Speciation in *Callitriche* (Plantaginaceae): the allopolyploid origin of *C. platycarpa*

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Running title: Allopolyploid speciation in *Callitriche*

Genomic *in situ* hybridisation (GISH) experiments involving hybridising labeled genomic DNA from the diploid species *C. stagnalis*, *C. obtusangula* and *C. cophocarpa* to chromosome spreads of the tetraploid *C. platycarpa* ($2n=4x=20$) show that *C. stagnalis* and *C. cophocarpa* DNA each hybridise with a different ten chromosomes of the *C. platycarpa* complement, whereas *C. obtusangula* DNA does not differentiate between the two genomes. We conclude on the basis of this and other evidence that *C. platycarpa* is likely to be an allotetraploid derivative of *C. cophocarpa* and *C. stagnalis*. This work demonstrates the successful application of genome in-situ hybridization (GISH) to the study of polyploid evolution in the genus *Callitriche*.

Keywords: *Callitriche cophocarpa*, *Callitriche stagnalis*, genome in situ hybridization, polyploidy, water starworts

21 Introduction

22 The genus *Callitriche* L. contains ca 75 species of terrestrial, amphibious or aquatic herbs (Lansdown,
 23 2008). For a long time they formed the only genus in the family Callitrichaceae (Cronquist, 1981), but
 24 evidence from plastid and nuclear DNA sequences indicates they are sister to another aquatic genus,
 25 *Hippuris*, and are better assigned to the Plantaginaceae (=Veronicaceae), in tribe Callitricheae
 26 Dumort. (Tank *et al.*, 2006). The flowers are unisexual and reduced, lacking a perianth, and are
 27 located in the axils of the distal cauline leaves. Depending on species, pollination is by wind or water
 28 (Philbrick & Anderson, 1992). Reported sporophytic chromosome numbers range from $2n=6$ to
 29 $2n=40$, with a putative base number of $x=5$, a pattern of variation that has invited hypotheses of
 30 recurrent polyploidy and aneuploidy (Philbrick & Les, 2000).

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32 The present study focuses on the origin of *C. platycarpa* Kütz., a tetraploid species with $2n=20$
 33 chromosomes distributed across north-western Europe (Lansdown, 2008). Possible progenitors
 34 include those diploid European species with $2n=10$ chromosomes, of which there are five
 35 (Lansdown, 2008). Three are widely distributed: i) *C. cophocarpa* Sendtn., from northern and eastern
 36 Europe; ii) *C. stagnalis* Scop., from across the whole of Europe; and iii) *C. obtusangula* Le Gall, from
 37 central and western Europe; and two are much more restricted, chiefly Mediterranean species: iv) *C.*
 38 *regis-jubae* Schotsman, from Spain, Sardinia and N. Africa; and v) *C. lenisulca* Clavaud., which occurs
 39 as scattered populations chiefly across the northern coast of the Mediterranean.

40

41 Two competing hypotheses to explain the origin of the tetraploid *C. platycarpa* have been proposed:
 42 i) an autopolyploid origin from *C. cophocarpa* (Savidge, 1958; Schotsman, 1967); and ii) an
 43 allopolyploid origin from the hybrid between *C. cophocarpa* and *C. stagnalis* (Savidge, 1958, 1960;
 44 Martinsson, 1985).

45

Evidence from DNA amount per chromosome is consistent with either hypothesis and also argues against the involvement of *C. obtusangula* (Pijnacker & Schotsman, 1988). More recent analysis using flow cytometry (Prančl *et al.*, 2014) is also consistent with either an autopolyploid or allopolyploid hypothesis. Evidence for an autopolyploid origin of *C. platycarpa* from *C. cophocarpa*, a proposal strongly favoured by Schotsman (1967), is based partly on its morphological similarity and partly on its partial sterility: apparently 25% of pollen of *C. platycarpa* is sterile and few fruits are produced by the female flowers (Savidge, 1958): p.114). Evidence for *C. platycarpa* being an allopolyploid derivative of the hybrid between *C. cophocarpa* and *C. stagnalis* derives from a number of sources, detailed as follows.

- 1) The putative parents are sympatric in eastern Europe (Schotsman 1967; Lansdown 2008).
- 2) *C. platycarpa* is morphologically intermediate between these two species (Savidge, 1960).
- 3) The allozyme complement of *C. platycarpa* is a summation of that present in *C. stagnalis* and *C. cophocarpa* (Byczkiewicz *et al.*, 2007) and also appears to rule out the involvement of *C. obtusangula*, the other $2n=10$ diploid with a widespread distribution in western Europe (Demars & Gornall, 2003).
- 4) The meiotic behaviour of the hybrid *C. platycarpa* $\times$ *cophocarpa* ($2n=15$), in which chromosomes form one quadrivalent, three bivalents and five univalents at meiosis, suggests that the genome of *C. cophocarpa* is homologous to half that of *C. platycarpa* (Savidge, 1960). In this explanation of pairing behaviour, it is proposed that the five gametic chromosomes from *C. cophocarpa*, pair with five of the ten gametic chromosomes from *C. platycarpa* to form the three bivalents and one quadrivalent.
- 5) Recent studies of isozyme phenotypes of *C. platycarpa* in both Britain (Demars & Gornall, 2003) and Poland (Byczkiewicz *et al.*, 2007) have shown that it may exhibit fixed heterozygosity at a number of loci, which is consistent with an allopolyploid origin if the alternative explanations of

sampling error caused by clonal growth or tetrasomic inheritance can be ruled out. Furthermore the species has a regular meiosis, forming ten bivalents and no multivalents (Savidge, 1958).

The aim of the present study was to conduct genome in-situ hybridisation (GISH) experiments (Schwarzacher *et al.*, 1989; Stace & Bailey, 1999) to test the genome homologies of *C. platycarpa* with its diploid relatives, viz. *C. stagnalis*, *C. cophocarpa* and *C. obtusangula*, and thereby distinguish between the autopolyploid and allopolyploid hypotheses of Schotsman (1967) and Savidge (1960), respectively.

Materials and Methods

Plant material

Samples of *Callitriche* species were collected from the localities listed in Table 1. Genomic DNA from each of the three diploid species (*C. cophocarpa*, *C. stagnalis* and *C. obtusangula*) was used to probe chromosome spreads of the tetraploid *C. platycarpa*.

Chromosome preparations

Young, actively growing root tips 0.5-1 cm long were pre-treated with 0.2 mM 8-hydroxyquinoline at 4 °C overnight, fixed in 3:1 ethanol (96%): acetic acid (100%) and stored at 4 °C. For chromosome counts, root tips were treated with 5M HCl at room temperature for 10 minutes and transferred to 70% industrial methylated spirit (IMS). Root tips were squashed in aceto-orcein on a clean slide and examined under a microscope.

For fluorescent and genomic *in situ* hybridization (FISH and GISH), roots were treated with protoelytic enzyme mixture containing 3% (w/v) pectinase from *Aspergillus niger* (Sigma P4716), 1.8% (w/v) cellulase from *Aspergillus niger* (Calbiochem, 21947), 0.2% (w/v) 'Onozuka' RS cellulase (Yakult Pharmaceutical Industry Co.) and 0.5% viscozyme (Novo Nordisk AIS) in 10 mM citric acid/tri-

sodium-citrate buffer. Squash preparations were made in 60 %(v/v) acetic acid as described in Schwarzacher & Heslop-Harrison (2000).

Genomic DNA extraction, probe preparation and labelling

Total genomic DNA was extracted from 200—700 mg of live plant material of *C. stagnalis*, *C. obtusangula* and *C. cophocarpa* frozen in liquid nitrogen, following the CTAB protocol of Doyle & Doyle (1990). DNA from each species was sheared to 300-1000bp fragments by adding ~5µg of DNA to 500µl 250 mM sodium hydroxide and incubating at room temperature for 20 minutes before precipitation with ethanol and resuspension in water. Labelling with biotin-16-dUTP and digoxigenin-11-dUTP (Roche Diagnostics) followed the BioPrime DNA Labeling System or Array CGH Genomic Labeling System (Invitrogen) as described by the manufacturer.

Fluorescent and genomic in situ hybridisation (FISH and GISH)

Chromosome preparations were re-fixed in alcohol- acetic acid (3:1), treated with 10mg/ml RNase and optionally with 5µg/ml pepsin and finally incubated in 4% freshly made or saline buffered paraformaldehyde solution as described by Schwarzacher and Heslop-Harrison (2000). The hybridization mixture contained 50% (v/v) formamide, 20% (w/v) dextran sulphate, 2x SSC (saline sodium citrate: 0.3M NaCl, 0.03M sodium citrate), 100—200 ng probe, 0.025µg of salmon sperm DNA, 0.125% (w/v) SDS (sodium dodecyl sulphate) and 0.125mM EDTA (ethylenediamine-tetraacetic acid). Probe combinations involved DNAs of pairs of species from the three under study. 40ul of probe mixture was applied to each slide and covered with a plastic cover slip. Chromosomes and probe DNA were denatured together on a Hybaid Omniblock at 75-80°C for 8 minutes and slowly cooled to 37°C and allowed to hybridise over night at. Stringent washes were carried out both with, or without 20% (v/v) formamide, in 0.1 x SSC at 42°C that allowed DNA molecules with 80-85% or 70-75% homology, respectively, to remain as hybrids. Slides were blocked with bovine serum albumin and incubated with streptavidin conjugated to Alexa594 (2.0µg/ml, Molecular Probes) and

antidigoxigenin conjugated to FITC (fluorescein isothiocyanate, 4µg/ml, Roche Diagnostics) for 1 hour at 37°C. After washing in 4xSSC with 0.2% (v/v) Tween 20, preparations were stained with DAPI (4',6-diamidino-2-phenylindole, 4µg/ml in McIlvaine's buffer, pH 7.0) and mounted in antifade solution (Citifluor) using a 22x40 No. 0 coverslip.

Image analysis

FISH and GISH chromosome slides were analysed on a Zeiss epifluorescence microscope equipped with a colour CCD camera (ProgRes C12, Optronics, model S97790) or Nikon ECLIPSE N80i fluorescent microscope (Nikon, Japan) equipped with a DS-QiMc monochromatic camera (Nikon). Each metaphase, was imaged using three different filter sets with green, blue and UV excitation respectively to visualise the red and green FISH signal and the cyan DAPI signal. Monochromatic images were assigned specific colours corresponding to the colour of the fluorochrome. Images were overlaid and analyzed using Adobe Photoshop CS4 (Adobe Systems, San Jose, California) or NIS-Elements BR3.1 software (Nikon), using only those functions that are applied to all pixels of the image.

Results

Chromosome numbers

Chromosome numbers were established from mitotic spreads of the root-tips (Table 1).

GISH experiments

Genomic DNA from *Callitriche stagnalis* consistently hybridised with ten of the twenty chromosomes in *C. platycarpa*; similarly, genomic DNA from *C. cophocarpa* also hybridised with ten chromosomes in *C. platycarpa*. When the genomic DNA probes were used in combination, ten of the twenty chromosomes of *C. platycarpa* fluoresced in red with the biotin-labelled probe DNA from *C. stagnalis* and a different ten fluoresced green with the digoxigenin-labelled probe DNA from *C. cophocarpa*.

(Fig. 1). In contrast, genomic DNA from the other diploid, *C. obtusangula*, showed very weak and diffuse hybridisation to all chromosomes.

Discussion

Our GISH experiments strongly suggest that the tetraploid genome of *C. platycarpa* is a product of genome duplication following interspecific hybridisation, most likely between *C. cophocarpa* and *C. stagnalis*. We can rule out the participation of the other common diploid with ten chromosomes, viz. *C. obtusangula*, because its genomic DNA showed very weak and diffuse hybridisation to all chromosomes in spreads of *C. platycarpa*. Furthermore, Pijnacker & Schotsman (1988) reported that pollen from *C. obtusangula* is rejected on the stigmas of *C. stagnalis* and vice versa, by way of inhibition of germination and tube growth, so it is unlikely to be involved in hybridisation with that species. Genome size data (1-C values) from the flow cytometry results of Prančl *et al.* (2014) would also preclude this species from being one of the parents.

The only other European candidate parental taxa with $2n=10$ chromosomes are *C. regis-jubae* and *C. lenisulca*. According to Lansdown (2008), the former is closely related to *C. stagnalis* and the latter to *C. cophocarpa*. We cannot yet rule out their involvement entirely but it seems unlikely given that both are essentially Mediterranean species, neither is particularly common and the evidence outlined in the Introduction does not implicate them.

A study of *rbcl* DNA sequences from Polish material showed that *C. platycarpa* was similar to *C. stagnalis* but different from *C. cophocarpa* which, assuming maternal inheritance of plastid DNA, suggests that *C. stagnalis* is the female parent (Byczkiewicz *et al.*, 2007) in that part of Europe. Whether it is the same in the British Isles remains to be seen: before any conclusions can be drawn about the direction of hybridisation or the number of speciation events, it will be necessary to establish the extent of infra-specific variation in all the taxa.

Hybridisation appears to be relatively rare in *Callitriche* (Martinsson, 1991; Prančl *et al.*, 2014). It seems to occur chiefly among species capable of wind-pollination, hence with an increased chance of outcrossing (Schotsman, 1982). A report of *C. cophocarpa* × *stagnalis* (Martinsson, 1991), a taxon that must have existed prior to genome duplication if our conclusions regarding the origin of *C. platycarpa* are correct, was questioned by Lansdown (2008) on the grounds that the voucher material is indistinguishable from *C. stagnalis*. There appear to be only two reliably recorded European hybrids. One is that between *C. cophocarpa* and *C. platycarpa* (= *C. ×vigens* K. Martinsson), which occurs where the parental ranges overlap or come close (Lansdown, 2008; Prančl *et al.*, 2014). A second case involves Czech plants with 2n=29 chromosomes, suggestive of *C. hamulata* (2n=38) as one parent and an as yet unidentified diploid (2n=10) as the other (Prančl *et al.*, 2014).

The colonisation of the British Isles by *C. platycarpa* provides an example of the presence of what is essentially a hybrid taxon in the absence of one of its parents. Another British aquatic taxon similarly orphaned is *Potamogeton pectinatus* × *vaginatus* (= *P. ×bottnicus* Hagstr.), which occurs in the Tweed valley without its Scandinavian *P. vaginatus* parent (Preston, Hollingsworth & Gornall, 1998; King *et al.*, 2001).

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Table 1. *Callitriche* accessions used in the study and their sporophytic chromosome numbers (2n) where counted.

C. cophocarpa Sendtn.

Denmark, Zealand, Taarbaek drainage, WNW of forest superintendent's residence, Dyrehaven, NNW of Klampenborg Station (55.7899°N, 12.5804°E). *N. Jacobsen & Marian Oergaard*, 3rd September 2013.

C. obtusangula Le Gall

England, Leics., Saddington reservoir outflow (52.5173°N, 1.0221°W). *Gornall*, September 2012 (LTR). 2n = 10.

England, Leics., Market Harborough, River Jordan (52.4783°N, 0.9097°W). *Gornall*, September 2012 (LTR). 2n = 10.

C. stagnalis Scop.

England, Leics., Charnwood Forest, near Copt Oak, (52.7016°N, 1.2719°W). *Gornall*, October 2012 (LTR). 2n = 10.

C. platycarpa Kütz.

England, Leics., Launde Abbey ponds (52.6312°N, 0.8234°W). *Gornall*, September 2012 (LTR). 2n = 20.

England, Leics., Bradgate Park, Roecliffe Rd gate (52.6917°N, 1.2214°W). *Gornall*, October 2012 (LTR). 2n = 20.

258 Figure 1. Early metaphase spread of twenty chromosomes from *C. platycarpa* showing ten
259 chromosomes hybridised with biotin-labeled *C. stagnalis* DNA probe (in red), and the remaining ten
260 chromosomes hybridised with digoxigenin-labeled *C. cophocarpa* DNA probe (in green), overlaid
261 onto the DAPI counterstained spread. Scale bar = 10µm.

262