with revised circumscriptions) if molecular data contain evidence that one group of hexaploids, matching the description of *J. bufonius* s. str., has a different evolutionary history from hexaploids independently arisen or arising from tetraploids belonging to *J. minutulus*. *Juncus minutulus* would therefore constitute a species comprising two ploidy levels, while *J. bufonius* would comprise morphologically distinct plants with a separate evolutionary history. The observations reported here indicate that if there is any group of plants within *J. bufonius* s.l. that consistently differs from the rest, it probably encompasses certain hexaploid plants. During the course of this study, certain individuals or populations were identified as hexaploid based on morphology and flow cytometry never disproved this (in the dataset, some of them were multivariate outliers). These plants had values of quantitative characters near the high extremes, frequently grew in taller vegetation, had relatively few flowers and often flowered chasmogamously. Of course, this could be induced ecologically, but the fact that none of these plants were tetraploid is intriguing. This did not work vice versa, since it was never certain that a plant was tetraploid. Most "tetraploid-looking" material turned out to be hexaploid.

By expressing the opinion that the two ploidy levels of *J. bufonius* s.l. cannot be safely identified using traditional diagnostic characters, it is not suggested that their existence be ignored, quite the opposite. The topic should not be put to rest if only for the reason that it offers an opportunity to study two related (although how closely remains to be seen) polyploid cytotypes that interact closely while being strongly reproductively isolated due to their cleistogamous mode of pollination. Putative differences in phenology and ecological preferences make *J. bufonius* a suitable candidate for studying polyploid dynamics in both natural and controlled systems. Further research, especially focusing on phylogenetic relationships, is desirable.

Conclusion

Polyploidy, as opposed to agmatoploidy, is the primary source of the karyological differences observed within the *J. bufonius* group. Populations of *J. bufonius* s.l. sampled consisted of tetraploid or hexaploid individuals, with mixed-ploidy populations occurring regularly. In cross-validated classification discriminant analyses of cytometrically defined groups, nearly half of all individuals were misclassified. This is in accord with the opinion of previous authors that tetraploid and hexaploid individuals of *J. bufonius* s.l. cannot be

reliably distinguished from each other using morphological characters, contrary to the conclusions of some previous studies. The overlap in values of quantitative characters is far greater than previously reported suggesting that *J. minutulus* is morphologically separable. With the present state of knowledge, it is recommended that tetraploids and hexaploids of the *J. bufonius* group are pooled together under the name *J. bufonius* L. as two cytotypes of a single polymorphic species, a pragmatic treatment already proposed and defended by some authors.

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Souhrn

Byl proveden screening velikosti jaderného genomu u ca 2400 rostlin z více než 120 převážně středoevropských lokalit skupiny *Juncus bufonius*. Vedle diploidního stupně, korespondujícího se známými diploidními druhy (v našem případě *J. ranarius, J. hybridus* a *J. sorrentini*), byly nalezeny dva polyploidní stupně, odpovídající tetraploidům a hexaploidům, kteří jsou některými autory klasifikováni jako samostatné druhy *J. minutulu*s a *J. bufonius* s. str. Vztah mezi obsahem jaderné DNA a počtem chromosomů byl ověřen počítáním chromosomů. Polyploidie, a nikoli agmatoploidie, tedy stojí za pozorovanou karyologickou variabilitou. Hodnoty 2C obsahu jaderné DNA diploidů, tetraploidů a hexaploidů jsou ca 0.65, 1.18±2.8 % potažmo 1.84±1.6 % pg 2C DNA. Žádný další cytotyp nebo statisticky významná variabilita v obsahu jaderného genomu nebyla nalezena. Za účelem zhodnocení užitečnosti dosud publikovaných morfologických znaků pro určování *J. minutulus* a *J. bufonius* s. str. byly zaznamenány hodnoty sedmi květních (přes 10 měření na květ a 30 na rostlinu) kvantitativních znaků a tři vegetativní pro 358 vyvinutých rostlin známé ploidie ze 47 lokalit. Různorodé ordinační a shlukovací metody nepoukázaly na žádnou shlukovou strukturu v datech. Kanonická diskriminační analýza a postupný výběr proměnných označily délku vnitřních okvětních lístků, průměrnou šířku tobolky a následně průměrnou délku tobolky za nejužitečnější znaky pro odlišení ploidních úrovní; křížově ověřená klasifikační diskriminační analýza nicméně určila 45 % objektů chybně. Jiné analýzy tento výsledek potvrzují. Žádný nový morfologický znak, pomocí něhož by bylo možné úspěšně odlišovat tetraploidy od hexaploidů nalezen nebyl. Nezávisle tudíž potvrzujeme názor některých předchozích autorů, že druh *J. bufonius* L. je nejlépe klasifikovat jako jediný variabilní druh zahrnující dva cytotypy, které od sebe pomocí dosud navržených morfologických znaků nelze rozeznat, alespoň dokud nebude k dispozici přesvědčivý důkaz pro opačné tvrzení.

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Appendix 1. – Non-comprehensive list of localities included in this study. Many localities with small populations or immature plants at the time of the visit are not listed even if they were analyzed cytometrically. Localities marked with an asterisk were included in the morphometric study. Locality numbers were semi-arbitrarily assigned in increasing order during the course of the study. LD designates samples from the seed collection of L. Záveská Drábková, G those collected in Georgia. Latitude and longitude are given for each locality where available. CR: – Czech Republic, SK – SK.

1 49.0105°, 13.5797°, CR: Kvilda, 6x. **3*** 49.90700°, 14.32 08°, CR: Varadov, 4x. **13** 49.74169°, 14.1514°, CR: Oboříště, 6x. **14** 50.15939°, 14.4013°, CR: Praha, 6x. **15*** 49.99581°, 14.4004°, CR: Praha, 6x. **16*** 50.01864°, 14.3951°, CR: Praha, 6x. **17*** 49.90724°, 14.3208°, CR: Veselí nad Lužnicí, 4x. **18** 50.49 256°, 13.67 844°, CR: Most, 2x *(J. ranarius)*, 6x. **19** 49.63552°, 13.9344°, CR: Vysoká u Příbramě, 6x. **20** 49.6944°, 14.0533°, CR: Dubno, 6x. **21** 49.6893°, 14.0555°, CR: Dubno, 6x. **22** 49.68813°, 14.0559°, CR: Dubno, 6x. **25** 48.969°, 17.8734°, CR: Starý Hrozenkov, 6x. **26** 48.98404°, 17.8953°, CR: Žítková, 6x. **28** 49.60317°, 14.4828°, CR: Doublovičky, 6x. **29** 49.18657°, 14.7024°, CR: Veselí nad Lužnicí, 6x. **30** 49.15925°, 14.7124°, CR: Vlkov, 6x. **31** 49.15711°, 14.7125°, CR: Vlkov, 6x. **32** 49.15578°, 14.7102°, CR: Vlkov, 6x. **33** 49.15452°, 14.7096°, CR: Vlkov, 6x. **39*** 49.76365°, 13.882°, CR: Malá Víska, 4x, 6x. **55** 41.49631°, 15.9161°, Italy Foggiamare, 2x (*J. hybridus*). **59** 49.17078°, 14.7153°, CR: Veselí nad Lužnicí, 6x. **60** 49.16617°, 14.7156°, CR: Veselí nad Lužnicí, 6x. **61** 49.16431°, 14.7156°, CR: Vlkov, 6x. **62** 49.16136°, 14.7153°, CR: Vlkov, 6x. **63** 49.1605°, 14.714°, CR: Vlkov, 6x. **64** 49.16044°, 14.7127°, CR: Vlkov, 6x. **65** 49.16022°, 14.7088°, CR: Vlkov, 6x. **66** 49.69411°, 13.9296°, CR: Brdy, 6x. **67** 49.69003°, 13.9005°, CR: Brdy, 6x. **68** 49.689°, 13.8996°, CR: Brdy, 6x. **69*** 49.692°, 13.8847°, CR: Tok, 4x. **81** 46.52033°, 23.3178°, Romania: Muntele Mare, 4x. **87*** 49.78837°, 14.1754°, CR:

Dobříš, 6x. **88*** 49.84217°, 13.9015°, CR: Hořovice, 6x. **89*** 49.13626°, 19.3534°, SK: Chočské vrchy . 6x. **102*** 50.06617°, 12.7885°, CR: Louka, 4x. **123** 48.9918°, 17.9016°, CR: Žítková, 6x. **124** 48.96832°, 17.891°, CR: Žítková, 6x. **130*** 49.82382°, 14.2264°, CR: Voznice, 4x, 6x. **131** 50.06779°, 14.4114°, CR: Praha, 6x. **132*** 48.41408°, 21.7258°, SK: Malá Bara, 6x. **133** 48.41042°, 21.7252°, SK: Malá Bara, 6x. **134** 48.38233°, 21.8576°, SK: Strážne, 6x. **135** 48.34511°, 21.8349°, SK: Veľký Kamenec, 6x. **137** 48.8185°, 21.9568°, SK: Vinné, 6x. **138** 48.59904°, 21.4575°, SK: Slanská Huta, 6x. **139** 48.50053°, 22.0533°, SK: Latorica most, 6x. **140** 48.49865°, 22.0558°, SK: Latorica most, 6x. **141** 48.49344°, 21.9387°, SK: Soľnička, 6x. **142*** 48.49165°, 21.9377°, SK: Soľnička, 6x. **143** 48.35869°, 21.8082°, SK: Veľký Kamenec, 6x. **146*** 49.83076°, 14.1649°, CR: Voznice, 4x, 6x. **147*** 45.54106°, 25.2944°, Romania: Zarnesti, 6x. **148*** 45.49506°, 25.1736°, Romania: Casa de Vanatoare Piatra Craiului, 6x. **149*** 45.49964°, 25.1465°, Romania: Valea Dâmboviţa, 6x. **150*** 45.51863°, 25.0748°, Romania: Valea Dâmboviţa, 6x. **151*** 45.44239°, 25.0449°, Romania: Muntele Iezer Păpuşa, near Cabana Voina, 6x. **154*** 50.70163°, 15.7055°, CR: Pec pod Sněžkou, 6x. **162** 49.89904°, 14.2746°, CR: Řitka, 6x. **165*** 49.8847°, 14.2538°, CR: Mníšek pod Brdy, 6x. **166*** 49.64794°, 13.7569°, CR: Padrťské rybníky, 6x. **170** 49.96474°, 13.7638°, CR: Skryje, 6x. **171*** 49.89848°, 14.3504°, CR: Klínec, 6x. **172*** 49.82299°, 14.1879°, CR: Voznice, 4x, 6x. **173*** 49.82383°, 14.186°, CR: Voznice, 4x, 6x. **174*** 49.98696°, 14.767°, CR: Mukařov (Louňovice), 4x, 6x. **175*** 49.985°, 14.7681°, CR: Mukařov (Louňovice), 6x. **177*** 49.97674°, 14.788°, CR: Vyžlovka, 4x, 6x. **180*** 49.9079°, 14.9315°, CR: Benátky, 4x, 6x. **181** 49.93414°, 14.9937°,, CR: Horní Kruty, 6x. **182*** 50.11481°, 14.4167°, CR: Praha, 4x, 6x. **184*** 49.91702°, 14.3335°, CR: Jíloviště, 4x. **185** 50.46988°, 13.4282°, CR: Chomutov, 4x, 6x. **187** 50.55464°, 13.392°, CR: Boleboř, 4x. **188** 50.55631°, 13.4018°, CR: Boleboř, 4x. **189*** 50.59117°, 13.4269°, CR: Hora svaté Kateřiny, 6x. **190*** 50.44182°, 14.6341°, CR: Mšeno, 6x. **191** 50.46068°, 14.66°, CR: Lobeč, 6x. **192*** 50.46331°, 14.6735°, CR: Lobeč, 6x. **193*** 48.18693°, 24.31°, Ukraine: Kvasy, valley of Black Tisza river, 6x. **194*** 48.18237°, 24.3394°, Ukraine: Chornohora massif, 6x. **195*** 48.16474°, 24.3435°, Ukraine: Chornohora massif, 6x. **196** 48.16842°, 24.4145°, Ukraine: Mount Petros (Chornohora massif), 6x. **197** 48.03081°, 24.5927°, Ukraine: Chornohora massif, 6x. **198*** 47.99781°, 24.5701°, Ukraine: Chornohora massif, 6x. **199*** 48.00997°, 24.5279°, Ukraine: Chornohora massif, 6x. **200*** 49.75758°, 13.9384°, CR: Jince, 4x, 6x. **201*** 49.90457°, 14.2859°, CR: Černolice, 6x. **202** 50.82459°, 14.1723°, CR: Maxičky, 4x, 6x. **203*** 50.82885°, 14.1653°, CR: Maxičky, 4x. **204*** 50.82939°, 14.1645°, CR: Maxičky, 4x, 6x. **205*** 49.76038°, 13.9486°, CR: Jince, 4x. **207*** 49.71679°, 13.9229°, CR: Obecnice, 4x, 6x. **209** 49.65608°, 15.8963°, CR: Radostín, 4x, 6x. **212** 49.91457°, 14.8871°, CR: Vlkančice, 4x, 6x. **217** 42.96293°, 9.4486°, Corsica: Macinaggio, 6x. **218** 42.65852°, 9.1588°, Corsica: Agriates Desert, 6x. **219*** 42.44163°, 8.8434°, Corsica: Foret de Bonifatu, 6x. **220** 42.28269°, 8.8709°, Corsica: Foręt d'Aďtone, 4x. **221** 42.29801°, 9.149°, Corsica: Corte, 4x. **222*** 41.40642°, 9.2111°, Corsica: Santa Manza Plage, 2x (*J. sorrentini, J. hybridus*), 4x, 6x. **G37** 41.68142°, 43.573611°, Georgia Bakuriani, 6x. **300** 40.34196°, 20.39816°, Albania, 6x. **LD78** Sweden Narvik, 6x. **LD107** Sweden: Ystad, 6x. **LD151** Canada: Presqu'ile Provincial Park in Northumberland county (Ontario), 6x. **LD244** CR: Střeleč u Libuně, 6x. **LD248** CR: Chlumy, 6x. **LD322** Denmark: Jutland, Billund International Airport, 4x. **LD323** Denmark: Jutland, Billund International Airport, 4x. **LD372** Germany: Berlin, 6x. **LD405a** Sweden: Saltefjel, 6x. **LD419** Morocco: Distr. Ouazzane: 25 km S of Ouazzane (=Wazzan), 6x. **LD428** CR: Chlumy, 4x. **LD437** Cyprus: Lemessol (Akrotiri), 2x, 4x, 6x. **LD438** Cyprus: Lemessol (Akrotiri), 2x, 4x, 6x. **LD439** Cyprus: Lemessol (Akrotiri), 2x, 4x, 6x.