SYSTEMATICS AND PHYLOGENY

Intricate evolutionary history of *Callitriche* (Plantaginaceae) taxa elucidated by a combination of DNA sequencing and genome size

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Abstract The widespread aquatic plant genus *Callitriche* is taxonomically very challenging, but noteworthy in many evolutionary aspects including a high overall diversity, extensive phenotypic plasticity, remarkable reproductive systems and a large variation in ploidy levels and chromosome numbers. We conducted a multi-level systematic study on 346 individuals of 25 taxa from 21 mostly European countries. Flow cytometric estimation of genome size, chromosome counting and direct sequencing of ITS and *trnT-trnL* DNA markers combined with RFLPs of the ITS region were applied in order to unravel the phylogenetic relationships among *Callitriche* taxa and to clarify the origin of polyploid species and hybrids. Additionally, ITS sequences from a recent worldwide phylogenetic study of the genus were included for comparison. We demonstrate that most of the traditionally recognized European *Callitriche* taxa are well defined by a combination of genome size and molecular markers. Several species showed remarkable intraspecific genetic variation; previously unknown cryptic taxa were revealed within *C. stagnalis*, *C. truncata* and North American *C. heterophylla*. The origin of selected polyploid taxa was investigated in detail. Diploid *C. cophocarpa* was confirmed to be the parental species of tetraploid *C. platycarpa*, but we did not find direct evidence for the putative allopolyploid origin of this species. The complex of *C. brutia* included three taxa; of these, *C. hamulata* is probably an allooctoploid derivative of *C. brutia* var. *brutia* and *C. cophocarpa*/*C. platycarpa*. The third member, *C. brutia* var. *naftolskyi*, was newly reclassified at the subspecies level; for the first time, chromosome numbers are provided for this poorly known taxon. For a single triploid sample, our results suggested an autopolyploid origin from *C. stagnalis*. Four *Callitriche* hybrids were revealed, two of which are newly described and validated here as *C.* ×*nyrensis* and *C. brutia* nothosubsp. *neglecta*. A tentative intrageneric concept of two sections (*Callitriche*, *Pseudocallitriche*) is adopted, with the need for a more detailed evaluation in the future.

Keywords diversity; hybridization; molecular identification; NeighborNet analysis; phylogenetic analysis; polyploidy

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Virtually all fields of biology rely on a solid framework of systematic classification, based primarily on phylogenetic relationships among organisms and their morphological and genetic differentiation. A detailed knowledge of the living objects we work with is essential for drawing any scientific hypotheses and conclusions, allowing to explore nature in a broader ecological context (Guerra-García & al., 2008; Ruggiero & al., 2015). Phylogenetic research enables us to better understand the evolutionary mechanisms responsible for the origin of the observed variation and the emergence of new species (e.g., Alix & al., 2017). Nevertheless, the existing, genetically determined biodiversity often remains neglected, being not reflected in conspicuous morphological characters. Despite the difficult detection of such cryptic taxa, studying them in an integrative research approach can bear substantial implications for evolutionary theories, biogeography, as well as for nature conservation (Bickford & al., 2007).

Among angiosperms, the genus *Callitriche* L. (water-starwort; Plantaginaceae Juss. sensu Albach & al., 2005) is exceptional in a number of evolutionary aspects. With ca 75 recognized species (Hassemer & Lansdown, 2018), it is one of the most diversified genera of aquatic plants. Water-starworts are considered taxonomically extremely challenging, which is mainly due to their reduced morphology (Schotsman, 1967; Lansdown, 2008), an extensive phenotypic plasticity (Schotsman, 1954; Jones, 1955; Martinsson, 1996) and the complex evolutionary history of particular taxa (e.g., Philbrick & Les, 2000; Demars & Gornall, 2003; Lansdown, 2006a; Ito & al., 2017). In total, 11 different chromosome numbers ranging from $2n = 6$ to $2n = 40$ are currently reported in the genus, including five ploidy levels (summarized in Prančl & al., 2014). The genus is also remarkable for its highly diversified pollination strategies including anemogamy (the dispersal of pollen by the wind), epihydrogamy (the spread of pollen across the water surface) and hypohydrogamy (underwater pollination through wettable exine-reduced pollen), combined with

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various modes of selfing (in fact, geitonogamy; Schotsman, 1982; Philbrick & Anderson, 1992; Philbrick & Bernardello, 1992; Martinsson, 1996).

The genus has been thoroughly studied using morphology (predominantly based on minute fruit and floral characters) and chromosome counting (e.g., Fassett, 1951; Mason, 1959; Schotsman, 1967, 1977; Philbrick, 1994; Lansdown, 2006b, 2008; Bean, 2007). However, molecular or cytogenetic approaches were rarely employed to examine the evolutionary relationships of *Callitriche* taxa. Two larger phylogenetic studies of the genus are available: Philbrick & Les (2000) and Ito & al. (2017). The former included 20 taxa from Europe and North America, using the *rbcL* plastid gene marker; however, the relationships among some taxa included in the study remained largely unresolved. The latter study involved 22 taxa from six continents, applying nuclear (ITS) and plastid (*matK*, *rbcL*) DNA regions. That study outlined basic phylogenetic relationships within *Callitriche*, but did not solve any formal intrageneric classification nor did the authors provide a taxonomic evaluation of the ascertained intraspecific genetic variation. Although polyploidy was detected in 19 of 35 taxa for which the chromosome numbers are known (Prančl & al., 2014), the evolutionary origins of particular polyploid taxa have remained entirely unknown. The only exception is the European species *C. platycarpa* Kütz., which has been repeatedly confirmed to be an allotetraploid derivative of the diploid parental species *C. cophocarpa* Sendtn. and *C. stagnalis* Scop. (Bączkiewicz & al., 2007; Schwarzacher & al., 2017). Also the impact of hybridization on the overall *Callitriche* diversity is poorly known. To date, only one interspecific hybrid has been formally described (*C.* ×*vigens* K.Martinsson, i.e., the primary triploid hybrid of *C. cophocarpa* and *C. platycarpa*; Martinsson, 1991). Although hybridization appeared to be relatively rare in *Callitriche*, direct evidence of this assumption using molecular markers was still lacking.

Recently, flow cytometry has been successfully utilized to distinguish central European *Callitriche* taxa, manifesting genome size as a suitable independent character that can serve as a basic marker to recognize taxonomic entities within the genus (Prančl & al., 2014). That study also revealed a previously unknown hybrid of the putative parents *C. hamulata* Kütz. ex W.D.J.Koch and *C. cophocarpa*, indicating that hybridization in this genus could be more frequent than hitherto assumed, but had remained elusive using the traditional morphological approach.

In Europe, 14–15 native (Table 1) and 3 rare introduced species are reported (Lansdown, 2006a, 2008). Water-starworts occur in almost all types of aquatic habitats, but prefer shallow waters including small temporary wetlands such as puddles on forest paths or various vernal pools. While most aquatic plants generally show a relatively wide range of distribution, limited taxonomic differentiation, and low infra-specific genetic variation (Santamaría, 2002), many *Callitriche* taxa are endemics of relatively small geographic regions (see Table 1). Intraspecific taxa have been described within three European species, including both diploids (*C. hermaphroditica* L., *C. truncata* Guss.) and polyploids (the complex of *C. brutia* consisting of hexaploid *C. brutia* Petagna var. *brutia*, *C. brutia* var. *naftolskyi* (Warb. & Eig) Lansdown with unknown chromosome number [until recently classified at the species level or treated as an unresolved taxon] and octoploid *C. hamulata*, recently re-evaluated as *C. brutia* var. *hamulata* (Kütz. ex W.D.J.Koch) Lansdown; Lansdown, 2006a; Lansdown & al., 2017). There is also indication that some species show wide morphological variation in some parts of Europe and may contain several cryptic taxa (e.g., *C. stagnalis* in Spain and *C. hermaphroditica* in Russia; Lansdown, 2008). Recently, many new records of *Callitriche* taxa had been reported, especially in the Mediterranean area, which is considered as a species diversity centre of the genus in Europe (Lansdown & Strid, 2011; Lansdown & al., 2016, 2017). All aforementioned facts illustrate the need to investigate the evolution of *Callitriche* species in more detail and suggest an indisputable potential for elucidating the processes that shape the evolution of aquatic plants as well as of angiosperms in general.

This article provides a molecular and cytogenetic study of European *Callitriche* taxa using flow cytometry and chromosome counting combined with direct sequencing of nuclear ribosomal (ITS) and plastid (*trnT-trnL*) DNA regions, complemented by restriction fragment length polymorphism (RFLP) of ITS to clarify the origin of several hybrid taxa. We investigated the genetic variation among and within particular species, specifically focusing on hybridization processes and phylogenetic relationships among neglected and morphologically poorly characterized taxa. We compared our ITS data with the results of a recent worldwide phylogenetic study on *Callitriche* (Ito & al., 2017). In addition, we discuss the evolutionary origins of polyploids in *Callitriche* and newly describe two previously undetected hybrids.

■ MATERIALS AND METHODS

Field sampling. — Plant samples were collected in 19 European countries and include all native European taxa except of *C. transvolgensis* Tzvelev and *C. truncata* subsp. *fimbriata* Schotsman, which are extremely rare and restricted to a small area of the Volga river delta. In addition, we included eight samples of European species that were collected in other continents, i.e., *C. stagnalis* from Australia and the U.S.A. (introduced), *C. hamulata* from the U.S.A. (introduced), *C. palustris* L. from the U.S.A. (native to both Eurasia and North America), four samples of *C. heterophylla* Pursh from the U.S.A. (considered to be closely related to *C. palustris*; Philbrick & Les, 2000; Ito & al., 2017), and *C. muelleri* Sond. from Australia (regarded as a sister to the remaining *Callitriche* taxa, being possibly the most ancestral water-starwort species; Ito & al., 2017). The initial determination of the samples followed the taxonomic treatments of Lansdown (Lansdown, 2008; Lansdown & al., 2017; with the exception of *C. hamulata*, see below) and Bean (2007). The subspecies of *C. heterophylla* were identified on the basis of the width of the ripe fruits (cf. Lansdown, 2009), if these were

Table 1. List of native European *Callitriche* taxa.

Taxon	2*n*	Distribution	Growth habit	Pollination	Key morphological characters
Section *Pseudocallitriche*					
C. hermaphroditica L.	6	Boreal Europe and Asia, boreal and temperate areas of North America (subsp. *macrocarpa* is more abundant in the northern part of the range)	Submersed	Submerged, hypohydrogamy, contacter	Leaves translucent, lingulate, 1-veined, leaf rosettes absent; peltate scales absent; bracts absent; pollen grains colourless; fruits broadly winged, 1.2–1.7 × 1.1–1.7 mm (subsp. *hermaphroditica*) or 1.5–2.4 × 1.6–2.8 mm (subsp. *macrocarpa*)
C. transvolgensis Tzvelev	?	Russia (Volgograd region)	Submersed	Submerged, hypohydrogamy	Leaves translucent, lingulate, 1-veined, leaf rosettes absent; peltate scales absent; bracts absent; pollen grains colourless; fruits longer than wide, 2.2–2.4 × 1.6–1.8 mm, winged only or mainly at apex
C. truncata Guss.	6*	Coastal areas of W Europe and Mediterranean (subsp. *occidentalis*); coastal areas of middle and E Mediterranean, introduced in Chile and Argentina (subsp. *truncata*); Volgograd region of Russia (subsp. *fimbriata*)	Usually submersed	Submerged, hypohydrogamy	Leaves translucent, lingulate, 1-veined, leaf rosettes absent; bracts absent, peltate scales absent; pollen grains colourless; fruits wider than long, 0.9–1.5 × 1.1–1.9 mm, subsessile or shortly pedunculate, narrowly winged (subsp. *truncata*), wings absent (subsp. *occidentalis*) or wings composed of a fringe of whitish fibrils (subsp. *fimbriata*)
C. pulchra Schotsman	8	Greece (island of Gavdos), Cyprus, N Libya	Submersed	Submerged, hypohydrogamy	Leaves translucent, lingulate, 1-veined, leaf rosettes absent; peltate scales absent; bracts absent; pollen grains colourless; fruits wider than long, 1.4–1.8 × 1.6–2.2 mm, all shortly pedunculate, ± broadly winged
C. lusitanica Schotsman	8	Iberian Peninsula, Sardinia, Sicily, Greece (island of Lesvos), Israel, NW Africa	Amphibious	Aerial/ epihydrogamy/ submerged, contacter	Leaves translucent, lingulate, leaf rosettes with irregular venation sometimes present; stem scales of 7–9 cells; bracts absent; pollen grains whitish to pale yellow; fruits 1–1.4 × 1.2–1.9 mm, narrowly to broadly winged
Section *Callitriche*					
C. cribrosa Schotsman	8	Iberian Peninsula, central Italy, NW Africa	Amphibious	Aerial/ epihydrogamy	Leaves up to 11.7 mm wide, often more than 11-veined, lingulate leaves usually absent; stem scales of 3–4 cells; bracts present, often forked; pollen grains yellow, filaments up to 9.4 mm; fruits 1.4–1.7 × 1.4–1.8 mm, ± broadly winged, greyish
C. cophocarpa Sendtn.	10	Central, N and E Europe	Amphibious	Aerial/ epihydrogamy	Leaves up to 6 mm wide, 1–5-veined, often lingulate; stem scales of 6–10 cells (most often 8); bracts present; pollen grains yellow, filaments up to 8.3(–12) mm, female and male flowers generally separated on different branches; fruits 0.9–1.2 × 0.9–1.1 mm, unwinged or narrowly winged, brown

(Continues)

Table 1. Continued.

Taxon	2*n*	Distribution	Growth habit	Pollination	Key morphological characters
C. lenisulca Clavaud	10	European & Asian Mediterranean, NE coast of the Black Sea	Amphibious	Aerial/ epihydrogamy/ submerged, contacter	Leaves up to 4.0 mm wide, 1–3-veined, often lingulate; stem scales of 8–16 cells; bracts present; male and female flowers generally in alternating pairs along stem, pollen grains yellow, filaments up to 2.3 mm, anthers small, <0.5 mm in diameter; fruits 1.1–1.4 × 1.3–1.6 mm, unwinged or very narrowly winged, pale brown to brown, generally occurring in every second pair of axils
C. obtusangula Le Gall	10	W and S Europe, NW Africa	Amphibious	Aerial/ epihydrogamy	Leaves up to 7 mm wide, 1–5(–7)-veined, often lingulate, wider leaves often rhombic; stem scales of 6–10 cells; bracts present; pollen grains yellow, elongate-ellipsoid and curved, filaments up to 7.6 (–12.3) mm long; fruits 1.1–1.8 × 1.1–1.7 mm, ellipsoid, usually longer than wide, unwinged (without even a ridge), pale brown
C. regis-jubae Schotsman	10	Iberian Peninsula, Sardinia (?), NW Africa	Amphibious	Aerial/ epihydrogamy, contacter	Leaves up to 4.1 mm wide, 1–5-veined, lingulate leaves sometimes present; stem scales of 7–10 cells; bracts present; pollen grains yellow, filaments up to 1.5 mm, anthers small, <0.6 mm in diameter; fruits 1–1.4 × 1.2–1.6 mm, wider than long, pedunculate, pale brown to pale maroon
C. stagnalis Scop.	10	Most of Europe, NW Africa and Macaronesia, Middle East (?); introduced in North America, Japan, Australia, New Caledonia and New Zealand	Amphibious	Aerial/ epihydrogamy	Leaves up to 9 mm wide, 1–7-veined, fresh-green, lingulate leaves usually absent; stem scales of 7–10 cells (most often 8); bracts present; pollen grains yellow, filaments up to 5.3(–8.5) mm; fruits 1.2–1.6 × 1.2–1.7 mm, broadly winged, pale brown to greyish
C. palustris L.	20	Europe (predominantly central, N and E), Asia & North America (predominantly boreal and temperate); introduced in Australia	Amphibious	Aerial/ epihydrogamy/ submerged, internal geitonogamy	Leaves up to 4.5 mm wide, 1–5-veined, fresh-green, lingulate leaves often present; stem scales of 8–16 cells; bracts often absent; pollen grains yellow, filaments up to 2.9(–3.8) mm, often <1 mm with aborted anthers, also styles often aborted; fruits 0.9–1.4 × 0.7–1 mm, obovate, longer than wide, brown-black, often without rests of styles on the top
C. platycarpa Kütz.	20	NW Europe, NW Spain, S Italy, Aegean Islands	Amphibious	Aerial/ epihydrogamy	Leaves up to 9 mm wide, 1–5(–7)-veined, ± deep green, lingulate leaves sometimes present; stem scales of 7–10 cells (most often 8); bracts present; pollen grains yellow to bright yellow, ellipsoid to bluntly triangular, filaments up to 7.8(–15.5) mm; fruits 1.2–1.7 × 1.2–1.6 mm, narrowly winged, brown
C. brutia Petagna	28**	W, NW and SW Europe, NW Africa, Middle East (?), introduced in Australia and New Zealand (var. *brutia*); Sardinia, Sicily, Capraia Island, Aegean Islands, Israel, Syria, N Africa (var. *naftolskyi*)	Amphibious	Submerged, hypohydrogamy, contacter	Leaves up to 3.8 mm wide, 1–3(–5)-veined, lingulate leaves often present, usually not expanded on notched apices; stem scales of 7–19 cells; bracts caducous; pollen grains colourless, lacking exine, filaments <1.2 mm, styles strongly reflexed; fruits 1–1.5 × 1–1.6 mm, with rests of styles appressed to side of fruit, ± orbicular, shiny, narrowly winged, sessile when submerged and with long peduncles up to 12 mm when terrestrial (var. *brutia*) or ± wider than long, matt, narrowly to broadly winged with undulate margin, always pedunculate (var. *naftolskyi*)

(Continues)

Table 1. Continued.

Taxon	2*n*	Distribution	Growth habit	Pollination	Key morphological characters
C. hamulata Kütz. ex W.D.J.Koch***	38	W, N & central Europe, Greenland, Kamchatka; introduced on the W coast of North America	Amphibious	Submerged, hypohydrogamy, contacter	Leaves up to 5.4 mm wide, 1–5-veined, lingulate leaves often present, often expanded on notched apices; stem scales of 9–19 cells; bracts caducous; pollen grains colourless, with rudimentary exine, filaments <1.2 mm, styles strongly reflexed; fruits 1–1.5 × 1–1.4 mm, with rests of styles appressed to side of fruit, ± orbicular, shiny, narrowly winged, sessile or very rarely on peduncles up to 2.6 mm

Distribution data were adopted mainly from Lansdown (2008) and Lansdown & al. (2017), with additional information from Mason (1959), Morita & Lee (1998), Philbrick & al. (1998), Bean (2007), Hassemer & O'Leary (2018) and Volkova & al. (2020). Diagnostic characters are based on the most relevant taxonomic studies (Schotsman, 1967; Lansdown, 2008; Lansdown & al., 2017) and our observations. Ploidy levels associated with particular chromosome counts: 2*x* (2*n* = 6,8,10), 4*x* (2*n* = 20), 6*x* (2*n* = 28), 8*x* (2*n* = 38). Contacter – pollination takes place through the contact between stigma and anther (details in the text); internal geitonogamy – the pollen tubes germinate directly inside an anther (that does not open at all) and grow through the vegetative tissue of filament and node to reach the adjacent ovaries (Philbrick & Bernadello, 1992).

* the chromosome number is only known for *C. truncata* subsp. *occidentalis*

** the chromosome number is only known for *C. brutia* var. *brutia*

*** *C. hamulata* is sometimes classified as *C. brutia* var. *hamulata* (Kütz. ex W.D.J.Koch) Lansdown, see Discussion for details

available. A single sample of *C. heterophylla* var. *bolanderi* (Hegelm.) Fassett (collection no. C14-144, Appendix 1 and suppl. Table S1) was collected out of the known distribution range of that subspecies (cf. Fassett, 1951). As the determination of that sample was not entirely clear, we refer to it as "cf. *bolanderi*".

Two samples of *Hippuris vulgaris* L. were included as an outgroup; this genus is sister to *Callitriche* (Albach & al., 2005) and was also used as an outgroup in previous phylogenetic analyses of water-starworts (Philbrick & Les, 2000; Ito & al., 2017).

The sampling was carried out to embrace materials from the widest possible range of aquatic habitats and covering a wide range of morphological variation. If necessary, multiple individuals were collected from several populations, especially when the presence of multiple species or hybrids was suspected. In total, 346 *Callitriche* individuals from 180 localities were obtained (for locality details, see Fig. 1, Appendix 1 and suppl. Table S1). Voucher specimens are preserved in the herbarium of Charles University, Prague (PRC).

Flow cytometry. — Genome size was estimated for 330 of 346 plants using flow cytometry (FCM). Of these, genome sizes of 149 individuals were taken from our previous cytometric study (Prančl & al., 2014), and 181 samples were newly analyzed from fresh plant material (see suppl. Table S1) using the identical procedure and laboratory equipment. Fresh material was not available for the remaining 16 samples. The sample preparation followed the simplified two-step procedure described by Doležel & al. (2007). Samples were analyzed individually, using propidium iodide (PI) as a fluorescent stain. Additionally, a simultaneous analysis of *C. brutia* var. *brutia* and *C. brutia* var. *naftolskyi* (bulked sample of two individuals in a single run) was performed to confirm differences between the genome sizes of both taxa. In this case, the sample was stained using 4,6-diamidino-2-phenylindole (DAPI) to achieve a higher resolution of peaks.

If possible, each sample was analysed 2 or 3 times on different days to account for random measurement error; if the range of variation of the repeated measurements exceeded a 2% threshold, the outlying value was discarded and the sample re-analysed. Histograms were evaluated using the FloMax software v.2.4d (Partec) or FlowJo 10 (TreeStar). In total, exact genome size (i.e., calculated as the mean of the repeated measurements) was estimated for 195 individuals, for which repeated analyses of appropriate quality were available (147 newly analyzed and 48 taken from the previous study). Only these repeatedly measured individuals were used for the calculation of the genome size statistics of particular taxa (see below).

The genome size was expressed as the ratio of the mean fluorescence of the sample and the internal standard. *Bellis perennis* L. was selected as a primary reference standard as it has a similar, but non-overlapping genome size with the majority of the samples studied (2C = 3.96 pg, Leong-Škorničková & al., 2007; because several different genome size values are reported for *Bellis perennis*, we adopted a 2C-value that was calibrated via simultaneous analyses of *Bellis* with

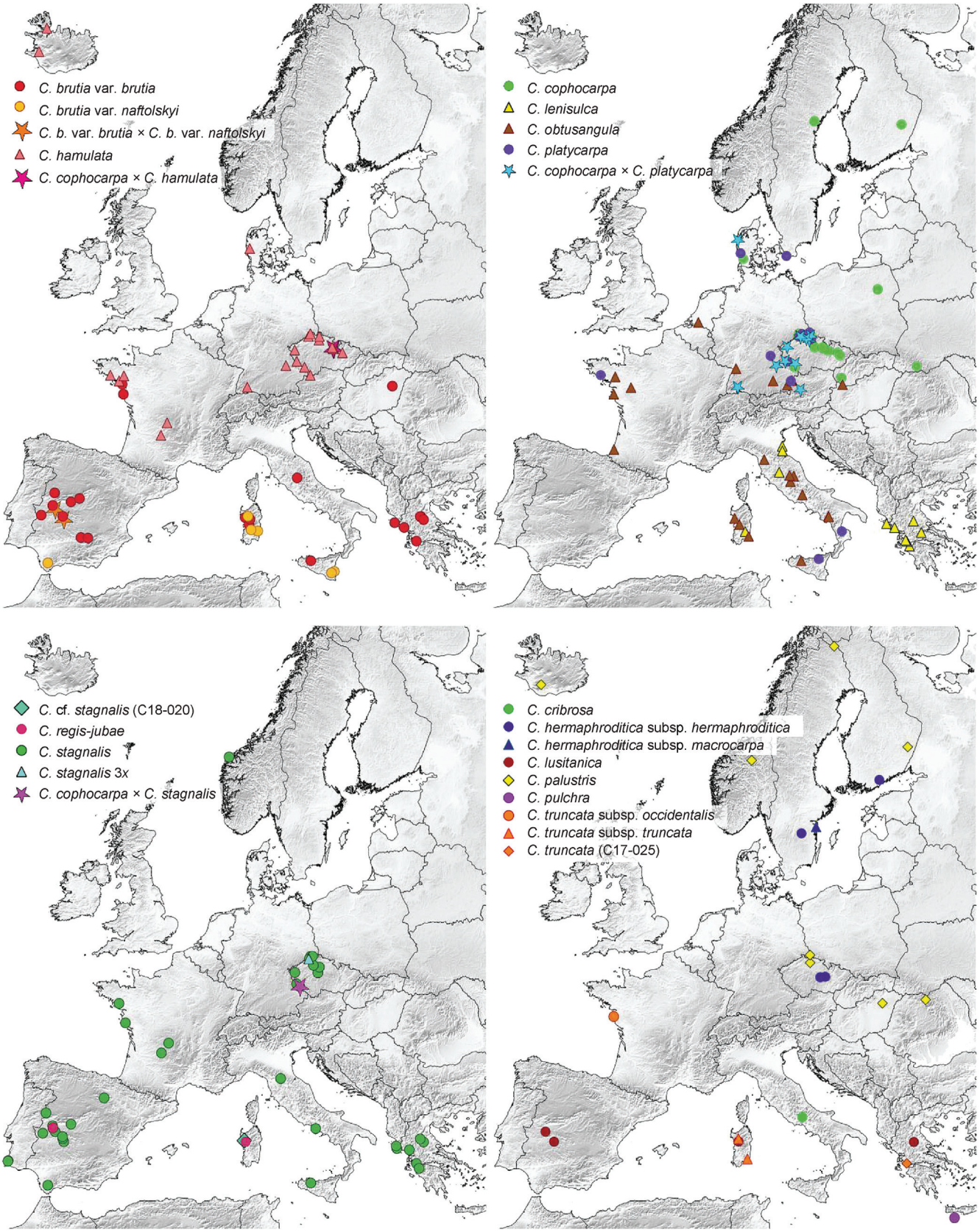

Fig. 1. Maps showing the locations of the *Callitriche* samples. Samples collected in the U.S.A. and Australia are not included. Due to numerous overlapping localities, individual taxa are depicted in four separate maps.

the second standard used in this study, *Glycine max*). *Glycine max* (L.) Merr. 'Polanka' (2C = 2.50 pg; Doležel & al., 2007) served as a reference standard for *C. heterophylla*, *C. obtusangula* Le Gall and *C. palustris* because the genome sizes of these taxa overlapped with that of *Bellis perennis*. The extent of the total variation in intraspecific genome size was calculated as a percentage of the difference between the highest and lowest genome size values and expressed as a percentage of the minimum.

To compare the recent results with our previous genome size estimations and to gain the most accurate genome size values, we extended the dataset for genome size statistics with 132 additional samples from our previous study (Prančl & al., 2014) (suppl. Table S1). These samples, mostly originating from central Europe, are not formally included in the present paper (as they were not sequenced), but their mean genome sizes estimated from the repeated measurements have been used. In total, genome size statistics of particular taxa were calculated using the combined dataset of 327 samples (including 147 newly analyzed samples and 180 genome size values published in the previous study).

Chromosome counts. — Selected plants were cultivated in a garden tank until they formed adventive roots on their stems, which were used for chromosome counting. Alternatively, plants were cultivated on wet mud in pots in a greenhouse and chromosomes were counted using shoot apical meristem and the youngest leaves emerging in the centre of the leaf rosettes.

The meristematic tissue was pre-treated in a saturated aqueous solution of p-dichlorobenzene at room temperature for approximately three hours, then fixed in a freshly prepared 3 : 1 mixture of 96% ethanol and acetic acid and stored at −20°C until further processing. Before chromosome preparation, the material was macerated in a 1 : 1 mixture of ethanol and hydrochloric acid for 10 s, then transferred onto a microscope slide. Non-meristematic tissues were removed, and the meristem was stained in a drop of lacto-propionic orcein, covered with a coverslip and squashed. The preparations were examined under an Olympus BX 51 microscope equipped with a DP-71 Olympus digital camera with the DP Controller imaging software v.3.1 (Olympus). Only slides on which at least five mitoses were found were considered.

Our previous study provided chromosome counts for eight *Callitriche* taxa growing in central Europe (Prančl & al., 2014). In this study, we determined chromosome numbers for additional eight samples belonging to seven taxa, which were not included in the previous study. For the remaining species included in this study, we were not able to obtain/cultivate usable material.

Molecular procedures. — In total, 224 *Callitriche* individuals including samples from 180 populations, and 2 individuals of the outgroup *Hippuris vulgaris* were subjected to molecular analyses. A single sample was sequenced from the majority of populations that were homogeneous morphologically and also proved to be invariable in genome size. Several samples were processed from populations that were assumed to be mixed on the basis of genome sizes and/or morphology, and also for some populations that included individuals of putative hybrid origin. Total genomic DNA was extracted from silica gel-dried leaf tissue according to a sorbitol extraction method (Štorchová & al., 2000). The internal transcribed spacer region of nuclear ribosomal DNA (containing ITS1, 5.8S rDNA and ITS2) was amplified using primers ITS F (King & al., 2001) and ITS 4 (White & al., 1990); the *trnT-trnL* plastid intergenic spacer was amplified using primers a and b (Taberlet & al., 1991). The ITS region was amplified as described in Kaplan & Fehrer (2004); PCR conditions for the *trnT-trnL* region followed Fehrer & al. (2007) except that *Taq* DNA polymerase and PCR Blue buffer from Top-Bio (Vestec, Czech Republic) were used. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced at GATC Biotech (Konstanz, Germany) / Eurofins Genomics (Ebersberg, Germany) using the PCR primers in one or both directions depending on read quality.

Sequences of the ITS region of several samples showed polymorphisms, i.e., superimposed peaks and occasionally shifts. This was especially true for several samples of putative hybrids (namely *C. cophocarpa* × *C. stagnalis*, *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi* and *C. hamulata* × *C. cophocarpa/platycarpa*) and for some samples of *C. hamulata*. For these samples, cloning and RFLP analysis were applied to make final identifications and documentations of hybrid identity. In the case of the first two aforementioned hybrids, multiple individuals showed the same patterns of polymorphisms; therefore, only one sample of each hybrid was selected (C15-084-03 and C16-013, respectively) and cloned as described in Fehrer & al. (2009). Eight clones were sequenced for each sample, and the parental copies were identified. A single clone (C15-084-03-x5) was recombinant and has been discarded. For *C. hamulata* and the putative hybrid *C. hamulata* × *C. cophocarpa/platycarpa*, the peaks corresponding to the polymorphisms were very small so that a too high number of clones would have to be sequenced to retrieve the underrepresented copies. Therefore, these samples were subjected to RFLP analysis. Based on the putative parental sequences, diagnostic restriction sites were identified that distinguished all species except *C. platycarpa* and *C. cophocarpa*, whose sequences were identical (see below). RFLPs of ITS were performed using a double digest with *Bam*HI (G'GATC_C) and *Bsi*WI (C'GTAC_G) enzymes (Fisher Scientific, Pardubice, Czech Republic). *Bam*HI cuts only *C. cophocarpa* and *C. platycarpa* once; *Bsi*WI cuts only *C. brutia* var. *brutia* once, and both cut *C. brutia* var. *naftolskyi* once resulting in three fragments of distinguishable size. Restriction digests were performed with 10 units of *Bam*HI and 3 units of *Bsi*WI according to the manufacturer's instructions using approx. 250 ng of PCR product in overnight digests. Products were separated on 2% agarose gels with 200 ng of DNA size standard. In total, 18 samples of 6 taxa were subjected to RFLPs, covering all putative parental species and samples representing the majority of the observed intraspecific genetic variation.

All sequences were submitted to GenBank (accession numbers MN091382–MN091622 [ITS], MN091980–MN092205 [*trnT-trnL*]). For a detailed list, see Appendix 1 and supplementary Table S1.

Molecular data analyses. — Sequence electropherograms were edited manually using Chromas v.1.45 (Technelysium, Australia) and aligned by hand in Bioedit v.7.0.9.0 (Hall, 1999) (for alignments, see suppl. Appendices S1, S2). Additive nucleotide polymorphisms in the ITS region were coded using the IUPAC nucleotide ambiguity codes. For the ITS dataset, available sequences from the study of Ito & al. (2017) were retrieved from GenBank and added to the alignment (see suppl. Appendix S1). Additionally, the individual ITS variants of the hybrids resulting from cloning were included. Before performing phylogenetic analyses, the number of samples for both ITS and *trnT-trnL* datasets were reduced in an effort to cover the whole molecular variation and a representative geographic range for all taxa. Samples containing nucleotide polymorphisms were excluded from the ITS dataset in order to prevent branch collapses (with a few exceptions such as *C. hamulata* samples, of which all sequences showed at least some polymorphisms). The final ITS dataset consisted of 73 of our samples (including eight clones) and 35 sequences from GenBank (suppl. Appendix S3). The final *trnT-trnL* dataset included of 90 accessions (suppl. Appendix S4); no corresponding data of this region were available in GenBank. All our samples included in the ITS dataset were also included in the *trnT-trnL* dataset. Since both trees were mostly congruent (see below), we also analyzed a concatenated dataset, consisting of 65 accessions that were included in both ITS and *trnT-trnL* trees.

Indel coding for both datasets was performed with FastGap v.1.2 (Borchsenius, 2009) based on the simple method of Simmons & Ochoterena (2000). Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian analyses (BA). Prior to analyses, the model of molecular evolution best fitting the data was determined for all datasets with Modeltest v.3.5 (Posada & Crandall, 1998). For ITS and the concatenated dataset, a TrN+Γ model was found in hierarchical likelihood ratio tests (hLRTs). ML analysis was performed with MEGA v.X (Kumar & al., 2018) using a Tamura-Nei model and gamma distribution with 5 discrete rate categories. All sites, extensive subtree-pruning-regrafting and a very strong branch swap filter were used. Bootstrap support was computed using 1000 replicates. Bayesian analyses were conducted with MrBayes v.3.2.6 (Ronquist & al., 2012), six substitution rates and gamma distribution as priors. Analyses were run with the default settings for 2.5 million generations (ITS) or 1 million generations (for the smaller concatenated dataset), sampling every 1000th tree. All indicators suggested that convergence between the different runs was achieved. The first 25% of trees were discarded as burn-in, and the rest of the trees were summarized. For *trnT-trnL*, a TVM+Γ model was found to best represent the data. A transversion model is not implemented in MEGA, it was replaced by the most similar one, a general time reversible model. For BA, 1.5 million generations were needed to reach convergence. Other parameters were the same as before.

To visualize the reticulate relationships among the species studied, two datasets (ITS and the concatenated dataset of ITS and *trnT-trnL*) were subjected to NeighborNet analysis performed with SplitsTree4 v.4.14.8 (Huson & Bryant, 2006), applying uncorrected *p* distances with ambiguities handled as average. Bootstrap support was calculated with 1000 replicates. For these datasets, all 224 of own sequenced samples were included, but the sequences from Ito & al. (2017) were omitted because polymorphisms were obviously not scored and evaluated in that study, and *trnT-trnL* was examined only in our study.

■ RESULTS

Genome size and chromosome counts. — Genome size was determined for all species included in this study except *C. pulchra* Schotsman, for which we did not have living plants. In total, 24 taxa of *Callitriche* were analyzed (Table 2, Fig. 2). The majority of species differs clearly in nuclear DNA content. The differences in genome size are insignificant only for the pairs of *C. regis-jubae* Schotsman–*C. stagnalis*, *C. obtusangula*–*C. palustris* and *C. brutia* var. *naftolskyi*–*C. platycarpa*. The detected 2C-values varied 7.36-fold from 1.21 pg in the Australian species *C. muelleri* up to 8.90 pg in *C. hamulata* (Fig. 2). Monoploid genome sizes (1Cx-values) were also highly variable, ranging 3.16-fold from 0.61 pg in *C. muelleri* to 1.93 pg in *C. obtusangula*. Flow cytometry was for the first time applied to estimate the genome size of five European and two non-European species (*C. brutia*, *C. cribrosa* Schotsman, *C. lusitanica* Schotsman, *C. regis-jubae*, *C. truncata*; *C. heterophylla*, *C. muelleri*). Additionally, cytotype variation was detected within *C. brutia* and *C. truncata*. In *C. brutia*, two cytotypes with similar, but non-overlapping cytotypes correspond well with two subordinate taxa, *C. brutia* var. *brutia* (lower genome size) and *C. brutia* var. *naftolskyi* (larger genome size; difference between means 4.6%). The simultaneous analysis of these two taxa confirmed the difference, resulting in a bifurcated peak. The case of *C. truncata* is more complicated, because three clearly different cytotypes were revealed among plants that fit morphologically to this species. From these, the cytotype with the largest genome size corresponds to *C. truncata* subsp. *occidentalis* (Rouy) Schotsman, the second to subsp. *truncata* and the third, with the lowest DNA content, represented by one population from Greece (C17-025), is not clearly attributable to any subspecies (see Discussion). The mean genome sizes differed by 14.5% (subsp. *truncata*–subsp. *occidentalis*), 13.8% (Greek *truncata*–subsp. *truncata*) and even by 30.3% (Greek *truncata*–subsp. *occidentalis*). We managed to count the chromosome number only for subsp. *occidentalis* ($2n = 6$). In contrast, two subspecies recognized within *C. hermaphroditica*, i.e., subsp. *hermaphroditica* and subsp. *macrocarpa* (Hegelm.) Lansdown, are indistinguishable using FCM.

Table 2. Summary of flow cytometric genome size estimations.

Taxon	2*n*	Ploidy level	N	2C ± SD	2C range	Var (%)	1Cx	Mean chromosome size	Standard
***C. truncata* (Greece)**	6?	2*x*	1	1.88	–	–	*0.94*	*0.31*	B
C. truncata* subsp. *truncata	6	2*x*	1	2.14	–	–	1.07	0.36	B
C. truncata* subsp. *occidentalis	**6**	2*x*	1	2.45	–	–	1.23	0.41	B
C. hermaphroditica subsp. *hermaphroditica*	6	2*x*	7	1.96 ± 0.03	1.92–2.01	4.69	0.98	0.33	B
C. hermaphroditica* subsp. *macrocarpa	**6**	2*x*	1	2.01	–	–	1.01	0.34	B
C. lusitanica	**8**	2*x*	3	1.83 ± 0.01	1.82–1.84	1.10	0.92	0.23	B
C. cribrosa	8	2*x*	1	3.62	–	–	1.81	0.45	B
C. muelleri	**10**	2*x*	2	1.21 ± 0.01	1.20–1.21	0.83	0.61	0.12	B
C. regis-jubae	**10**	2*x*	2	2.99 ± 0.02	2.97–3.01	1.35	1.50	0.30	B
C. stagnalis	**10**	2*x*	51	3.00 ± 0.03	2.94–3.08	4.76	1.50	0.30	B
C. cophocarpa* × *C. stagnalis	10?	2*x*	14	3.12 ± 0.01	3.10–3.14	1.29	*1.56*	*0.31*	B
C. cophocarpa	**10**	2*x*	39	3.20 ± 0.04	3.11–3.26	4.82	1.60	0.32	B
C. lenisulca	**10**	2*x*	10	3.63 ± 0.03	3.58–3.69	3.07	1.82	0.36	B
C. obtusangula	**10**	2*x*	26	3.86 ± 0.06	3.71–3.93	5.93	1.93	0.39	G
autotriploid *C. stagnalis*	15?	3*x*	1	4.55	–	–	*1.52*	*0.30*	B
C. ×*vigens* [*C. cophocarpa* × *C. platycarpa*]	**15**	3*x*	19	4.66 ± 0.04	4.62–4.72	2.16	1.55	0.31	B
C. palustris	**20**	4*x*	24	3.90 ± 0.05	3.75–3.96	5.60	0.98	0.20	G
C. heterophylla* var. cf. *bolanderi	20	4*x*	1	4.05	–	–	1.01	0.20	G
C. platycarpa	**20**	4*x*	27	6.19 ± 0.06	6.06–6.33	4.46	1.55	0.31	B
C. brutia* var. *brutia	**28**	6*x*	17	5.86 ± 0.04	5.81–5.96	2.58	–	0.21	B
C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi	**28**	6*x*	2	5.96 ± 0.02	5.94–5.98	0.67	–	0.21	B
C. brutia* var. *naftolskyi	**28**	6*x*	5	6.13 ± 0.03	6.10–6.19	1.48	–	0.22	B
C. cophocarpa × *C. hamulata*	**29**	6*x*	16	7.63 ± 0.06	7.56–7.78	2.91	–	0.26	B
C. hamulata	**38**	8*x*	56	8.90 ± 0.09	8.73–9.15	4.81	–	0.23	B

Taxa, for which the genome size is estimated here for the first time, are in bold.
2*n* – chromosome number; values in bold indicate taxa, for which the chromosomes were counted in this study or in Prančl & al. (2014); values indicated by "?" were estimated on the basis of 2C-values, chromosome numbers for these taxa are unknown. 2C ± SD – mean genome size (2C-value) in pg of DNA ± standard deviation. 2C range – minimum and maximum 2C-values. Var (%) – difference between minimum and maximum expressed as % of the minimum. 1Cx – monoploid genome size in pg of DNA calculated from the mean 2C-value and the ploidy level; if the ploidy level is only estimated by flow cytometry (i.e., DNA ploidy level), the values are in italics; for some taxa, the 1Cx value cannot be meaningfully calculated due to aneuploid chromosome counts. Mean chromosome size – theoretical value calculated from the mean 2C-value and the chromosome number. Standard – internal standard (B = *Bellis perennis*, G = *Glycine max* 'Polanka').

Two previously unknown taxa of putative hybrid origin were revealed. The first one (C15-084) was found at a single locality in the Czech Republic, co-occurring with *C. stagnalis*. Individuals from this flowering, but non-fertile population showed intermediate genome size between *C. stagnalis* and *C. cophocarpa* and were therefore assumed to be the hybrid of these species. The identity of this hybrid was later confirmed by molecular analyses (see below). The other hybrid was found in two streams in Spain (C16-009, C16-013). These plants showed genome sizes at the upper end of the range of *C. brutia* var. *brutia*, but both possessed mostly underdeveloped fruits. These samples were assigned to *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi* based on results of the molecular analyses (see below).

For 11 taxa, genome sizes were published previously (Prančl & al., 2014). The current FCM data correspond well to those previously published. The only exception is *C. obtusangula*, for which two cytotypes with slightly different genome sizes were reported in the previous study, one including plants from Italy and the second represented by plants from north-western Europe. Our new data, including more samples of this species, suggest that although the genome size variation of the Italian samples is higher in comparison with samples from the rest of Europe, the genome size range of this species is rather continuous. Therefore, we consider all samples of *C. obtusangula* as belonging to a single cytotype.

Chromosome numbers quoted in other published sources were confirmed in six taxa studied (Fig. 3). For *C. brutia* var. *naftolskyi* ($2n = 28$), the chromosome number was determined for the first time.

Molecular phylogenetic analyses. — Phylogenetic trees reconstructed on the basis of the plastid *trnT-trnL* region show with strong support that the Australian species *C. muelleri* is sister to the remaining *Callitriche* taxa together with the outgroup *Hippuris vulgaris* (Fig. 4A). Also in the ITS tree, in which samples from the study of Ito & al. (2017) are included, *C. muelleri* results as the most basally branching *Callitriche* species, followed by *C. japonica* Engelm. ex Hegelm. forming a second branch, which is sister to the strongly supported clade consisting of the rest of the genus (Fig. 4B). In all datasets, including the tree reconstructed on the basis of concatenated data (*trnT-trnL* + ITS; Fig. 4C), the clade corresponding to the traditionally recognized sect. *Pseudocallitriche* (Hegelmaier, 1864; Philbrick & Les, 2000) is also well-supported. Other smaller groups having high support in all trees are the complex of *C. brutia*, the group of *C. cophocarpa*, *C. platycarpa* and *C.* ×*vigens* and the species pairs *C. truncata* + *C. hermaphroditica*, *C. palustris* + *C. heterophylla* (*C. palustris* group; also including *C. umbonata* Hegelm. in the ITS dataset) and *C. stagnalis* + *C. regis-jubae*. The clade of *C. lenisulca* Clavaud and *C. obtusangula* possesses high support in the *trnT-trnL* and concatenated trees, but it is not significantly supported in the ITS dataset. *Callitriche cribrosa* forms an isolated lineage with unclear relationships in all trees. The ITS dataset also contains some well-supported groups of species that were not included in the other trees such as clades of *C. compressa*

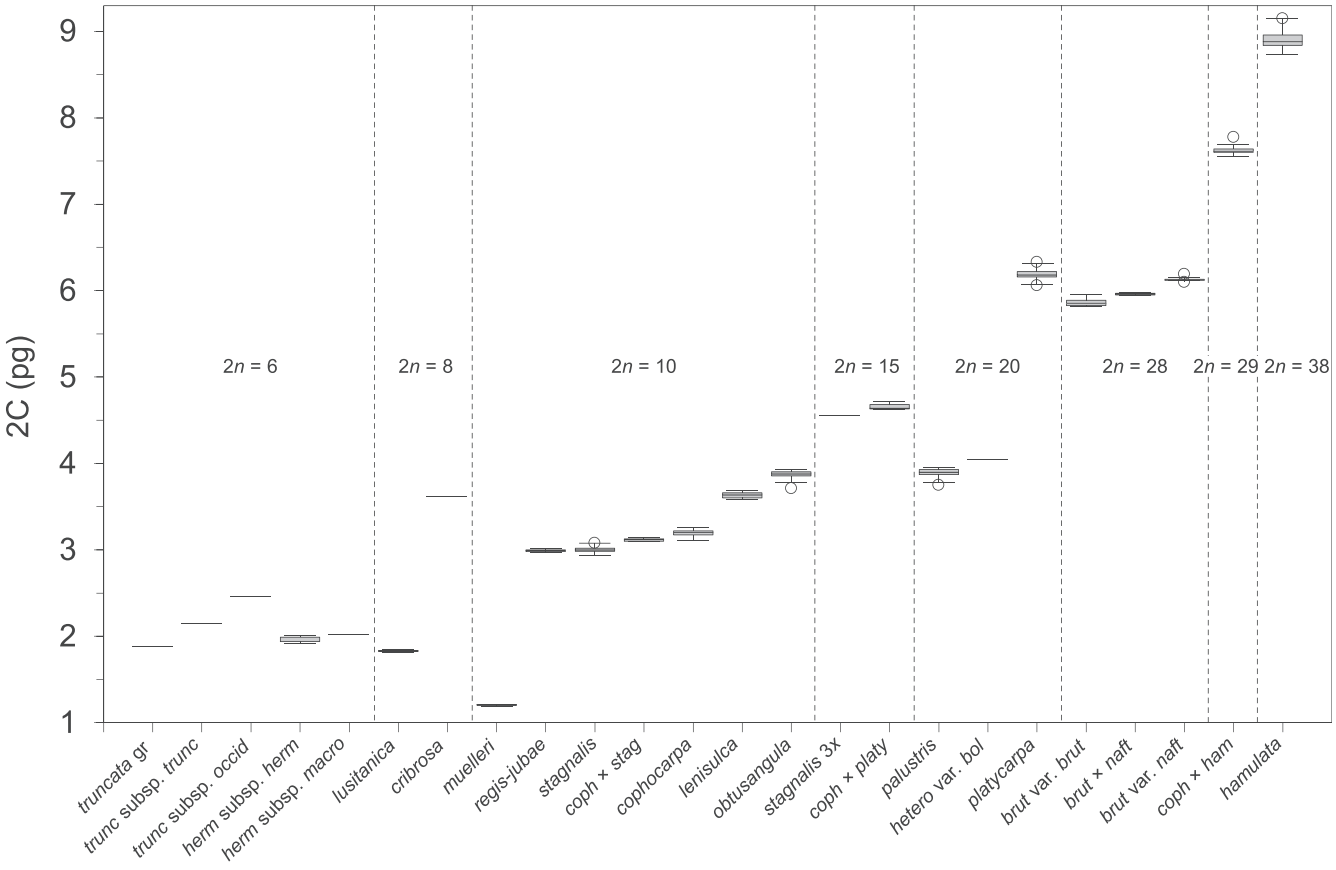

Fig. 2. Box-and-whisker plots showing the genome size variation (2C-values) of 24 *Callitriche* taxa. Taxa abbrevitions: *truncata* gr = *C. truncata* from Greece (C17-025); *trunc* subsp. *trunc* = *C. truncata* subsp. *truncata*; *trunc* subsp. *occid* = *C. truncata* subsp. *occidentalis*; *herm* subsp. *herm* = *C. hermaphroditica* subsp. *hermaphroditica*; *herm* subsp. *macro* = *C. hermaphroditica* subsp. *macrocarpa*; *coph* × *stag* = putative hybrid *C. cophocarpa* × *C. stagnalis*; *C. stagnalis* 3*x* = putative autotriploid *C. stagnalis*; *coph* × *platy* = *C. cophocarpa* × *C. platycarpa* [*C.* ×*vigens*]; *hetero* var. *bol* = *C. heterophylla* var. cf. *bolanderi*; *brut* var. *brut* = *C. brutia* var. *brutia*; *brut* × *naft* = putative hybrid *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi*; *brut* var. *naft* = *C. brutia* var. *naftolskyi*; *coph* × *ham* = putative hybrid *C. cophocarpa* × *C. hamulata*.

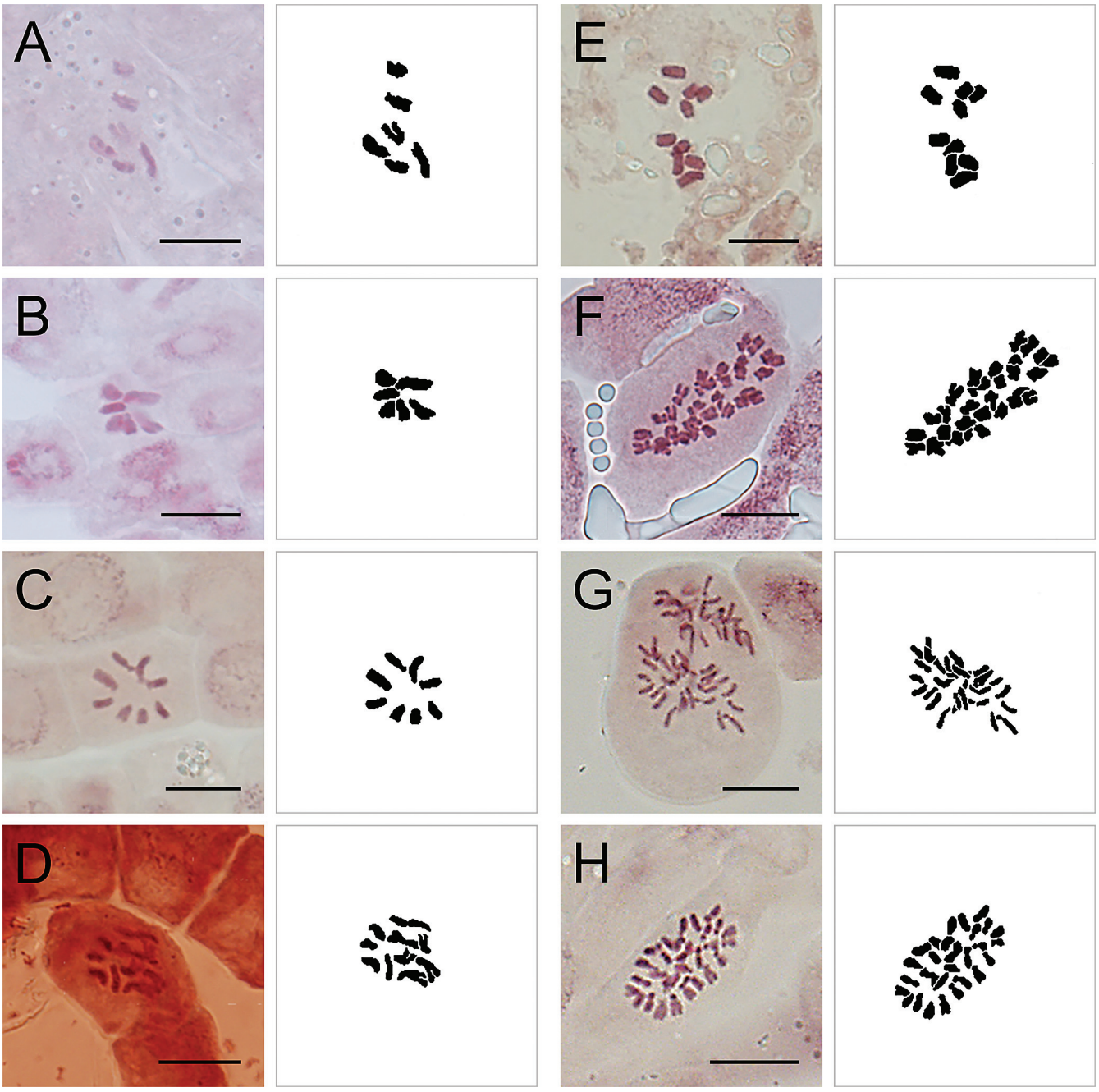

Fig. 3. Chromosomes (photograph of cytological preparation on the left with its interpretation on the right in each pair) of seven *Callitriche* taxa at mitotic metaphase in somatic cells, arranged according to increasing chromosome number: **A**, *C. hermaphroditica* subsp. *macrocarpa*, sample C17-051 (Sweden), 2*n* = 6; **B**, *C. truncata* subsp. *occidentalis*, sample C18-039 (France), 2*n* = 6; **C**, *C. lusitanica*, sample C17-015 (Greece), 2*n* = 8; **D**, *C. muelleri*, sample C15-093 (Australia), 2*n* = 10; **E**, *C. regis-jubae*, sample C16-016 (Spain), 2*n* = 10; **F**, *C. brutia* var. *naftolskyi*, sample C16-097 (Sardinia), 2*n* = 28; **G**, *C. brutia* var. *brutia*, sample C16-098 (Sardinia), 2*n* = 28; **H**, *C. brutia* var. *brutia*, sample C17-012 (Greece), 2*n* = 28. — Scale bar = 10 μm.

N.E.Br. + *C. lechleri* (Hegelm.) Fassett + *C. fehmedianii* Majeed Kak & Javeid or *C. sonderi* Hegelm. + *C. petriei* R.Mason. The Southern Hemisphere taxa *C. terrestris* subsp. *turfosa* (Bertero ex Hegelm.) Bacigalupo, *C. antarctica* Engelm. ex Hegelm. and *C. heteropoda* Engelm. ex Hegelm. end up forming a polytomy in the clade containing the *C. palustris* group (Fig. 4B).

Most of the traditionally recognized *Callitriche* species are well separated and supported in all trees, with several exceptions. The samples of *C. cophocarpa* and *C. platycarpa* share mostly identical ITS ribotypes (Fig. 4B). Plastid sequences of these two species differ only in one site except of two Italian accessions of *C. platycarpa*, which show slight differences (Fig. 4A). Likewise, *C. brutia* var. *brutia* and *C. hamulata* share an identical

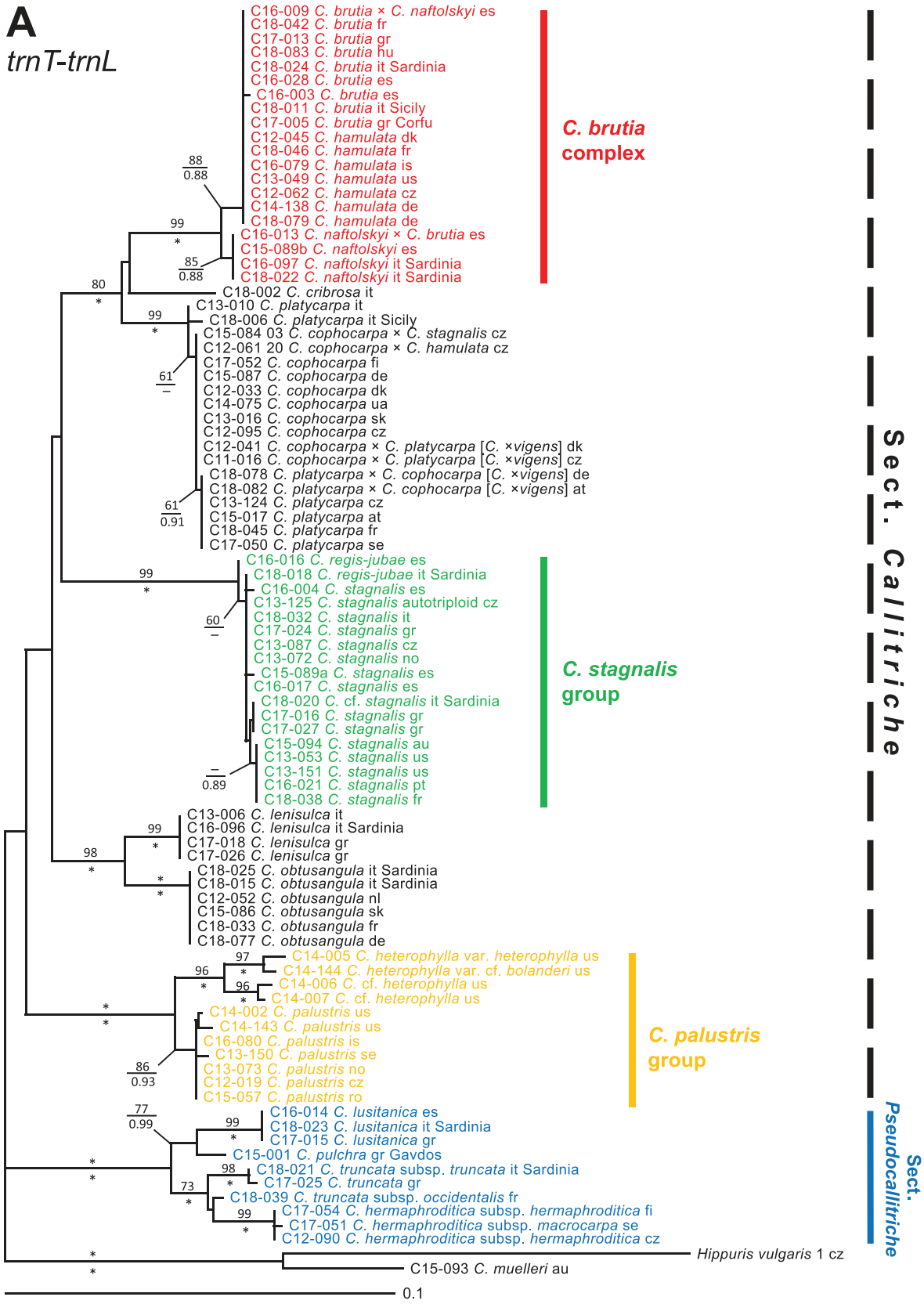

Fig. 4: For full caption, see Fig. 4C.

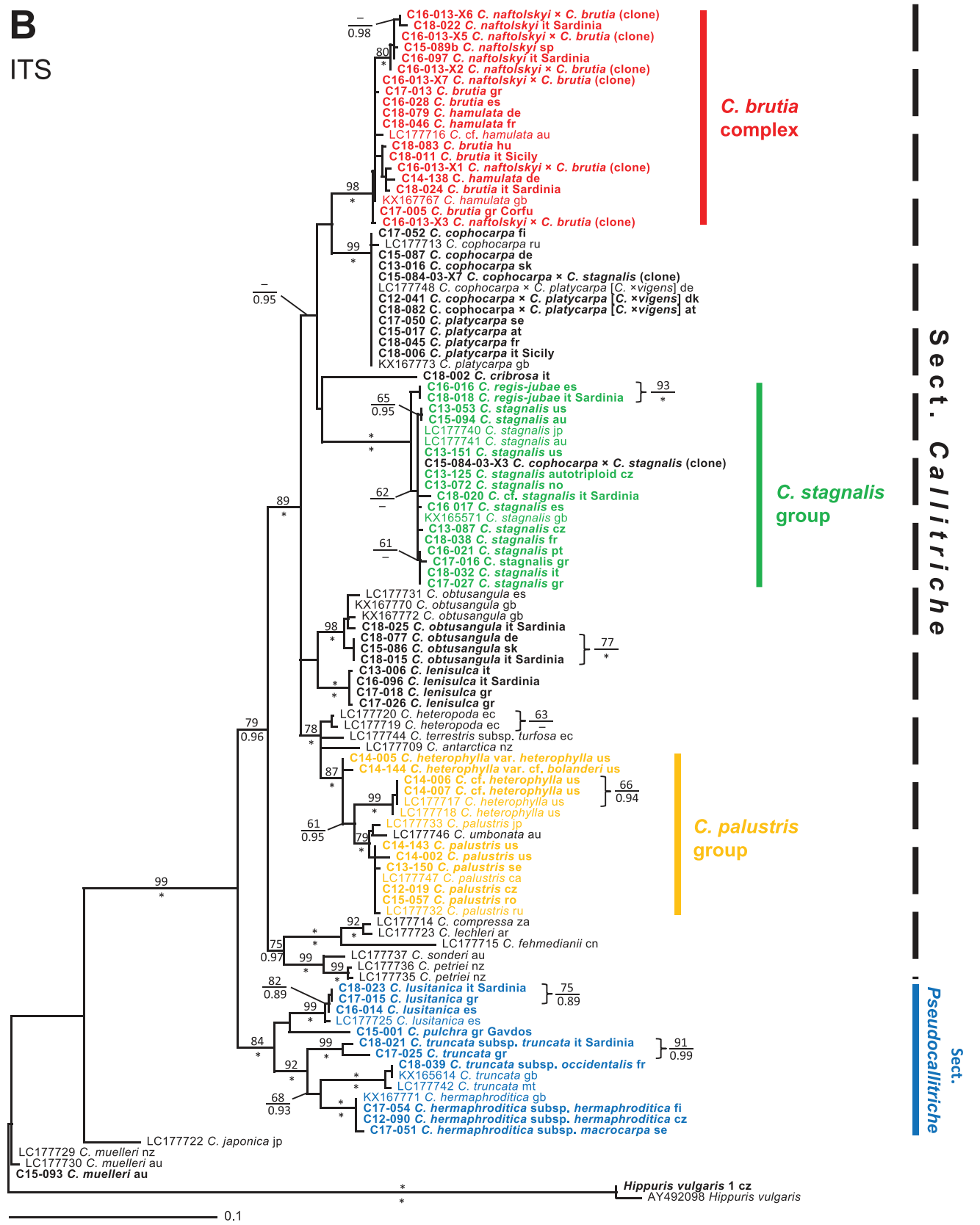

Fig. 4: Continued. For full caption, see Fig. 4C.

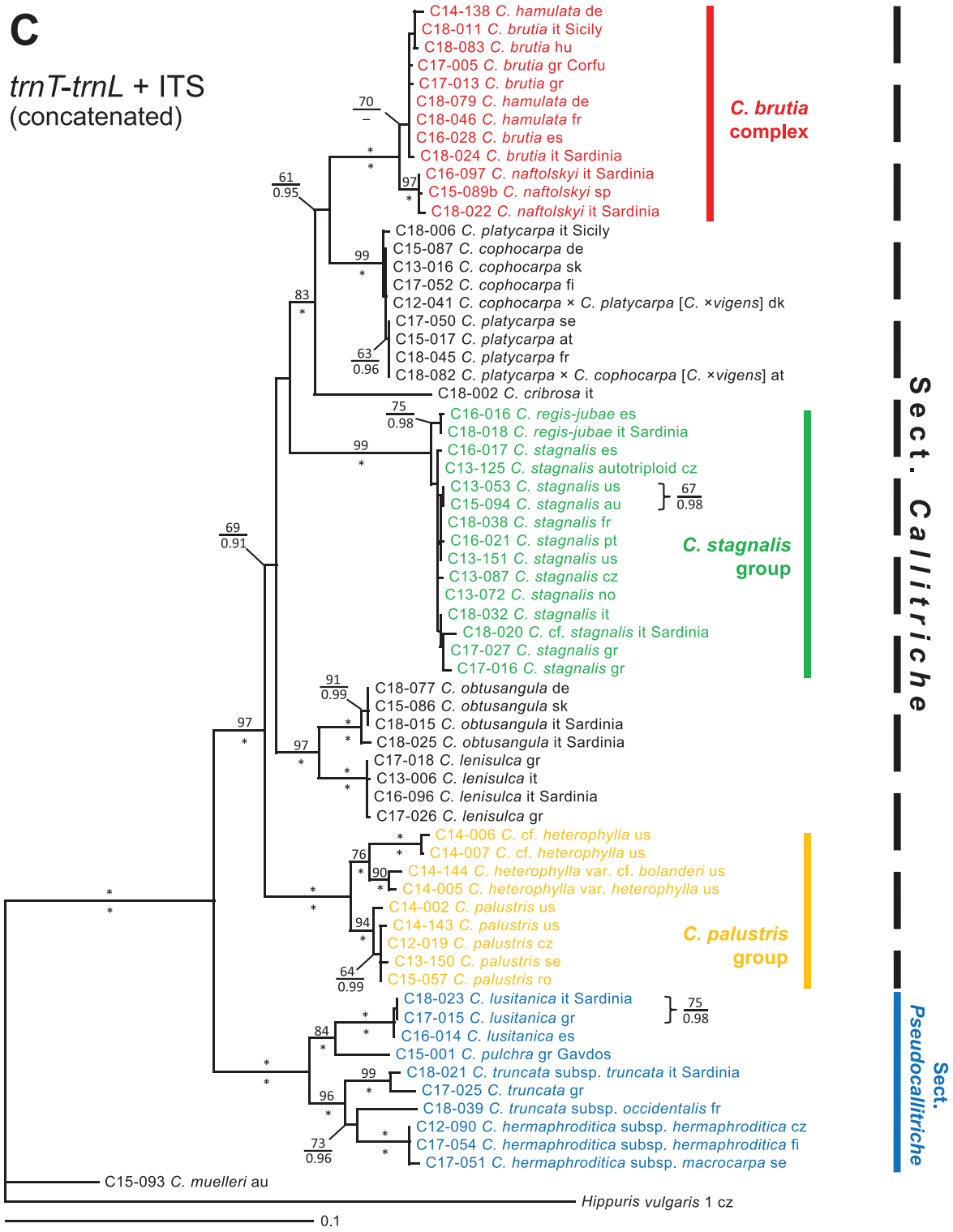

Fig. 4. Maximum likelihood (ML) trees of *Callitriche* species based on *trnT-trnL* (**A**), ITS (**B**) and on the concatenated dataset of ITS and *trnT-trnL* sequences (**C**). Bootstrap support values >60% (* = 100%) are indicated above branches, posterior probabilities >0.85 (* = 1.00) are given below branches. For simplification, *C. brutia* var. *brutia* and *C. b.* var. *naftolskyi* are listed as *C. brutia* and *C. naftolskyi*. In the ITS dataset, samples from this study are in bold, those from Ito & al. (2017) are in normal font. The sample LC177716-1 from Australia was originally listed as *C. brutia* var. *hamulata* by Ito & al., but according to Bean (2007), this taxon does not occur in that country; therefore it is labelled here as *C.* cf. *hamulata*. The sample LC177744 was originally listed as *C. turfosa*, but classified here as *C. terrestris* subsp. *turfosa*, following the recent treatment in *Flora Argentina* (Hassemer & O'Leary, 2018). For *C. cophocarpa* × *C. stagnalis* and *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi*, the ITS tree (B) includes cloned sequences matching those of the respective parents.

haplotype and are also indistinguishable on the basis of ITS sequences (Fig. 4A–C). Finally, both samples of *C. regis-jubae* are significantly supported as sister to *C. stagnalis* with ITS and in the concatenated tree, but the genetic distance between both species is very low, and only one sample of *C. regis-jubae* has also a slightly distinct *trnT-trnL* haplotype.

In general, plastid and ITS trees (Fig. 4A,B) are fairly congruent, resulting in high support of most main branches in the concatenated tree (Fig. 4C).

Intraspecific variation. — The majority of species show very little or no intraspecific genetic variation. On the other hand, molecular analyses confirmed differences between some previously known intraspecific taxa. In the complex of *C. brutia*, *C. brutia* var. *naftolskyi* is clearly distinguished from *C. brutia*/*C. hamulata* (Fig. 4A–C). Genetic differences, although slight, were revealed also between two recognized subspecies of *C. hermaphroditica*.

In *C. truncata*, three distinct genotypes were distinguished in all datasets (Fig. 4A–C), corresponding to the three groups revealed via flow cytometry (see above). Two of them from Sardinia and Greece form well-supported branches in both trees, while the branch including *C. truncata* subsp. *occidentalis* is sister to *C. hermaphroditica*, albeit with low support. Additional ITS sequences from Ito & al. (2017) group with *C. truncata* subsp. *occidentalis* with high support (Fig. 4B). The North American species *C. heterophylla* is another taxon in which surprisingly high genetic variation was revealed, forming two well-supported clusters in the *trnT-trnL* tree as well as in the concatenated tree (Fig. 4A,C). The topology of the ITS tree even suggests that this species is paraphyletic (Fig. 4B).

Hybridization. — While most ITS sequences show occasional polymorphic sites (small additional peaks) that appear to be singlets or are without any particular pattern, sequences of several samples show nucleotide polymorphisms that are additive for particular species pairs indicating hybridization (suppl. Appendix S1). Several species possess no polymorphisms (e.g., the diploid species *C. stagnalis*, *C. hermaphroditica*, *C. lusitanica*) or only sporadically (e.g., diploids *C. cophocarpa*, *C. lenisulca*, tetraploid *C. platycarpa*), whereas other species show numerous polymorphic sites in most sequences (diploid *C. obtusangula*, hexaploid *C. brutia*, octoploid *C. hamulata*). Three out of four putative hybrids (*C. cophocarpa* × *C. stagnalis*, *C. cophocarpa* × *C. hamulata*, *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi*) show clearly additive patterns (Fig. 5A,B). The remaining hybrid, *C.* ×*vigens*, shares an identical ITS sequence with both putative parents, *C. cophocarpa* and *C. platycarpa*, without any visible polymorphisms (Figs. 4B, 5A). Regarding plastid sequences, the hybrids *C. cophocarpa* × *C. stagnalis* and *C. cophocarpa* × *C. hamulata* show the haplotype of *C. cophocarpa* indicating that this species is the maternal parent (Fig. 4A). From 12 samples of *C.* ×*vigens*, 9 possess a haplotype identical with central and western European samples of *C. platycarpa*, whereas 3 samples have the same haplotype as *C. cophocarpa* (see suppl. Appendix S2); thus, this hybrid apparently is a result of reciprocal crosses. Similarly, one sample of *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi* (C16-009) shares the haplotype of *C. brutia* var. *brutia*, whereas the second (C16-013) shows the same haplotype as *C. brutia* var. *naftolskyi* (Fig. 4A).

Cloning of the hybrid *C. cophocarpa* × *C. stagnalis* retrieved ribotypes corresponding to each putative parent, whereas six ribotypes were revealed within *C. brutia* var. *brutia* × *C. brutia* var. *naftolskyi*, three clustering with var. *naftolskyi* in the ITS tree and three with the rest of the clade including *C. brutia* var. *brutia* and *C. hamulata* (Fig. 4B).

Closer inspection of the ITS electropherograms showed some very small additional peaks in sequences of *C. hamulata* that suggested a contribution from *C. cophocarpa*/*C. platycarpa* according to some readable diagnostic single nucleotide polymorphisms (SNPs) and one diagnostic 1 bp-indel leading to a frameshift. These small peaks were readable only in some samples of *C. hamulata* while lacking in *C. brutia*. In most samples of *C. hamulata*, only a part of the expected polymorphic sites was visible, but all predicted hybrid sites were present in at least some samples (suppl. Appendix S1). Additionally, three variable sites were revealed, shared by both *C. brutia* var. *brutia* and *C. hamulata*, in which most samples were hybridogenous. This pattern leads to a complex reticulate structure between *C. cophocarpa* and taxa of the *C. brutia* complex in the NeighborNet diagrams (Fig. 5A,B). The somewhat intermediate positions of the octoploid species *C. hamulata* along with heavily skewed ratios of peaks at polymorphic sites did not recommend a cloning approach; therefore, the *C. brutia* complex was additionally subjected to discriminating restriction digests.

RFLP analysis shows that the putative hybrid *C. cophocarpa* × *C. hamulata* exhibits a clearly additive pattern, combining bands from *C. hamulata* and *C. cophocarpa*/*platycarpa* (Fig. 6). One sample of *C. platycarpa* from Sicily (C18-006) shows a partial loss of the single restriction site, which is also detectable in all accessions of *C. hamulata* and their hybrid. The contribution of *C. cophocarpa*/*platycarpa* to the hybrid is more pronounced than that of *C. hamulata*. All samples of *C. hamulata* show a complex pattern suggesting the same origin of all samples with the strongest contribution from *C. brutia* var. *brutia*, but also additivity of bands with *C. brutia* var. *naftolskyi* and *C. cophocarpa*/*platycarpa* including a partially undigested band as in *C. platycarpa* (C18-006). This octoploid therefore shows an allopolyploid origin with detectable traces of three different taxa. However, *C. brutia* var. *brutia* shares all three bands characteristic for *C. brutia* var. *naftolskyi*, although two of them are weak and not clearly visible. Therefore, the involvement of *C. brutia* var. *naftolskyi* in the emergence of *C. hamulata* is not clear.

■ DISCUSSION

Divergence among and within *Callitriche* taxa. — Despite the general morphological similarity of water-starworts, most European *Callitriche* species are well-defined

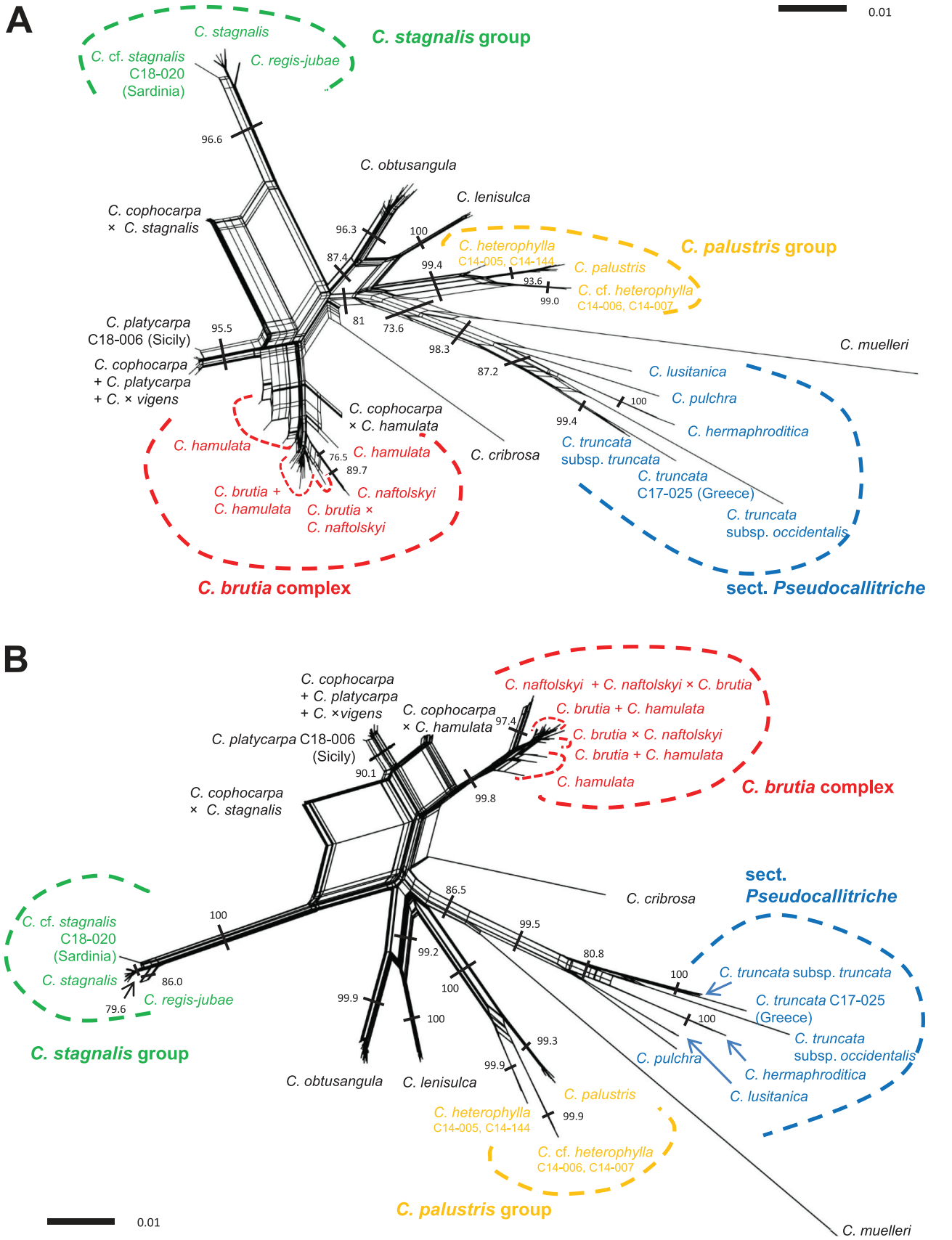

Fig. 5. NeighborNet analysis of *Callitriche* samples based on ITS sequences (**A**) and on the concatenated dataset of ITS and *trnT-trnL* (**B**). Bootstrap support for clusters is indicated next to the respective cluster delimitation; only values >70% are shown for main clusters.

by the combination of genome size, ITS and *trnT-trnL* markers. The only species that are difficult or even impossible to distinguish on the basis of direct sequences are the couples *C. cophocarpa–C. platycarpa* and *C. brutia* var. *brutia–C. hamulata* (see below). Our results also indicate that the western Mediterranean species *C. regis-jubae* is closely related to the broadly distributed *C. stagnalis*. Both species are indistinguishable by genome size, they are clearly separated only with ITS but not with *trnT-trnL* (Figs. 4A–C, 5A,B), and the genetic distance between the sister taxa is small. Based on these findings, *C. regis-jubae* is probably a recently diverged taxon, and one possible solution would be to reclassify it as a subspecies of *C. stagnalis*. At the current state of knowledge, we propose to keep *C. regis-jubae* at the species level because it is morphologically well distinguishable from *C. stagnalis* (see Table 1). Pollination modes also seem to be different for both taxa: in *C. regis-jubae*, pollination is referred to be obligatory geitonogamous, taking place through the direct contact between stigmata and anthers ("contacter"), whereas in *C. stagnalis*, the contact between male and female flowers does not occur ("non-contacter"; Schotsman, 1982). Both species occur sympatrically; therefore, the switch of *C. regis-jubae* to an autogamous (in fact, strictly geitonogamous) strategy could be indicative of reproductive isolation and may be one of the main reasons of their divergence. Nevertheless, further research is necessary to accurately assess the overall variation in the entire *C. stagnalis* group (see also below).

Within *C. truncata*, we revealed a surprisingly large genetic variation. The western European *C. truncata* subsp. *occidentalis* is so divergent (and even paraphyletic) in phylogenetic analyses based on plastid and nuclear markers as well as in genome size that it deserves to be classified at the species level (Fig. 4A–C). Two other samples of *C. truncata* from the study of Ito & al. (2017) also fall within this well-supported clade with little or no variation between accessions. The typical subspecies has been described from Calabria, Italy (Gussone, 1826), and it is reported also from the middle and eastern Mediterranean (Lansdown, 2008). In this study, we included two samples of *C. truncata* from Sardinia, genetically and cytometrically virtually identical (suppl. Appendix S1, S2, suppl. Table S1). One of them (C18-021) is fertile and shows typical characters of *C. truncata* subsp. *truncata*. Additionally, we collected a single sample in south-western Greece (C17-025) that is genetically and cytometrically clearly different from the Sardinian plants (Table 2, Figs. 2, 4A–C). The Greek plants, unlike the typical *C. truncata* subsp. *truncata*, have fruits with very narrow wings that are often not apparent on dried material. This population obviously represents a hitherto unknown cryptic taxon. It is clear that the entire *C. truncata* requires taxonomic revision and very probably also a reassessment of the nomenclature in connection with changes of taxonomic ranks. However, it would not be sensible to make any taxonomic changes until comparative material from a wider area can be investigated.

Two North American species included in our study (*C. heterophylla*, *C. palustris*) also show noticeable intraspecific variation. This is particularly evident in *C. heterophylla*, which clustered in two distinct groups in all datasets (Figs. 4A,C, 5B) and is not monophyletic with ITS (Figs. 4B, 5A). This species deserves a thorough taxonomic revision throughout its distribution area, since it probably contains several cryptic taxa.

In Europe, terrestrial plants commonly have diversity hotspots in the Mediterranean area and in high mountain ranges, especially the Alps (Myers & al., 2000; Väre & al., 2003). In

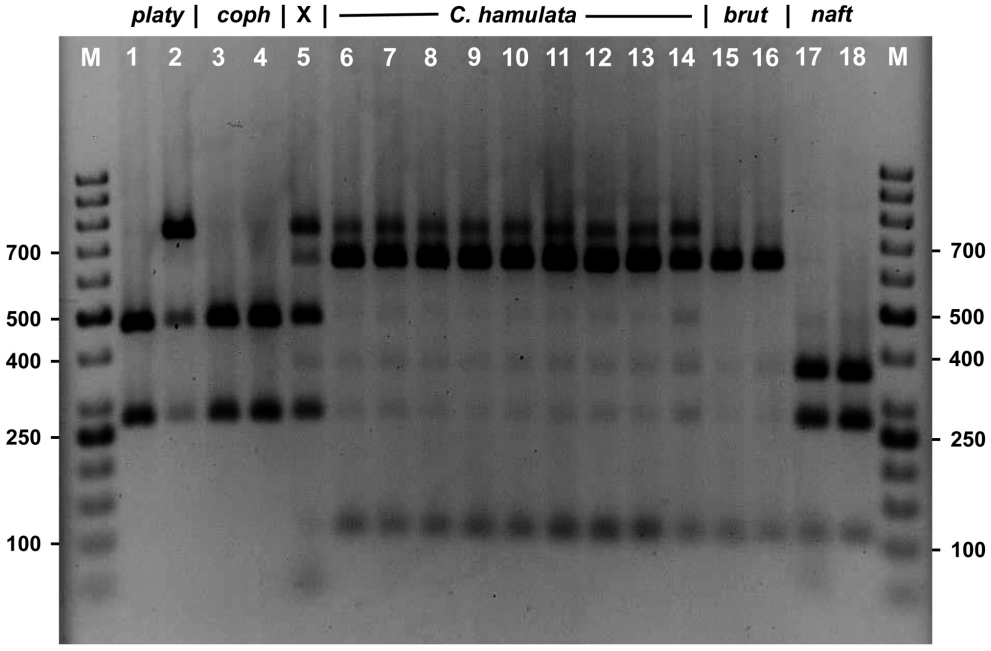

Fig. 6. RFLP analysis of 18 *Callitriche* samples. M = molecular size standard, *platy* = *C. platycarpa*, *coph* = *C. cophocarpa*, X = putative hybrid *C. cophocarpa* × *C. hamulata*, *brut* = *C. brutia* var. *brutia*, *naft* = *C. brutia* var. *naftolskyi*. Samples: 1, C14-139 (Germany); 2, C18-006 (Sicily); 3, C17-052 (Finland); 4, C12-063 (Czech Republic); 5, C12-061-20 (Czech Republic); 6, C12-062 (Czech Republic); 7, C13-077 (Czech Republic); 8, C13-132b (Germany); 9, C14-138 (Germany); 10, C14-077 (Austria); 11, C18-046 (France); 12, C12-045 (Denmark); 13, C16-079 (Iceland); 14, C13-049 (U.S.A.); 15, C15-091a (Spain); 16, C17-022 (Greece); 17, C15-089b (Spain); 18, C18-022 (Sardinia). See Appendix 1 and suppl. Table S1 for locality details.

contrast, there is typically no conspicuous variation among the numbers of aquatic plants reported from different parts of Europe (Chappuis & al., 2012). Despite this general view, our results suggest that the genetic diversity centre of *Callitriche* in Europe is situated in the Mediterranean area. Also, additional cryptic taxa may occur in the Mediterranean: a single sample of *C. stagnalis* from Sardinia (C18-020) is genetically similarly distant from the rest of *C. stagnalis* as *C. regisjubae* (Figs. 4C, 5B). These plants were collected young and without ripe fruits, yet it is apparent that at least some fruits are pedunculate, unlike all other samples of *C. stagnalis*. However, it is not appropriate to draw any conclusions on the basis of a single sample.

Our study shows a good agreement with that from Ito & al. (2017) because all species included in both studies clustered together (Fig. 4B) without any exceptions. The phylogenetic positions of other species from the study of Ito & al. (2017) that were not covered by our sampling, are difficult to assess. All ITS sequences from that study do not contain any additive polymorphisms, contrary to our data including numerous polymorphic sites. The phylogenetic positions of some species are rather surprising, namely the very close relationship between South African *C. compressa* and South American *C. lechleri*, as well as between Australian *C. umbonata* and the sample of *C. palustris* from Japan (Fig. 4B). Their relative genetic divergences, when compared with that of the remaining taxa, correspond rather to the subspecies than the species level.

In accordance with Ito & al. (2017), we propose to distinguish only one particular clade as sect. *Pseudocallitriche* and the main clade as a broadly defined sect. *Callitriche* (Figs. 4A–C, 5A,B). On the other hand, we leave the basally branching clades, including *C. muelleri*, *C. japonica* and a branch containing *C. compressa*, *C. lechleri*, *C. fehmedianii*, *C. petriei* and *C. sonderi*, without a formal assignment to taxonomic units. More species (especially from America, Africa and Asia) will need to be included to better resolve the classification of ancestral *Callitriche* species.

Polyploid origin of *Callitriche* species. — Four polyploid species are recognized in Europe (Tables 1, 2). From these, the evolutionary origin has been studied only in tetraploid ($2n = 20$) *C. platycarpa*. According to Philbrick & Les (2000), *C. platycarpa* shares an identical *rbcL* haplotype with *C. stagnalis*, contrary to the results of Ito & al. (2017), who suggested that *C. cophocarpa* is the maternal parent of *C. platycarpa*. Bączkiewicz & al.'s (2007) isozyme study on plant materials from north-western Poland and Schwarzacher & al.'s (2017) genomic in situ hybridization (GISH) on plant material from England consistently concluded that *C. platycarpa* is an allotetraploid formed by the diploid parental species *C. cophocarpa* and *C. stagnalis*. According to the Polish study, *C. stagnalis* is a maternal parent of *C. platycarpa*. Contrary to that study, we revealed that the plastid haplotype of all included samples of *C. platycarpa* is very similar (although not entirely identical) to that of *C. cophocarpa* (Fig. 4A). The ITS sequences of *C. platycarpa* are identical with those of *C. cophocarpa*, without any visible polymorphisms (Figs. 4B, 5A, suppl. Appendix S1). The only exception is a single sample from Sicily (C18-006), showing three additional polymorphisms corresponding to SNPs characteristic for both *C. cophocarpa* and *C. stagnalis*, but no visible polymorphisms on additional ca. 37 positions distinguishing these two species from each other. Two possible evolutionary scenarios can be inferred: (a) all samples of *C. platycarpa* included in our study are autotetraploids derived from *C. cophocarpa*, and the discrepancy to previous studies may be due to different material or different methods of inference; (b) at least some (if not all) samples are allotetraploids, but the contribution of *C. stagnalis* is not visible in electropherograms due to the process of concerted evolution in the ITS sequences (Arnheim, 1983; Elder & Turner, 1995). The latter scenario is also supported by flow cytometric results because the monoploid genome size (1Cx-value) of *C. platycarpa* is exactly intermediate between the values determined for *C. cophocarpa* and *C. stagnalis* (Table 2). However, we cannot rule out that some lineages of *C. platycarpa* can have different origins or arose recurrently from independent hybridization events, as is documented in many polyploid plant species (e.g., Soltis & Soltis, 1999). This may explain why plastid DNA of all 13 accessions of *C. platycarpa* included in our study corresponds to that of *C. cophocarpa* but none to *C. stagnalis*, in contrast to the findings of Philbrick & Les (2000) and Bączkiewicz & al. (2007). In the latter study, non-fertile plant material of three species (*C. cophocarpa*, *C. platycarpa*, *C. stagnalis*) was identified using chromosome counting (however, both *C. cophocarpa* and *C. stagnalis* have $2n = 10$) and sequencing of the *rbcL* plastid gene; the Polish sequences were subsequently compared with *rbcL* data published by the former study and the corresponding samples identified to fit the sequences. Therefore, it is worth noting that the correctness of the results of Bączkiewicz & al. (2007) is entirely dependent on the species identifications made by Philbrick & Les (2000).

The complex of *C. brutia* is taxonomically the most challenging polyploid complex among European *Callitriche*. Here we found that the hitherto poorly known Mediterranean taxon *C. brutia* var. *naftolskyi* is hexaploid ($2n = 28$) like *C. brutia* var. *brutia* (Fig. 3), and that it significantly differs from both *C. brutia* var. *brutia* and *C. hamulata* in genome size as well as in ITS and plastid DNA molecular analyses (Table 2, Fig. 4A–C). Recently, it was published that *C. b.* var. *brutia* and *C. b.* var. *naftolskyi* possess the same genome size (Prančl in Lansdown & al., 2017), but this information was reported by mistake, caused by confusion of seeds of both taxa, from which the genome size was established. The extent of the genetic divergence between *C. b.* var. *naftolskyi* and the rest of the group suggests it would be more appropriate to classify this taxon at a higher taxonomic rank. It is also worth mentioning that two hybrid samples between *C. b.* var. *naftolskyi* and *C. b.* var. *brutia* revealed by this study show significantly reduced fertility (see below). However, both taxa are morphologically very similar. Although *C. b.* var. *naftolskyi* differs from *C. b.* var. *brutia* in a number of features (Table 1), these are rather insignificant compared to the characters separating

most species within the genus (Lansdown & al., 2017). For the above-mentioned reasons, we recommend to classify both taxa at the subspecies level and designate the name *C. brutia* subsp. *naftolskyi* here at a new rank (see below). The remaining taxon of the aggregate, *C. hamulata*, differs from *C. brutia* by its octoploid chromosome number ($2n = 38$). We revealed that *C. brutia* subsp. *brutia* and *C. hamulata* share an identical plastid haplotype (Fig. 4A) and are also indistinguishable on the basis of ITS ribotypes (Fig. 4B). However, ITS sequences of some samples of *C. hamulata* show the weak admixture of another ribotype from *C. cophocarpa*/*C. platycarpa* (Fig. 5A,B). RFLP results indicate that the restriction pattern of *C. hamulata* can be that of a triple hybrid, showing bands of *C. brutia* subsp. *brutia*, *C. brutia* subsp. *naftolskyi* and *C. platycarpa* in all samples even though this contribution was hardly or not at all detectable in ITS sequences (Fig. 6). Because all accessions of *C. hamulata* share identical plastid DNA and also show very low variation in ITS and no variation in RFLP, it is likely that this species arose from a single polyploidization event. However, the exact evolutionary origin of particular taxa within the *C. brutia* complex remains a question for further research. With certainty, *C. brutia* subsp. *brutia* ($2n = 28$) is the maternal parent of *C. hamulata*. Both *C. cophocarpa* and *C. platycarpa* ($2n = 10$ or 20, respectively) can represent the second parental species, as the ITS sequences of both species are identical (Fig. 4B). On the other side, included samples of *C. cophocarpa* do not show a partial loss of the restriction site, which is visible in the RFLP pattern of *C. hamulata* and a single sample of *C. platycarpa* (C18-006; Fig. 6). The contribution of *C. brutia* subsp. *naftolskyi* ($2n = 28$), although suggested by the results of RFLP analysis, is not unequivocal. We should not forget that *C. brutia* is also a putative allopolyploid. The presence of weak bands corresponding to *C. brutia* subsp. *naftolskyi* in the banding patterns of *C. b.* subsp. *brutia* and *C. hamulata* indicate that *C. b.* subsp. *brutia* may contain a genetic contribution of *C. b.* subsp. *naftolskyi*. The partly missing/erased polymorphisms seen in ITS direct sequences of *C. hamulata* and the relatively weak bands corresponding to *C. platycarpa* and *C. brutia* subsp. *naftolskyi* in the RFLP analysis suggest that concerted evolution is indeed ongoing in *Callitriche* allopolyploids. In this case the homogenization went into the direction of *C. brutia* subsp. *brutia*. It should be further noted that while the pollen grains of *C. brutia* completely lack the exine (an adaptation for hypohydrogamy, see above), the exine is developed in *C. hamulata*, albeit strongly reduced (Cooper & al., 2000). This pattern also suggests that *C. hamulata* could be a hybridogenous species between *C. brutia* and some other species with normally developed exine (e.g., *C. platycarpa* and *C. cophocarpa*).

Lansdown (2006a) concluded on the basis of a detailed morphological study, that *C. brutia* (subsp. *brutia*) and *C. hamulata* are reliably distinguishable in the field only in the terrestrial state. Under such environmental conditions, *C. brutia* produces long-pedunculated fruits whereas the fruits of *C. hamulata* remain subsessile; when growing in water, both taxa are virtually indistinguishable. On the basis of their strong morphological similarity, he re-evaluated *C. hamulata* as a variety of *C. brutia*. In accordance with Lansdown, we did not observe any other reliable characters for distinguishing both taxa. However, we assume that the rank of variety is not appropriate for distinct allopolyploid taxa with different chromosome numbers. Allopolyploids with different evolutionary origins are usually classified at the species level, even if they share one or more parental species (e.g., Soltis & al., 2004; Kelly & al., 2013; Zou & al., 2015; Barker & al., 2016); in some cases, even the products of independent hybridization with an identical parental combination are being evaluated as separate species (mostly in apomictic genera, but also in allogamous species, e.g., Efimov & al., 2016). We also point out that *C. hamulata* and *C. brutia* subsp. *brutia*, if growing terrestrially, are easily recognizable. Both taxa occur sympatrically in western Europe (Schotsman, 1967; Lansdown; 2006a), but *C. hamulata* appears to be very rare or absent from the Mediterranean area, whereas *C. brutia* subsp. *brutia* is almost completely absent from central Europe (Kaplan & al., 2018a). With the current state of knowledge, we prefer the classification of *C. hamulata* as a separate species. However, a further in-depth study of the evolutionary relationships within the *C. brutia* complex may consider whether the species or the subspecies rank would be more appropriate.

Inter- and intraspecific hybridization. — Interspecific hybridization has so far been considered a rare phenomenon in *Callitriche*. This is mainly explained by the extraordinary differentiation of pollination systems across the genus, including various modes of (obligatory) geitonogamous pollination (Schotsman, 1982; Philbrick & Anderson, 1992; Martinsson, 1996). However, the only recognized and described hybrid, triploid *C. ×vigens* (*C. cophocarpa* × *C. platycarpa*), has been reported as relatively abundant in several areas of Europe (Martinsson, 1991; Kaplan & al., 2018a). Triploid plants are easily detectable using genome size (Table 2, Fig. 2), but their identification based on molecular sequences can be more tricky. Both putative parental species share an identical ITS ribotype, but differ slightly in *trnT-trnL* sequences (Fig. 4A, B). Most of the triploid samples included in our study have a haplotype identical to tetraploid *C. platycarpa*, which suggests these plants really belong to *C. ×vigens*. Three triploid samples (C11-016, C12-041, C13-108) share a haplotype identical to *C. cophocarpa* (suppl. Appendix S2). They probably represent the same hybrid combination, but we cannot exclude that at least some of these samples may actually be autotriploids of *C. cophocarpa*. Another triploid with different origin was recently found at a single locality in the Czech Republic (Prančl & al., 2014). In molecular analyses, this plant (C13-125) shows a sequence pattern identical to *C. stagnalis* in all trees (Fig. 4A–C), which confirms the original assumption that it is an autotriploid of *C. stagnalis*.

Three previously unknown hybrids were revealed based on additive patterns of ITS ribotypes (Fig. 5A). Two of these hybrids, *C. cophocarpa* × *C. stagnalis* and *C. brutia* subsp. *brutia* × *C. brutia* subsp. *naftolskyi*, are newly described below as *C. ×nyrensis* nothosp. nov. and *C. brutia* nothosubsp. *neglecta* nothosubsp. nov. (Fig. 7). The remaining hybrid ($2n = 29$),

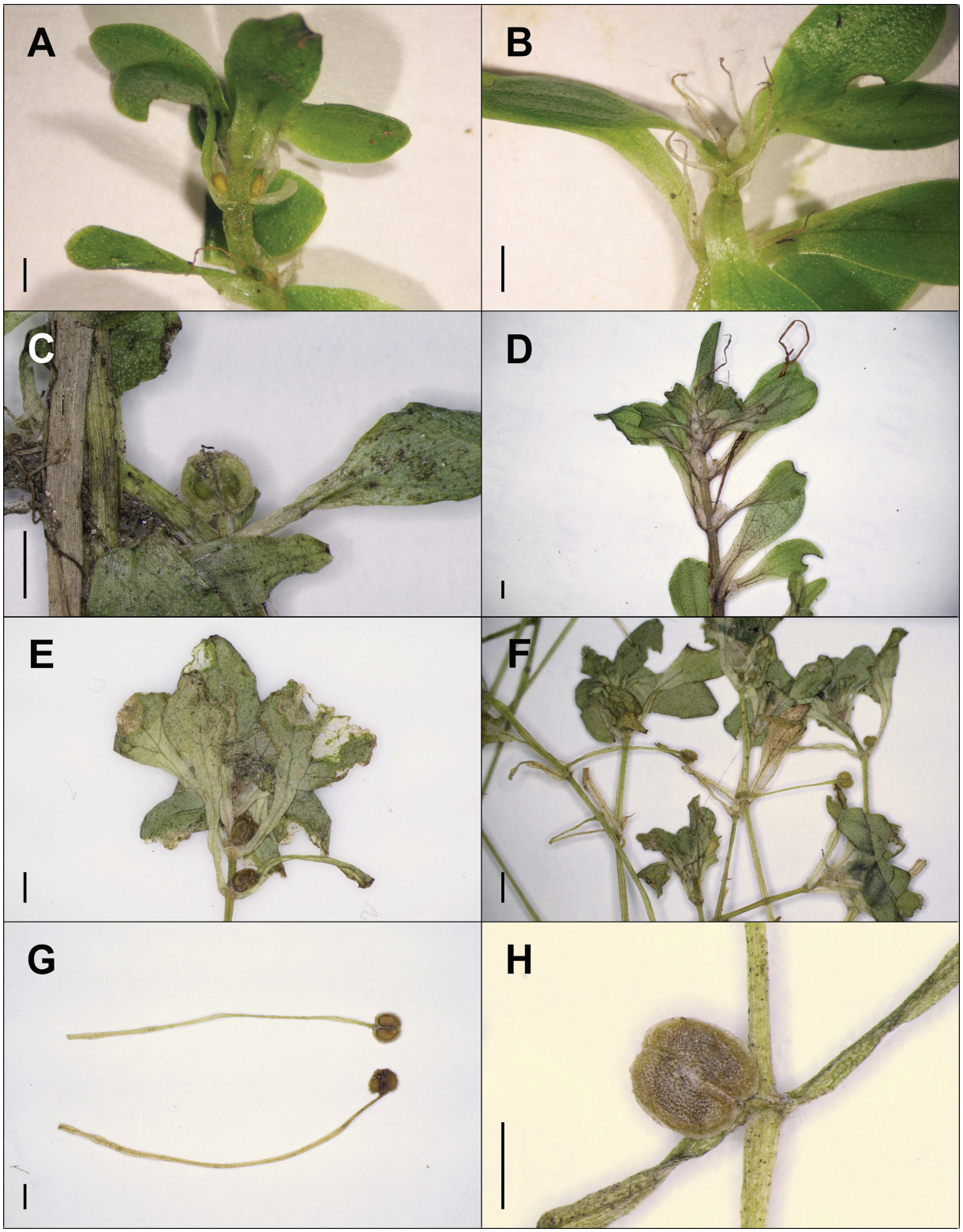

Fig. 7. Diagnostic features of two newly described hybrids. **A–D**, *Callitriche* ×*nyrensis* (*C. cophocarpa* × *C. stagnalis*): **A**, Leaf rosette with two reduced stamens in a single node, almost completely lacking filaments, surrounded by translucent bracts; **B**, Detail of female flowers, composed of two styles and a 4-locular (but bicarpellate) ovary; **C**, Fruit in the initial stage of development (ripe fruits never develop in this hybrid); **D**, Stem with female flowers and a single stamen (on the right in a leaf rosette). **E–H**, *Callitriche brutia* nothosubsp. *neglecta* (*C. brutia* subsp. *brutia* × *C. brutia* subsp. *naftolskyi*): **E**, Leaf rosette composed of leaves with characteristic sinuous venation; **F**, Stems with peduncles bearing under-developed fruits; **G**, Typical appearance of under-developed pedunculate fruits; **H**, Subsessile fruit lacking rests of styles. — Scale bar for all figures = 1 mm.

discovered in the Tichá Orlice river, Czech Republic, has been attributed to *C. hamulata* × *C. cophocarpa*, but *C. platycarpa* could not be excluded as a putative parental species (Prančl & al., 2014). Our study confirmed that ITS sequences of this hybrid represent a mixture of ribotypes of *C. hamulata*/*C. brutia* subsp. *brutia* and *C. cophocarpa*/*C. platycarpa*, and the haplotype of the hybrid is identical to *C. cophocarpa*, but differing from *C. platycarpa* in only a single nucleotide. Only *C. hamulata* and *C. cophocarpa* were found growing together with the hybrid in the river; therefore, these species are indeed the most probable parents. Nevertheless, we consider it more appropriate to postpone the description of this hybrid until the identity of the parents can be confirmed unequivocally.

Besides interspecific hybrids, also "pure" species often possess additive polymorphisms in ITS sequences, indicating intraspecific hybridization among particular, slightly different ribotypes. These polymorphisms were most often recorded in *C. brutia* subsp. *brutia*, *C. brutia* subsp. *naftolskyi*, *C. obtusangula* and *C. palustris*. It is interesting to compare the ITS variation within two widespread diploid species, *C. stagnalis* and *C. obtusangula*. Both species show significant intraspecific variation (Figs. 4B, 5A), but while most samples of *C. obtusangula* contain multiple polymorphic sites, no polymorphisms were found in *C. stagnalis* (suppl. Appendix S1). This may suggest that gene flow is efficiently ongoing among particular genotypes of *C. obtusangula*, whereas intraspecific recombination is rare or not occurring among individual variants of *C. stagnalis*.

New distribution information. — Our study contributes to the better understanding of the distribution of some taxa in Europe. During our fieldwork, we found *C. obtusangula* for the first time in Slovakia (C15-086). The discovered locality in the Danubian Lowland is linked to the previously known occurrence in the Lower Austrian Danube basin (Englmaier, 1985). We confirmed *C. brutia* subsp. *brutia* for Hungary (C18-083), which is probably the first unequivocally confirmed occurrence in the Pannonian Basin. The other intraspecific taxon of *C. brutia*, subsp. *naftolskyi*, was for the first time found in Spain (C15-089b). We also recorded *C.* ×*vigens* for the first time in Austria (C18-082) and *C. lusitanica* in continental Greece (C17-015). *Callitriche platycarpa* is a species with a distinctive European sub-Atlantic distribution, but very rarely occurring in the Mediterranean (Lansdown, 2006a, 2008; Lansdown & Strid, 2011; Prančl & al., 2014). We confirmed this species for the first time in Sicily (C18-006). *Callitriche lenisulca* has been referred to as a lowland species with a maximum elevation of 170 m and with all confirmed records from within 50 km of the sea (Lansdown, 2008). We found this species growing in Greece up to 78 km from the sea coast (C17-018) and at elevations of up to 650 m (C17-019). Finally, we managed to find the first recent occurrence of *C. cribrosa* in Italy (C18-002), where it has been probably last recorded in 1907 (Schotsman, 1977), and of *C. regis-jubae* for Sardinia (C18-018), where it has been recorded only once, in 1972 (Schotsman, 1973).

■ TAXONOMIC TREATMENT

Callitriche brutia subsp. ***naftolskyi*** (Warb. & Eig) Prančl, **stat. nov.** ≡ *Callitriche naftolskyi* Warb. & Eig in Repert. Spec. Nov. Regni Veg. 26: 84. 1929 ≡ *Callitriche brutia* var. *naftolskyi* (Warb. & Eig) Lansdown in Phytotaxa 313: 92. 2017 – Lectotype (designated by Lansdown & al. in Phytotaxa 313: 92. 2017): Israel, Sharon Plain, north-east of Tel Aviv, 23 Apr 1927, *Naftolsky 01853* (HUJ).

Note. – Morphological description and other details were provided by Lansdown & al. (2017).

Descriptions of new *Callitriche* hybrids

Callitriche* ×*nyrensis Prančl, **nothosp. nov.** [*C. cophocarpa* Sendtn. × *C. stagnalis* Scop.] – Holotype: Czech Republic; distr. Klatovy; Hamry: Úhlavský luh Nature Reserve, marsh with small pools on left bank of Úhlava river above bridge near settlement Hamerský Dvůr, 920 m N–NNW of church, alt. 529 m, 49°14′15.1″N, 13°09′27.0″E (WGS 84), 26 Jun 2016, *J. Prančl C16-051* (PRC barcode PRC 455760; isotypes: PR barcode PR 964819, PRA barcode PRA-00016236, PRC barcode PRC 455761).

Description. – Perennial amphibious herbs, producing floating rosettes when reaching the water surface, or semi-terrestrial. Stem much-branched, supported by water or prostrate and creeping when terrestrial, with scales of (6–)7–9 cells. Leaves narrowly oblanceolate to broadly spathulate, less often almost linear, 1–5-veined, up to 25 mm long, 1.1–5.2 mm wide, 2.5–11× longer than wide, narrower leaves shallowly notched at the apex, broader leaves obtuse. Bracts falcate, translucent, appearing whitish, 0.6–1.4 mm long, persistent. Flowers solitary in leaf axils, generally a pair of male flowers or a pair of female flowers in a pair of axils, often flowers of one sex are placed on separate stems or on different parts of the same stem. Styles usually erect, up to 5.6 mm long. Stamens with filaments strongly reduced before dehiscence, appearing sessile, usually completely covered by bracts, sometimes lengthening after anthesis, up to 4.2 mm long, anthers 0.3–0.6 mm wide; pollen bright yellow to sulphur-yellow, generally aborted, of irregular shape. Fruits not developed (plants sterile). Chromosome number probably diploid, $2n = 10$ (DNA ploidy level = $2x$).

Etymology. – The epithet *nyrensis* is derived from Nyra, the old name considered a Latin variant of Nýrsko, the town near which the hybrid was found.

Key characters. – The hybrid is intermediate between the parents, forming relatively broad leaves like *C. stagnalis*, but it is also capable of creating forms with narrow lingulate leaves like *C. cophocarpa*. The flower pattern of the hybrid resembles *C. cophocarpa*, generally having flowers of one sex placed on separate stems or on different parts of the same stem, but this pattern is not as regular as in *C. cophocarpa*. Also *C. platycarpa* is very similar; this species, however, does not occur in this part of the Czech Republic (Kaplan & al., 2018a). The hybrid can be

separated from all three species by malformed pollen and the peculiar appearance of undehisced stamens, which are mostly reduced to a small anther situated directly in the leaf axil, almost completely lacking filament (Fig. 7A). The hybrid also does not set fruits though it flowers abundantly. Nevertheless, the other hybrid, *C.* ×*vigens* (*C. cophocarpa* × *C. platycarpa*), possesses virtually the same floral characteristics like *C.* ×*nyrensis* (cf. Martinsson, 1991; Lansdown, 2008) and can only be reliably distinguished from it by the triploid ($2n = 15$) chromosome number. If *C. platycarpa* is allotetraploid with the diploid parental species *C. cophocarpa* and *C. stagnalis* (see above), *C.* ×*vigens* would have two chromosome sets corresponding to *C. cophocarpa* and one set of chromosomes corresponding to *C. stagnalis*. Therefore, the genetic composition of both hybrids may be similar.

Distribution. – *Callitriche* ×*nyrensis* is only known from a single locality in the Czech Republic. At this site it occurs together with *C. stagnalis* (C15-084-01), which is, however, much rarer there. The second parent, *C. cophocarpa*, was not found at the locality. *Callitriche* ×*nyrensis* is probably a rare hybrid. Both parental species have partly different ecological demands: whereas *C. cophocarpa* prefers permanent waters, *C. stagnalis* is typical for temporary habitats with shallow water (Kaplan & al., 2018a). In our previous cytometric paper (Prančl & al., 2014), we analyzed 150 populations of *C. cophocarpa* and 104 populations of *C. stagnalis* from central Europe, but only 8 of these populations hosted both species. Both species also frequently remained unflowering, especially in deeper or running water or in shaded habitats.

Additional specimens examined (paratypes). – Czech Republic; distr. Klatovy; Hamry: Úhlavský luh Nature Reserve, marsh with small pools on the left bank of Úhlava river above the bridge near the settlement Hamerský Dvůr, 920 m N–NNW of the church, alt. 529 m, 49°14′15.1″N, 13°09′27.0″E (WGS 84), 12 Sep 2015, *J. Prančl C15-068* (PRC barcode PRC 455762), 31 Oct 2015, *J. Prančl C15-084* (PRC barcodes PRC 455763–455766, PR barcodes PR 964820–964824, PRA barcodes PRA-00016237–16241). All paratypes were sampled non-flowering.

Callitriche brutia nothosubsp. ***neglecta*** Prančl, **nothosubsp. nov.** [*C. brutia* Petagna subsp. *brutia* × *C. brutia* subsp. *naftolskyi* (Warb. & Eig) Prančl] – Holotype: Spain; comm. Extremadura; prov. Cáceres; Jaraicejo: Almonte river below bridge of N-V road (Carretera de Extremadura), 1.7 km SSW of village, alt. 349 m, 39°38′46.7″N, 05°49′04.6″W (WGS 84), 3 May 2016, *J. Prančl, Z. Kaplan & P. Koutecký C16-013* (PRC barcode PRC 455758).

Description. – Amphibious herbs, producing floating rosettes when reaching the water surface, or semi-terrestrial. Stem much-branched, with scales of 8–16 cells, often irregular in outline. Leaves narrowly linear to broadly spathulate, 1–3-veined, often with sinuous venation, up to 10 mm long, 0.3–2.6 mm wide, 1.5–25× longer than wide, broader leaves usually very shallowly notched at the apex. Bracts apparently absent. Flowers solitary in leaf axils, generally a male flower opposed by a female. Styles up to 0.5 mm long, initially ± erect but soon becoming strongly reflexed, most styles very short. Stamens with filaments up to 0.4 mm long, anthers ca. 0.3 mm wide, appearing whitish. Peduncles 0−16(−30) mm long; fruits mostly undeveloped or underdeveloped, most often pedunculate, less often subsessile, well-developed fruits rare, 0.8–1.1 mm long × 0.8–1.1 mm wide, dark brown when mature, narrowly winged throughout, wing 0.02–0.07 mm wide, rests of styles not visible or apressed to side of fruit. Chromosome number probably $2n = 28$ (based on flow cytometric genome size analyses).

Etymology. – The epithet *neglecta* means "neglected", reflecting the fact that the true identity of this hybrid was not recognized in the field, but revealed on the basis of molecular analyses.

Key characters. – This hybrid differs from the parental subspecies in having most fruits undeveloped or small, not filled by well-developed seeds (Fig. 7G). One of the parents, *C. brutia* subsp. *naftolskyi*, always has pedunculate fruits, whereas *C. brutia* subsp. *brutia* forms subsessile fruits when growing in water, but pedunculate fruits when terrestrialised (Lansdown & al., 2017). The hybrid has most often long pedunculate fruits, but also sessile fruits are present on the same individuals. The fertility of the hybrid is not known, but at least some mericarps (although rare) seem to appear normally with fully developed seeds. Thus, it cannot be ruled out that the hybrid could be capable of breeding F2 offsprings or even backcrossing with the parents.

Distribution. – *Callitriche brutia* nothosubsp. *neglecta* is known only from two localities in Spain, both hosting rich aquatic vegetation (*Ranunculus peltatus* s.l., *Callitriche lusitanica*, *C. stagnalis* and many other species). The question is how often this hybrid can arise because all taxa of the *C. brutia* complex are believed to be strongly geitonogamous, and the pollen transfer is usually mediated through the direct contact of anther and stigma in adjacent leaf axils ("contacter", Schotsman, 1982). Both localities of the hybrid are situated in streams. While *C. brutia* subsp. *brutia* can grow in rivers and brooks (see the list of localities in suppl. Table S1), *C. brutia* subsp. *naftolskyi* typically grows in vernal pools and has never been found in running water (Lansdown & al., 2017). On the other hand, rivers and streams often provide shelter for rare and sterile hybrids that can spread and persist here even for thousands of years through vegetative propagation (e.g., King & al., 2001; Kaplan & Fehrer, 2009, 2011; Kaplan & al., 2018b; Prančl & al., 2018).

Additional specimen examined (paratype). – Spain; comm. Extremadura; prov. Badajoz; Herrera del Duque: Arroyo Pelochejo stream (tributary of Guadiana river), 650 m NNE of town, alt. 420 m, 39°10′40.2″N, 05°02′45.9″W (WGS 84), 2 May 2016, *J. Prančl*, *Z. Kaplan & P. Koutecký C16-009* (PRC barcode PRC 455759).

■ AUTHOR CONTRIBUTIONS

JP, ZK and JF made the design of the research; JP and ZK collected the samples; JP made cytometric analyses; VB, PC and JP produced molecular data and alignments; ML performed chromosome counting; JF and JP analyzed the molecular data; JP, ML and ZK prepared figures; JP wrote the manuscript, JF and ZK helped with preparing the manuscript. — JP, https://orcid.org/0000-0003-4308-0824; JF, https://orcid.org/0000-0002-0337-5444; ML, https://orcid.org/0000-0002-4612-3693; ZK, https://orcid.org/0000-0003-1707-7461

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Appendix 1. Voucher information and GenBank accession numbers (ITS, *trnT-trnL*). Taxa are listed in alphabetical order and further ordered by country and collection number. All sequences are published here for the first time. All voucher specimens are preserved in PRC. For more detailed locality information, see suppl. Table S1.

Taxon name + taxonomic authority, country, major political subdivision (if applicable), collector(s), collection number, ITS, *trnT-trnL*.

Callitriche brutia Petagna subsp. ***brutia*****:** France, reg. Pays de la Loire, *Prančl C18-041*, MN091383, MN091981; France, reg. Pays de la Loire, *Prančl C18-042*, MN091382, MN091980; Greece, *Prančl*, *Kaplan & Koutecký C17-012*, MN091389, MN091987; Greece, *Prančl*, *Kaplan & Koutecký C17-013*, MN091388, MN091986; Greece, *Prančl*, *Kaplan & Koutecký C17-017*, MN091387, MN091985; Greece, *Prančl*, *Kaplan & Koutecký C17-022*, MN091386, MN091984; 2, Greece, *Prančl*, *Kaplan & Koutecký C17-028*, MN091384, MN09198; Greece, Kerkyra (Corfu) island, *Gilli*, *Hofbauer*, *Reich & Sander C17-005*, MN091385, MN091983; Hungary, *Kaplan & Mesterházy C18-083*, MN091390, MN091988; Italy, reg. Sardegna (Sardinia), *Lansdown C16-098*, MN091395, MN091993; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Koutecký C18-024*, MN091392, MN091990; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Koutecký C18-026*, MN091391, MN091989; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Koutecký C18-011*, MN091393, MN091991; Italy, reg. Umbria, *Kaplan*, *Hanzlíčková & Koutecký C18-028*, MN091394, MN091992; Spain, prov. Badajoz, *Prančl*, *Kaplan & Koutecký C16-024*, MN091400, MN091998; Spain, prov. Cáceres, *Koutecký C15-091*, MN091403, MN092001; Spain, prov. Cáceres, *Prančl*, *Kaplan & Koutecký C16-005*, MN091401, MN091999; Spain, prov. Cáceres, *Prančl*, *Kaplan & Koutecký C16-006*, MN091402, MN092000; Spain, prov. Jaén, *Prančl*, *Kaplan & Koutecký C16-025*, MN091396, MN091994; Spain, prov. Jaén, *Prančl*, *Kaplan & Koutecký C16-026*, MN091397, MN091995; Spain, prov. Madrid, *Prančl*, *Kaplan & Koutecký C16-028*, MN091404, MN092002; Spain, prov. Salamanca, *Prančl*, *Kaplan & Koutecký C16-018*, MN091398, MN091996; Spain, prov. Toledo, *Prančl*, *Kaplan & Koutecký C16-003*, MN091399, MN091997. ***Callitriche brutia*** subsp. ***naftolskyi*** (Warb. & Eig) Prančl: Italy, reg. Sardegna (Sardinia), *Lansdown C16-097*, MN091405, MN092003; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Koutecký C18-016*, MN091407, MN092005; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Koutecký C18-022*, MN091406, MN092004; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Koutecký C18-009*, MN091409, MN092007; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Koutecký C18-010*, MN091408, MN092006; Spain, prov. Cádiz, *Koutecký C15-089b*, MN091410, MN092008. ***Callitriche brutia*** nothosubsp. ***neglecta*** Prančl [*C. b.* subsp. *brutia* × *C. b.* subsp. *naftolskyi*]: Spain, prov. Badajoz, *Prančl*, *Kaplan & Koutecký C16-009*, MN091411 MN092009; Spain, prov. Cáceres, *Prančl*, *Kaplan & Koutecký C16-013*, MN091412 + MN091413–MN091420 (ITS clones x1–x8), MN092010. ***Callitriche cophocarpa*** Sendtn.: Czech Republic, *Prančl C12-063*, MN091430, MN092020; Czech Republic, *Prančl C12-095*, MN091421, MN092011; Czech Republic, *Prančl & Kabátová C13-001*, MN091425, MN092015; Czech Republic, *Prančl C13-011*, MN091422, MN092012; Czech Republic, *Prančl & Kaplan C13-027*, MN091428, MN092018; MN091426, MN092016, Czech Republic, *Prančl & Kaplan C13-030*; Czech Republic, *Prančl & Kabátová C13-081*, MN091423, MN092013; Czech Republic, *Prančl & Kabátová C13-085*, MN091424, MN092014; Czech Republic, *Prančl & Kabátová C13-095*, MN091431, MN092021; Czech Republic, *Rydlo & Rydlo jr. C13-119*, MN091429, MN092019; Czech Republic, *Prančl C15-061*, MN091427, MN092017; Denmark, *Prančl & Kaplan C12-033*, MN091432, MN092022; Finland, reg. Etelä-Savo (Southern Savonia), *Prančl*, *Koutecký & Hanzlíčková C17-052*, MN091433, MN092023; Germany, Sachsen (Saxony), *Kačmar*, *Rydlo & Rydlo jr. C15-087*, MN091434, MN092024; Poland, Mazowieckie Voivodeship, *Trávníček & Kubátová C12-074*, MN091435, MN092025; Slovakia, *Prančl & Hrdinová C13-016*, MN091436, MN092026; Sweden, Västernorrland county, *Rydlo jr. C13-071*, MN091437, MN092027; Ukraine, Zakarpatska (Zakarpattia) oblast, *Kabátová C14-075*, MN091438, MN092028. ***Callitriche cophocarpa* × *Callitriche hamulata*** **(putative hybrid):** Czech Republic, *Prančl C12-061-04*, MN091439, MN092029; Czech Republic, *Prančl C12-061-20*, MN091440, MN092030; Czech Republic, *Prančl & Kabátová C13-092-04*, MN091441, MN092031; Czech Republic, *Prančl C12-065*, MN091443, MN092033; Czech Republic, *Prančl C12-066*, MN091442, MN092032; Czech Republic, *Prančl C15-060-12*, MN091444, MN092034. ***Callitriche cribrosa*** Schotsman: Italy, reg. Lazio, *Kaplan*, *Hanzlíčková & Koutecký C18-002*, MN091445, MN092035. ***Callitriche hamulata*** Kütz ex W.D.J.Koch: Austria, Oberösterreich (Upper Austria), *Hrdinová C14-077*, MN091446, MN092036; Czech Republic, *Prančl C12-062*, MN091455, MN092045; Czech Republic, *Prančl C12-073*, MN091450, MN092040; Czech Republic, *Prančl C12-091*, MN091447, MN092037; Czech Republic, *Prančl & Kaplan C13-028*, MN091452, MN092042; Czech Republic, *Prančl & Kabátová C13-077*, MN091451, MN092041; Czech Republic, *Prančl & Kabátová C13-084*, MN091449, MN092039; Czech Republic, *Chrtek jr. C13-086*, MN091448, MN092038; Czech Republic, *Rydlo & Rydlo jr. C13-117*, MN091454, MN092044; Czech Republic, *Prančl C15-059*, MN091456, MN092046; Czech Republic, *Prančl C15-062*, MN091453, MN092043; Denmark, *Prančl & Kaplan C12-045*, MN091457, MN092047; France, reg. Bretagne (Brittany), *Prančl C18-043*, MN091460, MN092050; France, reg. Bretagne (Brittany), *Prančl C18-046*, MN091459, MN092049; France, reg. Bretagne (Brittany), *Prančl C18-047*, MN091458, MN092048; France, reg. Nouvelle-Aquitaine, *Prančl C18-036*, MN091461, MN092051; France, reg. Occitanie, *Prančl C18-034*, MN091462, MN092052; Germany, Baden-Württemberg, *Prančl & Hanzlíčková C18-079*, MN091463, MN092053; Germany, Bayern (Bavaria), *Kabátová C13-132b*, MN091464, MN092054; Germany, Sachsen (Saxony), *Rydlo & Rydlo jr. C14-138*, MN091465, MN092055; *Iceland*, *Prančl C16-079*, MN091467, MN092057; Iceland, *Prančl C16-081*, MN091466, MN092056; U.S.A., Oregon, *Prančl & Kávová C13-049*, MN091469, MN092059; U.S.A., Oregon, *Prančl & Kávová C13-050*, MN091468, MN092058. ***Callitriche hermaphroditica*** *L. subsp*. ***hermaphroditica*****:** Czech Republic, *Prančl C12-090*, MN091470, MN092060; Czech Republic, Šumberová *C16-089*, MN091471, MN092061; Finland, reg. Uusimaa, *Prančl*, *Koutecký & Hanzlíčková C17-054*, MN091472, MN092062; Sweden, Östergötland county, *Svenson C13-127*, MN091473, MN092063. ***Callitriche hermaphroditica*** subsp. ***macrocarpa*** (Hegelm.) Lansdown: Sweden, Östergötland county, *Prančl*, *Koutecký & Hanzlíčková C17-051*, MN091474, MN092064. ***Callitriche heterophylla*** var. ***bolanderi*** **(Hegelm.) Fassett (cf.):** U.S.A., Colorado, *Majack C14-144*, MN091475, MN092065. ***Callitriche heterophylla*** Pursh var. ***heterophylla***: U.S.A., New Hampshire, *Hellquist & Callahan C14-005*, MN091476, MN092066; ***Callitriche heterophylla*** **(cf.):** U.S.A., New Hampshire, *Hellquist & Callahan C14-006*, MN091477, MN092067; U.S.A., New York, *Stevens C14-007*, MN091478, MN092068. ***Callitriche lenisulca*** Clavaud: Greece, *Prančl*, *Kaplan & Koutecký C17-018*, MN091485, MN092075; Greece, *Prančl*, *Kaplan & Koutecký C17-019*, MN091484, MN092074; Greece, *Prančl*, *Kaplan & Koutecký C17-020*, MN091479, MN092069; Greece, *Prančl*, *Kaplan & Koutecký C17-023*, MN091482, MN092072; Greece, *Prančl*, *Kaplan & Koutecký C17-026*, MN091483, MN092073; Greece, *Prančl*, *Kaplan & Koutecký C17-029*, MN091480,

Appendix 1. Continued.

MN092070; Greece, Kerkyra (Corfu) island, *Reich*, *Gilli*, *Hofbauer & Sander C17-003*, MN091481, MN092071; Italy, reg. Emilia-Romagna, *Trávníček & Kubátová C13-005*, MN091486, MN092076; Italy, reg. Emilia-Romagna, *Trávníček & Kubátová C13-006*, MN091487, MN092077; Italy, reg. Sardegna (Sardinia), *Lansdown C16-096*, MN091488, MN092078; Italy, reg. Toscana (Tuscany), *Trávníček & Kubátová C13-004*, MN091489, MN092079. ***Callitriche lusitanica*** Schotsman: Greece, *Prančl*, *Kaplan & Kouteckỳ C17-015*, MN091490, MN092080; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-023*, MN091491, MN092081; Spain, prov. Badajoz, *Prančl*, *Kaplan & Kouteckỳ C16-010*, MN091492, MN092082; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-014*, MN091493, MN092083. ***Callitriche muelleri*** Sond.: Australia, Queensland, *Jobson C15-093*, MN091494, MN092084; Australia, *Jobson C15-085*, MN091495, MN092085. ***Callitriche* ×*nyrensis*** Prančl [*C. cophocarpa* × *C. stagnalis*]: Czech Republic, *Prančl C15-084-02*, MN091496, MN092086; Czech Republic, *Prančl C15-084-03*, MN091497 + MN091498–MN091504 (ITS clones C15-084-03-x1–x4, x6–x8), MN092087; Czech Republic, *Prančl C15-084-06*, MN091505, MN092088; Czech Republic, *Prančl C15-084-07*, MN091506, MN092089. ***Callitriche obtusangula*** Le Gall: Austria, Oberösterreich (Upper Austria), *Hrdinová C14-079*, MN091508, MN092091; Austria, Oberösterreich (Upper Austria), *Hrdinová C14-081*, MN091509, MN092092; Austria, Oberösterreich (Upper Austria), *Prančl*, *Kouteckỳ & Hohla C15-019*, MN091507, MN092090; France, reg. Bretagne (Brittany), *Prančl C18-048*, MN091510, MN092093; France, reg. Nouvelle-Aquitaine, *Prančl C18-033*, MN091511, MN092094; France, reg. Pays de la Loire, *Prančl C18-040*, MN091513, MN092096; France, reg. Pays de la Loire, *Prančl C18-049*, MN091512, MN092095; Germany, Baden-Württemberg, *Prančl & Hanzlíčková C18-080*, MN091514, MN092097; Germany, Bayern (Bavaria), *Prančl & Hanzlíčková C18-077*, MN091515, MN092098; Italy, reg. Campania, *Trávníček & Kubátová C13-008*, MN091517, MN0920100; Italy, reg. Campania, *Trávníček & Kubátová C13-009*, MN091518, MN0920101; Italy, reg. Campania, *Trávníček C16-085*, MN091516, MN092099; Italy, reg. Lazio, *Trávníček & Kubátová C13-007*, MN091520, MN092103; Italy, reg. Lazio, *Kaplan*, *Hanzlíčková & Kouteckỳ C18-003*, MN091519, MN092102; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-012*, MN091524, MN092107; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-015*, MN091523, MN092106; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-019*, MN091522, MN092105; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-025*, MN091521, MN092104; Italy, reg. Toscana (Tuscany), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-031*, MN091525, MN092108; Italy, reg. Umbria, *Kaplan*, *Hanzlíčková & Kouteckỳ C18-029*, MN091527, MN092110; Italy, reg. Umbria, *Kaplan*, *Hanzlíčková & Kouteckỳ C18-030*, MN091526, MN092109; Netherlands, *Trávníček & Kubátová C12-052*, MN091528, MN092111; Slovakia, *Bubíková C15-086*, MN091529, MN092112. ***Callitriche palustris*** L.: Czech Republic, *Prančl & Trávníček C12-019*, MN091531, MN092114; Czech Republic, *Prančl C12-081*, MN091530, MN092113; Finland, reg. Etelä-Savo (Southern Savonia), *Prančl*, *Kouteckỳ & Hanzlíčková C17-053*, MN091532, MN092115; Hungary, *Kaplan & Mesterházy C18-084*, MN091533, MN092116; Iceland, *Prančl C16-080*, MN091534, MN092117; Norway, Trøndelag County, *Kabátová C13-073*, MN091535, MN092118; Romania, Bistri□a-Năsăud county, *Kabátová C15-057*, MN091536, MN092119; Sweden, Norrbotten county, *Kaplan C13-150*, MN091537, MN092120; U.S.A., Colorado, *Majack C14-143*, MN091538, MN092121; U.S.A., Maine, *Hellquist C14-002*, MN091540, MN092123; U.S.A., Maine, *Hellquist C14-003*, MN091541, MN092124; U.S.A., Maine, *Hellquist C14-004*, MN091539, MN092122; U.S.A., New York, *Stevens & Graham C14-008*, MN091542, MN092125. ***Callitriche platycarpa*** Kütz.: Austria, Oberösterreich, *Prančl*, *Kouteckỳ & Hohla C15-017*, MN091543, MN092126; Czech Republic, *Prančl C12-093*, MN091546, MN092129; Czech Republic, *Prančl & Kabátová C13-074*, MN091548, MN092131; Czech Republic, *Prančl & Kabátová C13-079*, MN091547, MN092130; Czech Republic, *Rydlo & Rydlo jr. C13-124*, MN091545, MN092128; Czech Republic, *Rydlo & Rydlo jr. C14-072*, MN091544, MN092127; Denmark, *Prančl & Kaplan C12-046*, MN091549, MN092132; France, reg. Bretagne (Brittany), *Prančl C18-045*, MN091550, MN092133; Germany, Bayern (Bavaria), *Knotek C12-077*, MN091551, MN092134; Germany, Sachsen (Saxony), *Rydlo & Rydlo jr. C14-139*, MN091552, MN092135; Italy, reg. Calabria, *Trávníček & Kubátová C13-010*, MN091553, MN092136; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-006*, MN091554, MN092137; Sweden, Skåne county, *Prančl*, *Kouteckỳ & Hanzlíčková C17-050*, MN091555, MN092138. ***Callitriche pulchra*** Schotsman: Greece, island of Gavdos, *Bazos & Lansdown C15-001*, MN091557, MN092140; Greece, island of Gavdos, *Bazos & Lansdown C15-002*, MN091558, MN092141; Greece, island of Gavdos, *Bazos & Lansdown C15-003*, MN091556, MN092139. ***Callitriche regis-jubae*** Schotsman: Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-018*, MN091559, MN092142; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-016*, MN091560, MN092143. ***Callitriche stagnalis*** Scop.: Australia, New South Wales, *Jobson C15-094*, MN091561, MN092144; Czech Republic, *Hadinec & Bauer C12-076*, MN091562, MN092145; Czech Republic, *Prančl C12-092*, MN091564, MN092147; Czech Republic, *Prančl C13-002*, MN091567, MN092150; Czech Republic, *Prančl C13-018*, MN091568, MN092151; Czech Republic, *Chrtek jr. C13-087*, MN091565, MN092148; Czech Republic, *Rydlo & Rydlo jr. C13-114*, MN091563, MN092146; Czech Republic, *Prančl C13-135*, MN091569, MN092152; Czech Republic, *Prančl C15-084-01*, MN091566, MN092149; France, reg. Bretagne (Brittany), *Prančl C18-044*, MN091570, MN092153; France, reg. Nouvelle-Aquitaine, *Prančl C18-035*, MN091571, MN092154; France, reg. Nouvelle-Aquitaine, *Prančl C18-037*, MN091572, MN092155; France, reg. Pays de la Loire, *Prančl C18-038*, MN091573, MN092156; Germany, Sachsen (Saxony), *Rydlo & Rydlo jr. C14-141*, MN091574, MN092157; Greece, *Prančl*, *Kaplan & Kouteckỳ C17-014*, MN091581, MN092164; Greece, *Prančl*, *Kaplan & Kouteckỳ C17-016*, MN091580, MN092163; Greece, *Prančl*, *Kaplan & Kouteckỳ C17-021*, MN091575, MN092158; Greece, *Prančl*, *Kaplan & Kouteckỳ C17-024*, MN091579, MN092162; Greece, *Prančl*, *Kaplan & Kouteckỳ C17-027*, MN091576, MN092159; Greece, Kerkyra (Corfu) island, *Gilli*, *Hofbauer*, *Reich & Sander C17-004*, MN091578, MN092161; Greece, Kerkyra (Corfu) island, *Hofbauer*, *Reich & Sander C17-008*, MN091577, MN092160; Italy, reg. Campania, *Kaplan*, *Hanzlíčková & Kouteckỳ C18-004*, MN091582, MN092165; Italy, reg. Sicilia (Sicily), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-013*, MN091583, MN092166; Italy, reg. Toscana (Tuscany), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-032*, MN091585, MN092168; Norway, Møre og Romsdal County, *Kabátová C13-072*, MN091586, MN092169; Portugal, reg. Algarve, *Prančl*, *Kaplan & Kouteckỳ C16-021*, MN091588, MN092171; Portugal, reg. Algarve, *Prančl*, *Kaplan & Kouteckỳ C16-022*, MN091587, MN092170; Spain, prov. Badajoz, *Prančl*, *Kaplan & Kouteckỳ C16-011*, MN091592, MN092175; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-002*, MN091595, MN092178; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-007*, MN091593, MN092176; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-008*, MN091594, MN092177; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-012*, MN091596, MN092179; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-015*, MN091597, MN092180; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-017*, MN091598, MN092181; Spain, prov. Cáceres, *Prančl*, *Kaplan & Kouteckỳ C16-027*, MN091599, MN092182; Spain, prov. Cáceres, *Kouteckỳ C15-090*, MN091600, MN092183; Spain, prov. Cádiz, *Kouteckỳ C15-089a*, MN091589, MN092172; Spain, prov. La Rioja, *Prančl*, *Kaplan & Kouteckỳ C16-001*, MN091601, MN092184; Spain, prov. Salamanca, *Prančl*, *Kaplan & Kouteckỳ C16-020*, MN091590, MN092173; Spain, prov. Toledo, *Prančl*, *Kaplan & Kouteckỳ C16-004*, MN091591, MN092174; U.S.A., Oregon, *Prančl & Kávová C13-053*, MN091602, MN092185; U.S.A., Washington, *Prančl & Kávová C13-151*, MN091603, MN092186. ***Callitriche stagnalis* (cf.):** Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-020*, MN091584, MN092167. ***Callitriche stagnalis* (autotriploid):** Czech Republic, *Rydlo & Rydlo jr. C13-125*, MN091604, MN092187; ***Callitriche truncata*** Guss.: Greece, *Prančl*, *Kaplan & Kouteckỳ C17-025*, MN091605, MN092188. ***Callitriche truncata*** subsp. ***occidentalis*** (Rouy) Schotsman: France, reg. Pays de la Loire, *Prančl C18-039*, MN091606, MN092189; ***Callitriche truncata*** Guss. subsp. ***truncata*****:** Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-014*, MN091608, MN092191; Italy, reg. Sardegna (Sardinia), *Kaplan*, *Hanzlíčková & Kouteckỳ C18-021*, MN091607, MN092190. ***Callitriche* ×*vigens*** K.Martinsson [*C. cophocarpa* × *C. platycarpa*]: Austria, Oberösterreich (Upper Austria), *Kaplan*, *Kouteckỳ & Lučanová C18-082*, MN091609, MN092192; Czech Republic, *Prančl & Kouteckỳ C11-016*, MN091616, MN092199; Czech Republic, *Prančl & Trávníček C12-021*, MN091617, MN092200; Czech Republic, *Hrdinová C13-068*, MN091613, MN092196; Czech Republic, *Prančl & Kabátová C13-082*, MN091614, MN092197; Czech Republic, *Prančl & Kabátová C13-083*, MN091610, MN092193; Czech Republic, *Rydlo & Rydlo jr. C13-108*, MN091615, MN092198; Czech Republic, *Rydlo & Rydlo jr. C13-115*, MN091611, MN092194; Czech Republic, *Rydlo & Rydlo jr. C13-116*, MN091612, MN092195; Denmark, *Prančl & Kaplan C12-041*, MN091618, MN092201; Germany, Baden-Württemberg, *Prančl & Hanzlíčková C18-078*, MN091619, MN092202; Germany, Bayern (Bavaria), *Kabátová C13-132a*, MN091620, MN092203. ***Hippuris vulgaris* L. (outgroup):** Czech Republic, *Anonym Hippuris 1*, MN091621, MN092204; Czech Republic, *Prančl Hippuris 2*, MN091622, MN092205.