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SHORT COMMUNICATION

Holocentric chromosomes may be an apomorphy of Droseraceae

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Abstract
Holocentric chromosomes have evolved in various plant and animal taxa, which suggests they may confer a selective advantage in certain conditions, yet their adaptive potential has scarcely been studied. One of the reasons may reside in our insufficient knowledge of the phylogenetic distribution of holocentric chromosomes across eukaryotic phylogeny. In the present study, we focused on Droseraceae, a carnivorous plant family with an unknown chromosomal structure in monotypic genera *Dionaea* and *Aldrovanda*, and a closely related monotypic family Drosophyllaceae. We used flow cytometry to detect holocentric chromosomes by measuring changes in the ratio of the number of G2 nuclei to the number of G1 nuclei in response to gamma irradiation and determined chromosomal structures in *Aldrovanda vesiculosa*, *Dionaea muscipula, Drosera tokaiensis*, and *Drosera ultramafica* from Droseraceae and *Drosophyllum lusitanicum* from Drosophyllaceae. We confirmed monocentric chromosomes in *D. lusitanicum* and detected holocentric chromosomes in all four Droseraceae. Our novel finding of holocentric chromosomes in monotypic genera *Aldrovanda* and *Dionaea* suggests that all Droseraceae may be holocentric, but to confirm that further research is needed due to previously reported conflicting results in *Drosera rotundifolia.*

Keywords *Aldrovanda* · *Dionaea* · *Drosera* · *Drosophyllum* · Flow cytometry · Gamma irradiation

Introduction

Holocentric chromosomes, which attach spindle microtubules to the kinetochore formed along most of their length (Cuacos et al. 2015), have evolved repeatedly in plants and animals (Melters et al. 2012; Bureš et al. 2013). Ever since holocentric chromosomes were recognized (Schrader 1935), many studies have focused on cytogenetics (e.g., Nordenskiöld 1963; Heckmann et al. 2011; Jankowska et al. 2015), cytogenomics (e.g., Marques et al. 2015; de Souza et al. 2018), cell biology (e.g., Wanner et al. 2015; Marques et al. 2016), genomics (e.g., d'Alençon et al. 2010), and other aspects of holocentric organisms, shedding light on structural and mechanistic differences between holocentric and monocentric chromosomes. But the question of why holocentric chromosomes appeared repeatedly over the course of evolution has been studied (Zedek and Bureš 2016; Márquez-Corro et al. 2018) or discussed (Wrensch et al. 1994; Talbert et al. 2008; Mandrioli and Manicardi 2012; Zedek and Bureš 2018) only rarely. Their repeated origin indicates that holocentric chromosomes confer some selective advantage, which may be a defense against centromere drive (Talbert et al. 2008; Zedek and Bureš 2016), an ability to rapidly change recombination rates via chromosomal rearrangements (Escudero et al. 2012), or tolerance to chromosome-breaking factors (Mandrioli and Manicardi 2012; Zedek and Bureš 2018).

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The adaptive role of holocentric chromosomes may be understudied because of their rarity in Eukaryotes (Mola and Papeschi 2006; Melters et al. 2012; Bureš et al. 2013). In animals, holocentric chromosomes have been documented in roundworms and in some groups of insects, mites, spiders, scorpions, and millipedes. In plants, holocentric chromosomes have been found in zygnematophycean algae; in the higher-plant families Cyperaceae and Juncaceae; and in the genera *Myristica* (Myristicaceae), *Chionographis* (Melanthiaceae), *Cuscuta* (Convolvulaceae), and *Drosera* (Droseraceae). Recent studies also found evidence for holocentric chromosomes in *Trithuria submersa* (Hydatellaceae; Kynast et al. 2014) and *Prionium serratum* (Thurniaceae; Zedek et al. 2016). It is possible that holocentric chromosomes are not rare at all and that their apparent rarity is an illusion caused by historical and methodical biases (discussed in detail in Zedek and Bureš 2018). Regardless, clear knowledge of the phylogenetic distribution of holocentric chromosomes is needed to understand their origin and adaptive potential.

Droseraceae is a dicot family of carnivorous plants containing approximately 200 species in three genera (Fig. 1): the genus *Drosera* (sundews) and two monotypic genera, *Aldrovanda* and *Dionaea*. The genus *Drosera* is distributed worldwide except Antarctica, *Dionaea muscipula* (Venus flytrap) occurs in the wetlands of North and South Carolina (USA), and *Aldrovanda vesiculosa* (waterwheel plant) is an aquatic species with scattered distribution in Africa, Australia, and Eurasia (Veleba et al. 2017). Sundews (*Drosera*) are considered holocentric because their chromosomes lack primary constriction (Kondo and Lavarack 1984; Sheikh and Kondo 1995; Hoshi and Kondo 1998), segregate in parallel orientation in anaphase (Kondo and Nontachaiyapoom 2008; Shirakawa et al. 2011a), and attach microtubules along their length (Kondo and Nontachaiyapoom 2008), and also because their chromosomal fragments are regularly inherited (Sheikh et al. 1995; Shirakawa et al. 2011a; Jankowska et al. 2015). Two recent studies reported monocentric chromosomes in four *Drosera* species (Shirakawa et al. 2011b; Demidov et al. 2014) and in *Dionaea muscipula* (Shirakawa et al. 2011b), but they used markers that were not suitable for a reliable distinction between holocentric and monocentric chromosomes (see Discussion for details). Therefore, the chromosomal structure of *Dionaea muscipula* remains unknown. Because the chromosomal structure of *Aldrovanda vesiculosa* is also unknown, it is difficult to assess whether holocentric chromosomes occurred in the common ancestor of the family or appeared in sundews after they diverged from the common ancestor (Fig. 1). Such an uncertainty makes

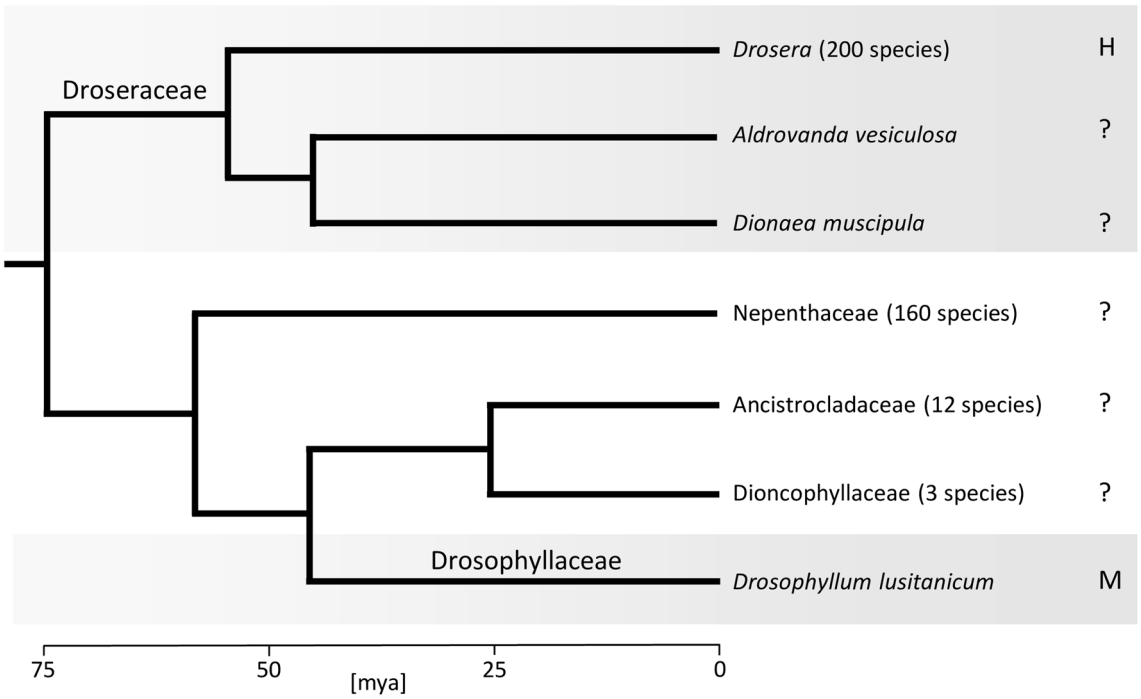


Fig. 1 Chromosomes in Droseraceae and closely related families. Current knowledge of chromosome types in Droseraceae and closely related families is shown next to the dated phylogenetic tree. *H* holocentric chromosomes, *M* monocentric chromosomes, ? unknown chromosomes. Timescale indicates millions of years before present day. Species from shaded clades were analyzed in the present study. The phylogenetic tree was adopted and simplified from Veleba et al. (2017). Numbers of species were taken from Angiosperm Phylogeny Website (Stevens 2017)

it difficult to address questions of the adaptive potential of holocentric chromosomes in comparative studies.

In the present study, we aimed to determine chromosomal structure in four species (*Aldrovanda vesiculosa*, *Dionaea muscipula*, *Drosera tokaiensis*, and *Drosera ultramafica*) representing all genera of Droseraceae. We also included the presumably monocentric species *Drosophyllum lusitanicum* (Hoshi and Kondo 1998) from the closely related monotypic family Drosophyllaceae (Veleba et al. 2017). To determine chromosomal structure, we combined ionizing irradiation with flow cytometry. Ionizing radiation (e.g., gamma or x-rays) has commonly been used to detect holocentric chromosomes ever since they were discovered because it induces chromosome fragments that are regularly inherited in holocentrics but not in monocentrics. However, previous studies combined ionizing radiation with microscopic observations (e.g., Nordenskiöld 1963; Murakami and Imai 1974; Sheikh et al. 1995; Jankowska et al. 2015).

The flow-cytometric (FCM) method for detecting holocentric chromosomes has been developed for plants and is completely independent of microscopic observations (Zedek et al. 2016). The method relies on the tolerance of holocentric chromosomes to fragmentation and has two steps: (1) induction of chromosomal fragmentation, e.g., by ionizing radiation, in meristematic tissues and (2) flow-cytometric measurements of tissues grown from irradiated meristems. Specifically, flow cytometry is used to count nuclei in the G1 (2C nuclei) and G2 (4C nuclei) phases of the cell cycle. In monocentric plants, the cell cycle is stopped in the G2 phase to prevent cell division with broken chromosomes (Preuss and Britt 2003; Culligan et al. 2004; Carballo et al. 2006), because fragments would otherwise be lost. By contrast, this problem is much smaller or does not exist at all in holocentric organisms because their chromosomal fragments are regularly inherited during cell division (Sheikh et al. 1995; Shirakawa et al. 2011a). As a result, the G2/G1 ratio differs between irradiated plants and non-irradiated control plants in monocentrics, but does not differ in holocentrics (Zedek et al. 2016; see also Methods for details). Because the FCM method for holocentric chromosomes detection is based solely on counting nuclei in G1 and G2 phases of the cell cycle, it does not require an internal standardization. This is an important technical difference from FCM estimation of the nuclear DNA content (C-value), which is a more common FCM application in plant sciences and may be biased by instrumental or sample-preparation fluctuations.

Materials and methods

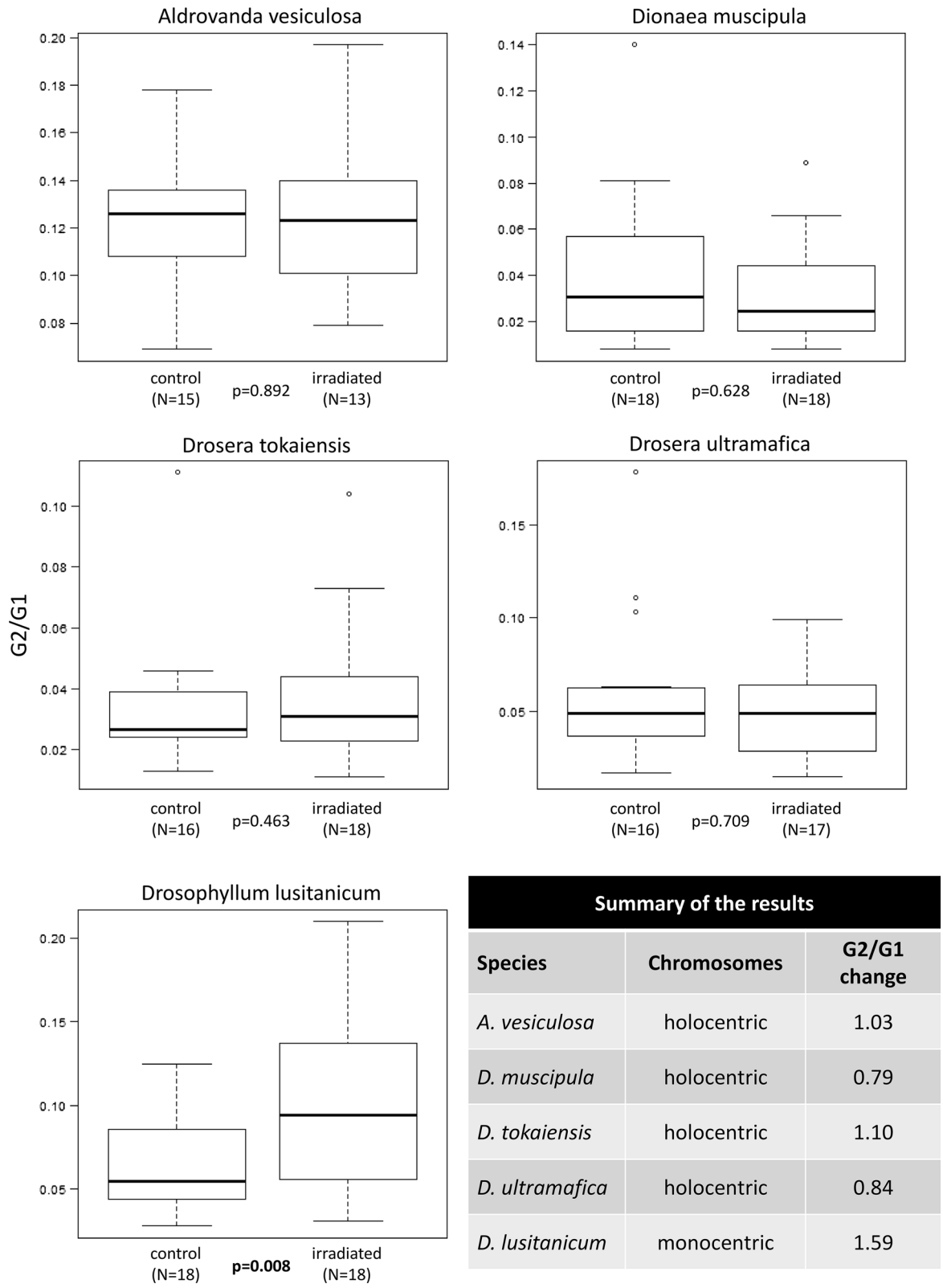
Species collection and cultivation

Specimens of analyzed species were obtained from the collection at the Institute of Botany of the Czech Academy of Sciences in Třeboň (*Aldrovanda vesiculosa*); from the in vitro collection at the Department of Experimental Biology, Masaryk University (*Drosera tokaiensis* and *Dionaea muscipula*); and from the private collections of Michal Kouba (in vitro culture of *Drosera ultramafica*) and David Švarc (seeds of *Drosophyllum lusitanicum*). *Aldrovanda vesiculosa* was cultivated outdoors in a 350 l container filled with CO_2 enriched water (Adamec 1997). *In vitro* cultures of *Drosera ultramafica*, *D. tokaiensis*, and *Dionaea muscipula* were cultivated in glass jars on agar with 1/3 strength Murashige and Skoog (MS) medium (Sigma Aldrich) with the addition of activated charcoal. Seeds of *Drosophyllum lusitanicum* were sterilized using a two-step procedure: First, seeds were submerged in 50% (v/v) ethanol with 3% H_2O_2 for 1 min; then seeds were immersed in 0.6% (v/v) sodium hypochlorite for 20 min. The seeds were washed 3 times with deionized sterilized water. The tips of the seeds were gently cut off with a razor to disrupt testa and induce germination. Then, the seeds were transferred to Petri dishes with agar containing the MS medium. After 2 weeks, seedlings were transferred and cultivated in vitro in glass jars with agar containing 1/3 MS medium and activated charcoal. Both seeds and in vitro cultures were placed in a growth chamber under the following conditions: 16 h light/8 h dark, 40 µmol m^{-2} s^{-1} at 23 °C.

Gamma irradiation and flow-cytometric (FCM) detection of holocentric chromosomes

Approximately half of the specimens (*A. vesiculosa*) or glass jars (remaining species) were randomly chosen for gamma irradiation with a 150 Gy dose (Cobalt-60, Bioster, Czech Republic) to induce chromosomal fragmentation, while the rest were kept as a control that was not exposed to gamma irradiation. As soon as the irradiated samples formed new tissues, typically after 2 weeks, we conducted FCM measurements.

Only the newly grown tissues from irradiated and non-irradiated control samples were subjected to FCM. We performed FCM analyses on a CyFlow ML flow cytometer (Partec, Germany) that was equipped with a UV-LED diode excitation source. We used a DAPI fluorochrome, applying a 2-step sample-preparation procedure (Otto 1990) and following the protocol developed for genome size measurements in Droseraceae (Veleba et al. 2017). We chose DAPI because it produces low background noise, but other fluorochromes, such as propidium iodide, may also be used. For each species, all of the irradiated and control samples were measured in a random order within a single day. For each sample, we measured 10,000 nuclei in total and we recorded the number of nuclei in the G1 and G2 peaks (FloMax software, Partec, Germany). The upper and lower boundaries of the G2 peak were always



Summary of the results		
Species	**Chromosomes**	**G2/G1 change**
A. vesiculosa	holocentric	1.03
D. muscipula	holocentric	0.79
D. tokaiensis	holocentric	1.10
D. ultramafica	holocentric	0.84
D. lusitanicum	monocentric	1.59

Fig. 2 Results of flow-cytometric determination of chromosomal structure. The comparison of G2/G1 ratio between control and irradiated plants is shown in box-plot graphs for each species. The significance of the Mann–Whitney *U* test is indicated by a *p* value below each graph. The value *N* refers to the number of measured samples. The table at the bottom right summarizes chromosomal structures that were determined for each species. The column "G2/G1 change" shows the average change in G2/G1 ratio in irradiated samples relative to non-irradiated controls and was calculated as the mean of G2/G1 in irradiated samples divided by the mean of G2/G1 in control samples

set manually and calculated as twice the upper and lower boundaries of the G1 peak to ensure comparability across samples. Finally, we calculated the G2/G1 ratio. Examples of flow histograms showing calculations of the G2/G1 ratios are supplied in Online Resource 1. Statistical differences between the G2/G1 ratios of the irradiated and control sample sets were tested using Mann–Whitney *U* tests.

Results

Using flow cytometry, we measured the G2/G1 ratio in 167 irradiated and control samples from *Aldrovanda vesiculosa* (13 irradiated and 15 control samples), *Drosera tokaiensis* (18 irradiated and 16 control samples), *Drosera ultramafica* (17 irradiated and 16 control samples), *Dionaea muscipula* (18 irradiated and 18 control samples), and *Drosophyllum lusitanicum* (18 irradiated and 18 control samples). The G2/G1 ratio of each sample is supplied in Online Resource 2. The results are summarized in Fig. 2.

Aldrovanda vesiculosa, with a previously unknown chromosomal structure, has been determined to be holocentric because its G2/G1 ratio did not increase in irradiated plants. Also *Drosera ultramafica* and *Drosera tokaiensis* did not show an increase in the G2/G1 ratio in irradiated plants and, therefore, their chromosomes were determined to be holocentric. *Drosophyllum lusitanicum* showed a significantly increased G2/G1 ratio in irradiated plants ($p = 0.008$, Mann–Whitney *U* test), which agrees with the expectation of monocentric chromosomes in this species. Moreover, the average G2/G1 ratio was 1.59 times higher in irradiated samples of *D. lusitanicum* relative to the control samples, which is above the previously suggested threshold of 1.5 for monocentric chromosomes (Zedek et al. 2016). However, *Dionaea muscipula*, which was also expected to be monocentric, did not show any difference between irradiated and control samples (Fig. 2), suggesting that its chromosomes are, in fact, holocentric.

Discussion

We confirmed monocentric chromosomes in *Drosophyllum lusitanicum* (Drosophyllaceae) and identified all four Droseraceae species as having holocentric chromosomes (Fig. 2). In particular, we found evidence for holocentric chromosomes in *Aldrovanda vesiculosa*, which is consistent with a previous study suggesting that primary constriction is missing in this species (Shirakawa et al. 2011a). We expected holocentric chromosomes in *Drosera tokaiensis* and *Drosera ultramafica* because sundews were identified as holocentric in previous studies (see Introduction). The finding of holocentric chromosomes in *Dionaea muscipula* (Fig. 2) contradicts previous reports that suggested monocentric chromosomes in this species (Hoshi and Kondo 1998; Shirakawa et al. 2011a).

However, the evidence for monocentric chromosomes in *D. muscipula* was based only on differential staining of mitotic metaphase chromosomes with chromomycin A3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI; Hoshi and Kondo 1998; Shirakawa et al. 2011a). In those papers, the authors observed weaker CMA and stronger DAPI signals in the central parts of chromosomes, which indicated localized centromeres. A similar observation also led to the suggestion of monocentric chromosomes in *Drosera regia* (Shirakawa et al. 2011b). However, these markers bind to GC (CMA)- and AT (DAPI)-rich regions in the minor groove of DNA and, therefore, are not inherently centromeric markers. Moreover, the same or very similar patterns of CMA and DAPI staining can also be seen in holocentric chromosomes of both plants (Guerra and García 2004) and animals (Kaur et al. 2012; Bardella et al. 2014). These observations strongly indicate that differential staining with DAPI and CMA is not a reliable marker to distinguish between holocentric and monocentric chromosomes.

Another marker that suggested monocentric chromosomes in *Drosera aliciae*, *D. binata*, and *D. rotundifolia* was the histone H2A phosphorylated at threonine 120 (Demidov et al. 2014). But the regular inheritance of induced chromosomal fragments in *D. rotundifolia* (Shirakawa et al. 2011a) is very strong evidence for holocentric chromosomes. Provided the specimens of *D. rotundifolia* analyzed by Demidov et al. (2014) and Shirakawa et al. (2011a) were not misidentified, these observations cast doubt on the reliability of H2AThr120ph as a marker for holo/monocentric distinction.

Although DAPI, CMA, and H2AThr120ph appear to be unreliable markers for distinguishing between holocentric and monocentric chromosomes, it is still possible that *D. muscipula* is monocentric and the flow-cytometric method has simply failed to detect it. In the two weeks that elapsed between irradiation and flow cytometry (see Methods), the chromosomal fragments may have repaired themselves enough so that the difference between irradiated and control plants would be lost. However, Shirakawa et al. (2011a) reported that *Drosera petiolaris* and *D. rotundifolia* that had been gamma-irradiated with 50 Gy showed chromosomal aberrations in more than 90% of cells 120 days after exposure. Similarly, doses of 5 and 30 Gy led to weeks-long persistence of chromosome abberations and fragments in *Chionographis japonica* (Tanaka and Tanaka 1977) and *Luzula elegans* (Jankowska et al. 2015), respectively. Because the dose of 150 Gy we

used for *D. muscipula* was much higher, it is reasonable to expect that a measurable difference between irradiated and control plants should not disappear.

Taking previous reports and our results together, we conclude that there is stronger evidence for holocentric than for monocentric chromosomes in Droseraceae. However, given the conflicting reports on mono/holocentrism in *Drosera rotundifolia* (see above) and possibility of switches between monocentrism and holocentrism even within a genus (see below), monocentric chromosomes in *Drosera* cannot be ruled out until more detailed analyses employing multiple methods are done. Because we identified holocentric chromosomes also in *Aldrovanda vesiculosa* and *Dionaea muscipula*, holocentrism may be an apomorphy of the entire family Droseraceae and we propose to consider this in future comparative studies addressing the evolutionary significance of holocentric chromosomes. However, it still remains unclear whether holocentric chromosomes are an ancestral or a derived state in the entire clade of Droseraceae and closely related families, because data on chromosome structure from Nepenthaceae, Ancistrocladaceae, and Dioncophyllaceae are lacking (Fig. 1).

Similar uncertainties about ancestral states are present also in other plant taxa in which holocentric chromosomes have been found, including algae (Charophyta; Godward 1966), basal angiosperms (family Hydatellaceae; Kynast et al. 2014), magnoliids (family Myristicaceae; Flach 1966), monocots (tribe Chionographidae from Melanthiaceae; Tanaka and Tanaka 1977), and eudicots (the genus *Cuscuta* from Convolvulaceae; Pazy and Plitmann 1994). Moreover, because back and forth transitions between holocentrism and monocentrism can happen (Melters et al. 2012; Escudero et al. 2016), it is possible that holocentric species are more common than currently thought but are intermingled with monocentric species at finer phylogenetic scales, e.g., within a genus as in *Cuscuta* (Pazy and Plitmann 1994) or within a family as in Melanthiaceae (Tanaka and Tanaka 1977). Although many studies conducted over past decades provided chromosome counts for approximately 70,000 plant species (ca 20–25% of plant species; Rice et al. 2015), only a minority of them inspected chromosome structure in order to determine whether chromosomes were monocentric or holocentric, and detailed sophisticated cytogenetic studies have been restricted to a few model taxa, among holocentrics mainly from Cyperaceae (e.g., Marques et al. 2015, 2016) and Juncaceae (e.g., Heckmann et al. 2011; Jankowska et al. 2015). We attempted to elucidate the ambiguity of chromosome types in Droseraceae, but further studies are needed to resolve the distribution of holocentric chromosomes in plants. Such studies should be based on the differential reaction of holocentric and monocentric organisms to chromosome-breaking factors (i.e., checking cell cycle responses or behavior of chromosomal fragments) as the tolerance to fragmentation is an undoubtable hallmark of holocentrism. Also, immunostaining of kinetochore proteins, such as CENH3, which should be distributed along the length of holocentric chromosomes, may shed more light into the chromosomal structure of Droseraceae and other families.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement No human participants or animals were involved in this research.

Information on Electronic Supplementary Material

Online Resource 1. Examples of flow histograms with the calculations of the G2/G1 ratios.
Online Resource 2. G2/G1 ratios of all analyzed samples.

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