Flora 257 (2019) 151415

Contents lists available at ScienceDirect

Flora

journal homepage: www.elsevier.com/locate/flora

Higher benefits of clonal integration in rhizome-derived than in frond-derived ramets of the tropical fern *Bolbitis heteroclita*

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ARTICLE INFO

Edited by Hermann Heilmeier

Keywords:
Clonal reproduction
Physiological integration
Resource sharing
Rhizomatous plant
Tropical seasonal forest

ABSTRACT

Clonal integration between connected ramets within genets can increase their survival and growth. Different clonal plants form ramets in a variety of ways, and a number of species form more than one type of ramets. We tested for the first time whether effects of clonal integration can differ between different types of ramets within the same species. We compared effects of severing connections between ramets (i.e. preventing clonal integration) on the survival and growth of ramets produced along rhizomes and at the tips of fronds in a natural population of the fern *Bolbitis heteroclita* in a tropical seasonal rainforest in southwestern China. Severance strongly reduced the survival of both rhizome-derived and frond-derived ramets but reduced survival and the growth of surviving ramets much more in rhizome- than in frond-derived ramets. Rhizome-derived ramets were larger, allocated less dry mass to belowground structures, and had greater cross-sectional area of connecting vascular tissue. These results suggest that clonal integration increases performance of both rhizome- and frond-derived ramets of *B. heteroclita*, but that benefits of integration are greater in rhizome-derived ramets. This may be due to greater conductance in the connections to rhizome-derived ramets.

1. Introduction

Many plants are capable of clonal growth, defined as the production of vegetative offspring that remain physically connected to the parent at least until the offspring establish (i.e., become able to directly acquire sufficient resources to survive on their own; de Kroon and van Groenendael, 1997; Brezina et al., 2006; Klimesova and Klimes, 2008). This results in a set of potentially independent but physically attached units, termed ramets, of the same genetic individual (Jackson et al., 1985; van Groenendael and de Kroon, 1991; Zhou et al., 2017). Forms of asexual reproduction in which offspring detach before establishment, such as apomixis or vivipary, offer greater potential for the dispersal of offspring, but clonal growth uniquely confers the potential for physiological integration of ramets via the transport of signals or of resources such as photosynthates, water, or nutrients through their connecting organs (Jackson et al., 1985; van Groenendael and de Kroon, 1991; de Kroon and van Groenendael, 1997). Such clonal integration has been widely shown to increase the individual and combined performance of ramets and clones in both terrestrial and aquatic ecosystems (Roiloa et al., 2013; Touchette et al., 2013; Luo et al., 2014; Chen et al., 2015; Adonsou et al., 2016; Duchoslavová and Weiser, 2017).

The extent of clonal integration and its effects on survival, growth, and reproduction vary between and within clonal plant species (Alpert, 1999; Pennings and Callaway, 2000; Roiloa et al., 2014a; Wang et al., 2017a,b). For example, different species show different effects of clonal integration on growth and competitive ability (Pennings and Callaway, 2000; Schwarzschild and Zieman, 2008; Xu et al., 2010; He et al., 2011). Different genotypes within species can show different capacities for resource sharing and induction of division of labor (Alpert, 1999; Alpert et al., 2003; Roiloa et al., 2014a).

One possible source of differences between clonal integration in different plant species is that different species grow clonally via different organs (e.g., Klimesova and Klimes, 2008). Many species grow clonally via roots, rhizomes, or stolons (Klimes et al., 1997; Xu et al., 2012; Song et al., 2013). Other organs of clonal growth include aerial stems or leaves that contact the ground and root (Klimes et al., 1997). A number of species have two forms of clonal reproduction (Klimes and Klimesova, 1999), such as both rhizomes and stolons (Dong and de

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https://doi.org/10.1016/j.flora.2019.06.001
Received 25 January 2019; Received in revised form 14 May 2019; Accepted 3 June 2019

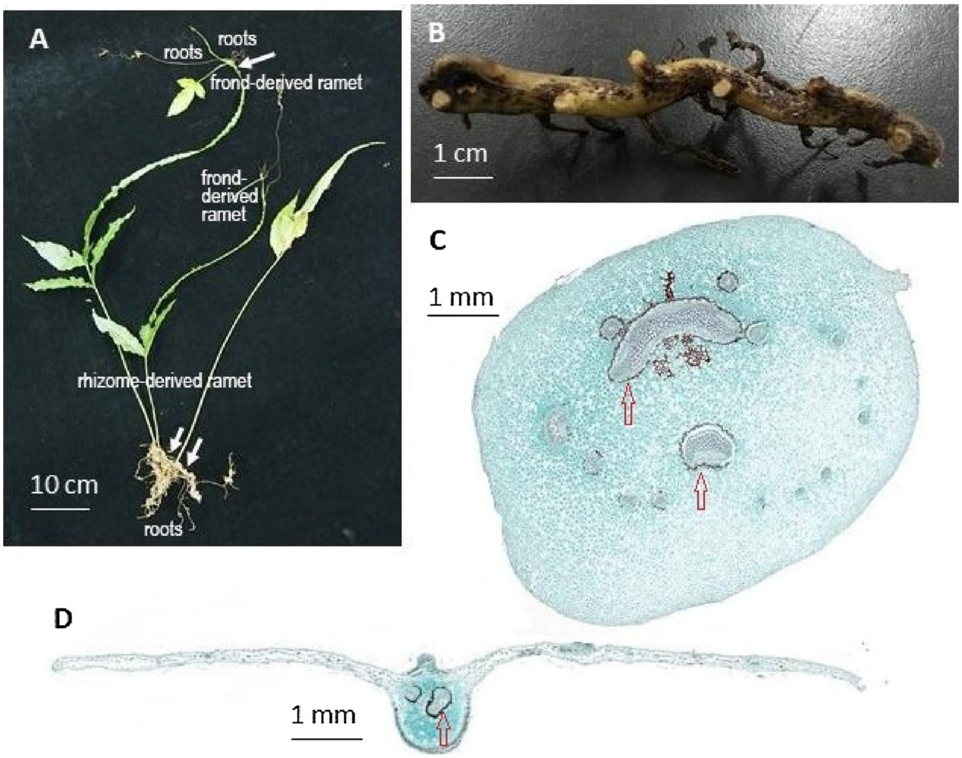

Fig. 1. Morphology and anatomy of *Bolbitis heteroclita*: (A) a group of three connected rhizome-derived ramets and two frond-derived ramets each connected to a frond, with arrows to show typical points of severance of frond- and rhizome-derived ramets; (B) rhizome with fronds and fine roots removed to show spacing of ramets; light micrographs of cross sections of (C) a rhizome at a point of severance of a rhizome-derived ramet and (D) a frond tip at a point of severance of a frond-derived ramet. Arrows show vascular tissue of rhizome and frond in C and D.

Kroon, 1994; Skálová et al., 1997; Sosnova et al., 2010). These various organs are likely to differ in characteristics that could affect clonal integration, such as conductance (i.e., flows of water, nutrients, and photosynthates at a given driving force for flow) and storage capacity (amount of nutrients and photosynthates that can be stored), so it seems plausible that species with different modes of clonal growth will tend to show different effects of integration (Song et al., 2013).

Nearly all work on clonal integration in plants so far has focused on species that grow clonally via either rhizomes or stolons (Klimesova and Klimes, 2008; Roiloa et al., 2014b; Lin et al., 2018; Wang et al., 2017a,b; Wei et al., 2018). A recent meta-analysis by Song et al. (2013) found no consistent difference between the effects of clonal integration in rhizomatous and stoloniferous species. However, no study appears to have tested for differences in effects of clonal integration between different types of ramet produced by the same species or to have tested effects of clonal integration on ramets produced on leaves.

To test whether effects of clonal integration differ between ramets produced on rhizomes and at the tips of leaves in the same species, we conducted a field experiment on a common fern, *Bolbitis heteroclita* (Presl) Ching, in a tropical forest in China. Most ferns grow clonally via rhizomes (Lu, 2007; Wolf et al., 2009; Guo et al., 2011). A few species of ferns also grow clonally via fronds, by forming foliar buds, or bulbils, that can develop roots (Moran, 2004; Mehltreter et al., 2010). In some cases, these buds detach before rooting and function as vagile propagules. In other cases, the buds root in the soil while still attached, allowing for potential clonal integration between the parental frond and the offspring ramet.

The use of a tropical fern in China as the study species enhanced the ecological interest of the test for several reasons. First, ferns, or members of the Polypodiopsida, are an important component of tropical and subtropical forest biodiversity (Zhu, 1992; Schneider et al., 2004; Watkins et al., 2010), especially in China, which has more than 2000 species of ferns, accounting for about one sixth of ferns worldwide (Yan et al., 2013; Zhou et al., 2016). Second, ferns play important roles in forest regeneration and nutrient fluxes (Mehltreter et al., 2010; Umana and Wanek, 2010). Third, ferns have rarely been studied for effects of clonal integration (Lu et al., 2015, 2016). To heighten the realism of the study, we conducted the experiment on a natural population in a relatively undisturbed habitat.

We hypothesized 1) that the rhizome-derived and frond-derived ramets of *B. heteroclita* are both physiologically integrated with their parental ramets, but 2) that the effect of clonal integration differs between the two types of ramets. We accordingly predicted 1) that severing the connection between an established offspring ramet and its parental ramet to prevent integration would decrease performance as measured by survival, accumulation of mass, and size of fronds in both rhizome-derived and frond-derived ramets, but 2) that the effects of severance on performance would differ between the two types of offspring. Since rhizomes seem likely to have greater conductance and storage capacity than fronds, one might expect that clonal integration would be more pronounced and that the negative effects of severance would be greater in rhizome-derived than in frond-derived ramets. However, the fronds of frond-derived ramets tend to be smaller than those of rhizome-derived ramets in *B. heteroclita*, suggesting that frond-derived ramets might depend more upon the parental ramet and thus suffer greater negative effects of severance.

2. Materials and methods

2.1. Site and species

The study was performed in a seasonal tropical rainforest in Xishuangbanna Tropical Botanical Garden (21° 54′ N, 101° 46′ E; 580 m a.s.l.), Menglun, southwestern Yunnan Province, China. Annual mean temperature at the garden is 21.8 °C. Annual precipitation is 1493 mm, of which 84% falls from May to October (Zhu, 1992). Fog occurs on a mean of 116 days per year and plays an important role in easing drought stress during the dry season (Liu et al., 2010). Soils are mainly acidic laterites, with a pH of 4.5–5.5. Species richness of woody plants and epiphytes is high (Zhang and Cao, 1995).

Bolbitis heteroclita is one of the most common ferns in this seasonal tropical rainforest (Lv et al., 2011; Fig. 1A). This perennial, evergreen, rhizomatous species grows on soil, rocks, and trees at elevations from 50 to 1500 m in much of southern China (Flora of China, http://www.

efloras.org/flora_page.aspx?flora_id = 2). The genus *Bolbitis* includes 20 species and 3 hybrids in China and belongs to the family Bolbitidaceae (Dong and Zhang, 2005). The rhizomes of *B. heteroclita* are robust, about 3 to 6 mm in diameter, and bear roots and sterile or fertile fronds about 1–3 cm apart (Fig. 1B). Sterile fronds on rhizomes are 15 to 80 cm long, with a stipe 10–30 cm long. Together with their attached rooted rhizome, these sterile fronds function as rhizome-derived ramets. Rhizomes have well-developed vascular tissue (Fig. 1C) that can transport resources and signals between ramets along the same rhizome. Sterile fronds often produce an apical bud that can grow into a new frond with adventitious roots. The tip of the parental frond generally bends down to the ground, allowing the offspring frond to root in the soil and function as a frond-derived ramet. Fronds borne at the tip of other fronds are 5 to 40 cm long and so mostly shorter than fronds borne directly on rhizomes. Like rhizomes, the midrib of frond tips is well vascularized and so potentially able to transport resources and signals between a parental frond and its offspring ramet (Fig. 1D). However, the cross-sectional area of the conducting tissue is less in frond tips than in rhizomes.

2.2. *Experimental design*

On 1 July 2014, we located three areas about 20–40 m apart where *B. heteroclita* was abundant and centered a 20 m × 30 m plot on each area. Within the three plots combined, we located all the rhizome-derived ramets that were rooted, were still connected by the rhizome to an older ramet, were the youngest ramet on the rhizome, and had not yet produced any offspring ramets. We then chose the 60 of these rhizome-derived ramets that were most similar in size for experimental use. We similarly located all the frond-derived ramets that were rooted, were situated at frond tips, were still connected to the parental frond, and had not yet produced offspring, and chose the 60 of these frond-derived ramets that were most similar in size. We measured the length of the frond of each selected ramet and randomly assigned half of the ramets of each type to each of two severance treatments, severed and intact.

Severance of clonal connections has been widely used to manipulate clonal integration and test for its effects (e.g., de Kroon and van Groenendael, 1997; Alpert et al., 2003; Song et al., 2013). One caveat associated with this method is that severance might affect plants in ways other than by preventing clonal integration, such as by inducing damage responses or increasing vulnerability to infection. Alpert (1991) directly tested this by comparing effects of severing developmentally similar ramets in a homogeneous environment, in which case integration would be expected to have no net effect through transfers of resources or signals, and in a heterogeneous environment; the finding was that severance had no effect on plant performance under uniform conditions and decreased performance under heterogeneous ones, suggesting that severance did not introduce confounding effects at least in the species studied.

To sever a rhizome-derived ramet, the rhizome was carefully exposed and cut halfway between the ramet and the next older ramet, and halfway between the ramet and the tip of the rhizome (Fig. 1A). In the intact treatment, the rhizome was similarly exposed but not cut. To sever a frond-derived ramet, the tip of the parental frond was cut about 1 cm away from the ramet in between the parental frond and the ramet (Fig. 1A).

2.3. *Measurements*

After 90 days, on 28 September 2014, we measured the maximum quantum yield of photosystem II (F_v/F_m) on a fully developed, healthy pinna of each surviving ramet (n = 36 for rhizome-derived ramets and 32 for frond-derived ramets) as an indicator of plant stress, using a portable fluorometer (FMS-2; Hansatech, Norfolk, UK; saturation pulse method). Measurements were conducted at 08:00–12:00 h after a dark adaptation period of at least 30 min (Lu et al., 2016). F_v/F_m is a widely used proxy for plant stress (e.g., Roiloa and Retuerto, 2006), which may be alleviated by clonal integration (e.g., You et al., 2014).

On 29 September 2014, we measured the length and width of the frond of each surviving ramet and separated each ramet into aboveground parts (frond) and belowground parts (rhizome and roots). Frond-derived ramets had roots but had not yet produced a rhizome as defined by visible elongation of an underground stem. We dried the plant parts at 70 °C for 48 h and weighed them. We measured both mass and size because, while net accumulation of mass may be a better measure of performance, size could be non-destructively, repeatedly measured to give a direct indication of growth during the treatment period.

To add information on the anatomy of the connections between rhizome- and frond-derived ramets and their parental ramets, four connections of each type were collected on 20 March 2018 from the areas used for the experiment and fixed in a standard solution of formalin, acetic acid, and ethanol (FAA) for 24 h. Rhizomatous connections were softened in a 1:1 (v:v) mixture of glycerin and ethanol after fixation. All samples were then dehydrated in a tertiary butanol series, embedded in paraffin, cut into 6–8 μm sections on a microtome, and stained with safranin and fast green.

2.4. *Data analysis*

We used a *t*-test to determine whether initial frond length differed between rhizome- and frond-derived ramets. Effects of initial frond length (covariate), severance (severed or intact), ramet type (rhizome- or frond-derived), and interaction of severance and ramet type on the survival of ramets were tested with logistic regression. Repeated-measure ANOVA was used to test effects of time (start and end of experiment, repeated measure), severance (fixed effect), ramet type (fixed effect), and interactions between factors on the frond length of ramets that survived. Two-way ANOVAs were used to test effects of severance, ramet type, and their interaction on F_v/F_m, final frond width, ratio of belowground to aboveground mass, and final total, aboveground, and belowground mass of ramets that survived. Measurements of mass and ratio of belowground to aboveground mass were log-transformed before analysis to remove heteroscedasticity and increase normality. Data on frond size and F_v/F_m did not require transformation. Statistical analyses were carried out with SPSS 19.0 (IBM, Armonk, NY, USA). Figures show untransformed data.

3. Results

If left intact, nearly all of the rhizome-derived ramets and about three-fourths of the frond-derived ramets survived (Fig. 2A). Consistent with our first prediction, severance reduced survival of both types of ramets by more than half (effect on survival, logistic regression: severance - $\chi^2 = 47.90$, $P < 0.001$). Consistent with our second prediction, severance reduced survival more in rhizome- than in frond-derived ramets (Fig. 2A; effects on survival, logistic regression: ramet type - $\chi^2 = 2.61$, $P = 0.9$; severance × type - $\chi^2 = 5.29$, $P = 0.02$).

Initial frond size was not related to mortality (Fig. 2; effect on survival, logistic regression: initial frond length, as covariate - $\chi^2 = 0.12$, $P = 0.7$). Across severed and intact treatments, initial frond length was about twice as great in rhizome-derived (mean ± SE: 21.2 ± 0.4 cm) as in frond-derived ramets (10.2 ± 0.3 cm; $t_{118} = 22.0$, $P < 0.001$).

Among ramets that survived, severance reduced final total mass more in rhizome-derived than in frond-derived ramets (Fig. 3A, Table 1). Severance decreased final belowground mass by about 50% in both types of ramets (Fig. 3C, Table 1), but decreased aboveground mass much more in rhizome- than in frond-derived ramets (Fig. 3D, Table 1). The ratio of belowground to aboveground biomass was not affected by severance (Table 1), but was about twice as high in frond-derived ramets as in rhizome-derived ramets (Fig. 3B). Severance had

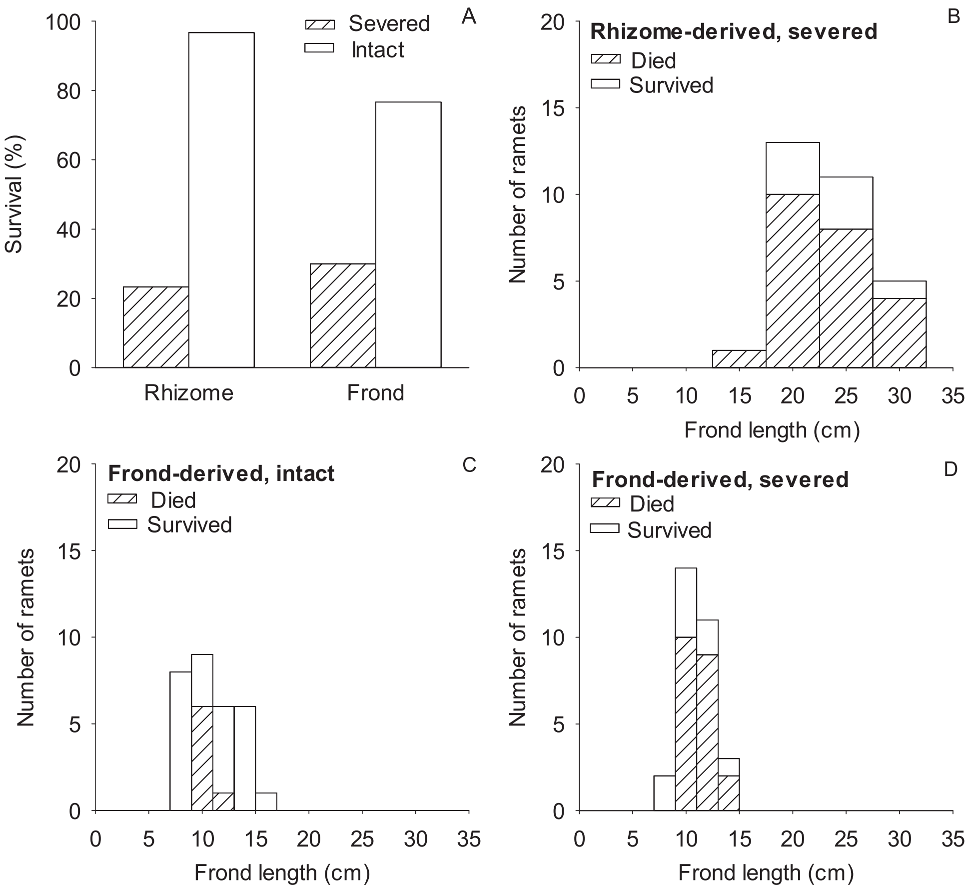

Fig. 2. Survival of severed and intact rhizome- and frond-derived ramets: (A) percent survival; survival as a function of initial frond length in (B) rhizome-derived ramets that were severed, (C) frond-derived ramets that were left intact, and (D) frond-derived ramets that were severed. Rhizome-derived ramets that were left intact are not shown because survival was 97%. See text for statistical analysis.

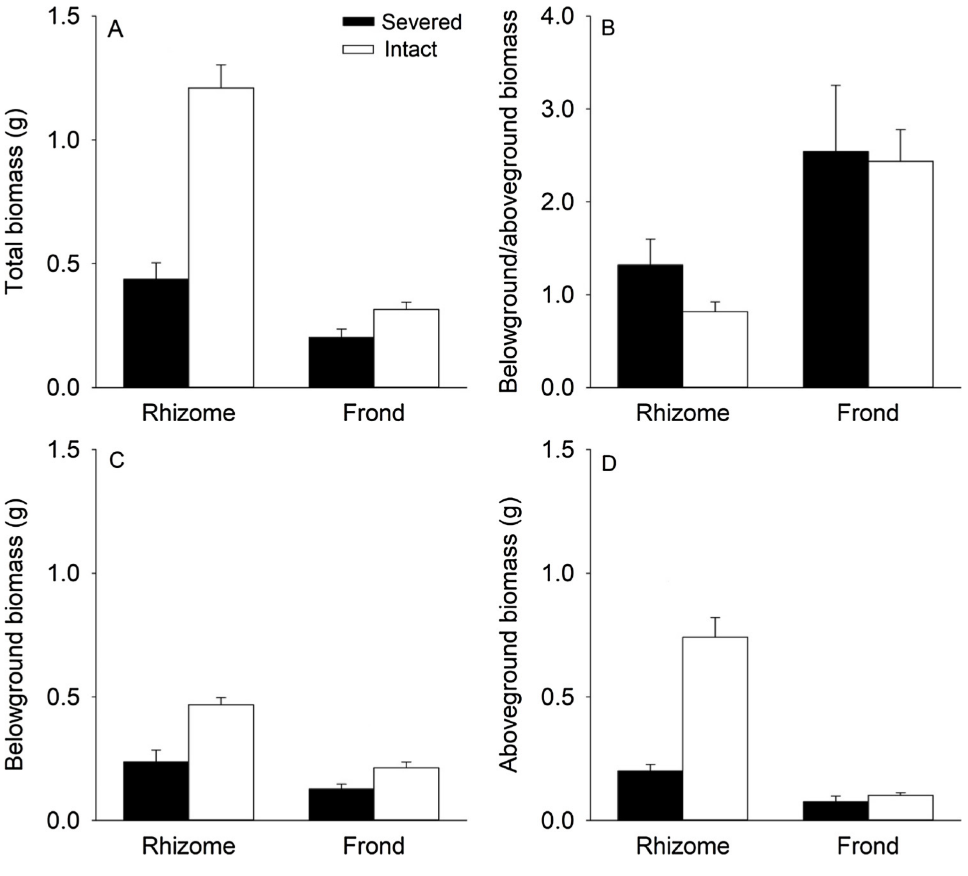

Fig. 3. Effects of ramet type (Rhizome ‐ rhizome-derived; Frond ‐ frond-derived) and severance on final biomass of ramets of *Bolbitis heteroclita*: (A) total, (B) ratio of belowground to aboveground, (C) belowground, and (D) aboveground. Bars show mean + SE (n = 7 for severed rhizome-derived ramets, 29 for intact rhizome-derived ramets, 9 for severed frond-derived ramets, and 23 for intact frond-derived ramets). See Table 1 for results from ANOVAs.

Table 1
Effects of ramet type (rhizome- or frond-derived) and severance on final total, aboveground, and belowground dry biomass, ratio of belowground to aboveground biomass, final frond width, and F_v/F_m of surviving ramets of *Bolbitis heteroclita.* Values are in bold if $P < 0.05$.

	Type (T)		Severance (S)		T × S	
	$F_{1,64}$	P	$F_{1,64}$	P	$F_{1,64}$	P
Total biomass	**73.34**	**< 0.001**	**34.25**	**< 0.001**	**5.27**	**0.003**
Aboveground biomass	**92.33**	**< 0.001**	**29.18**	**< 0.001**	**6.04**	**0.017**
Belowground biomass	**28.27**	**< 0.001**	**18.71**	**< 0.001**	0.89	0.350
Belowground/ aboveground	**18.67**	**< 0.001**	1.68	0.199	2.23	0.140
Frond width	**26.68**	**< 0.001**	**16.39**	**< 0.001**	**12.76**	**0.001**
F_v/F_m	**12.25**	**0.001**	0.03	0.87	0.12	0.726

no effect on F_v/F_m (Table 1), but F_v/F_m was significantly higher in rhizome-derived (0.784 ± 0.005) than in frond-derived ramets (0.754 ± 0.005).

Fronds increased in length during the experiment in all treatments, but increased much more in intact, rhizome-derived ramets than in other treatments (Fig. 4A, Table 2). Severance reduced growth in frond length by about 80% in rhizome-derived ramets, but did not reduce growth in frond length in frond-derived ramets (Fig. 4A). Final frond length and width showed qualitatively similar but quantitatively smaller effects of severance (Fig. 4B and C): frond length and width were about 40% less in severed than in intact rhizome-derived ramets but did not differ between severed and intact frond-derived ramets.

4. Discussion

As predicted, severance decreased performance of both rhizome- and frond-derived ramets of the fern *Bolbitis heteroclita*. In both types of ramets, cutting off the connection to the parental ramets strongly decreased survival. Results support the hypothesis that both types of ramets are physiologically integrated with their parental ramets and suggest that clonal integration can increase performance in natural populations of *B. heteroclita.*

This appears to be the first test for clonal integration of ramets derived from fronds in ferns or from leaves in any plants. The limited, previous studies on ramets derived from rhizomes in ferns consistently show positive effects of clonal integration on their performance (Lau and Young, 1988; Railing and McCarthy, 2000; Du et al., 2010; Lu et al., 2015, 2016). These include studies on natural populations of the terrestrial ferns *Lycopodium flabelliforme* (Lau and Young, 1988), *Diplopterygium digitatum* (Railing and McCarthy, 2000), and *D. glaucum* (Du et al., 2010). Lu et al. (2015, 2016) found that connection between rhizome-derived ramets contributed to both survival and growth of epiphytic ferns in a montane, tropical forest.

Preventing clonal integration had no effect on Fv/Fm in *B. heteroclita*. This was consistent with a previous study on the fern *D. glaucum* (Du et al., 2010). However, Lu et al. (2015, 2016) did find positive effects of clonal integration on F_v/F_m in the epiphytic ferns *Polypodiodes subamoena*, *Lepisorus scolopendrium*, and *Selliguea griffithiana*, and such positive effects have been reported in some angiosperms (e.g., Roiloa et al., 2014b).

Table 2
Effects of time, severance, and ramet type (rhizome- or frond-derived) on frond length of surviving ramets of *Bolbitis heteroclita.* Values are in bold if $P < 0.05$.

Effect	$F_{1,46}$	P
Time	**45.17**	**< 0.001**
Severance	**7.45**	**0.008**
Type	**90.66**	**< 0.001**
Severance × Type	**5.78**	**0.019**
Time × Severance	**9.73**	**0.003**
Time × Type	**12.79**	**0.001**
Time × Severance × Type	**11.01**	**0.001**

Also as predicted, effects of severance on performance differed between rhizome- and frond-derived ramets. Severance decreased survival and final net accumulation of mass more in rhizome-derived than in frond-derived ramets and decreased the final length and width of fronds only in rhizome-derived ramets. As far as we know, these results provide the first direct evidence that the benefits of clonal integration differ between forms of clonal reproduction within the same species. In *B. heteroclita*, clonal integration appears to increase performance more in rhizome-derived than in frond-derived ramets.

One possible explanation is that characteristics of the connecting organ determine potential benefits of clonal integration. For example, parental rhizomes are likely to have higher storage capacity and conductance than parental fronds. Dong et al. (2010) showed that stem internodes can provide resources that increase the growth of new ramets. Conductance of water between ramets may be an especially important aspect of clonal integration in ferns, which tend to have lower vascular water conductance than angiosperms, at least as measured in the stipes of fronds (Brodribb et al., 2005; Mehltreter et al., 2010; Watkins et al., 2010). Cross-sections of connections in *B. heteroclita* suggest that conductance from parental to offspring ramets is higher in rhizome-derived than in frond-derived ramets. Whether severed or connected, frond-derived ramets of *B. heteroclita* had a greater ratio of belowground to aboveground dry mass than rhizome-derived ramets despite having no rhizome. This could reflect greater allocation to acquisition of water in frond-derived ramets to compensate for more limited conductance of water from connected ramets.

A second explanation for the difference between the effects of clonal integration in different forms of clonal growth is that they have been selected to function differently, like different forms of reproduction more generally. Independent of connection to the parental ramet, rhizome-derived ramets of *B. heteroclita* had greater dry mass, longer fronds, and higher maximum quantum yield of photosystem II than frond-derived ramets. When left connected to the parental ramet, rhizome-derived ramets also had higher survival than frond-derived ramets. A number of clonal species that have more than one form of

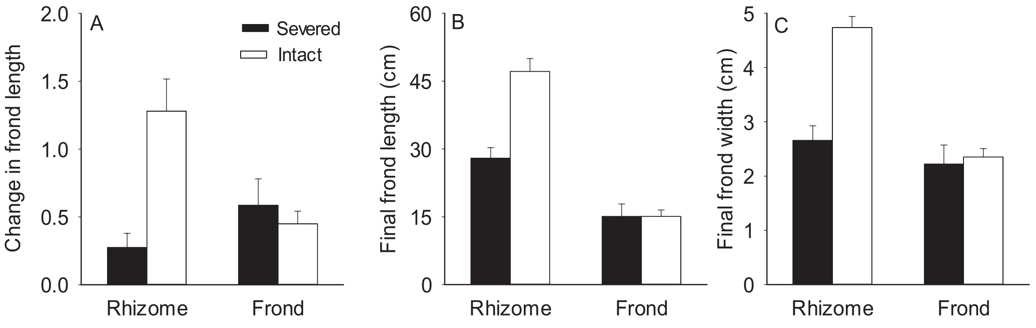

Fig. 4. Effects of ramet type (Rhizome – rhizome-derived; Frond – frond-derived) and severance on (A) relative change in frond length between initial and final measurements and (B) final frond length and (C) final frond width in surviving ramets of *Bolbitis heteroclita.* Bars show mean + SE (n = 7 for severed rhizome-derived ramets, 29 for intact rhizome-derived ramets, 9 for severed frond-derived ramets, and 23 for intact frond-derived ramets). See Tables 1 and 2 for results from ANOVAs.

clonal growth likely produce ramets of different sizes on different organs, though this has not been quantified, and this may reflect differential investment in different types of ramets (Jitka Klimesova, personal communication based on CLO-PLA, a database of clonal growth in plants, http://clopla.butbn.cas.cz). *Bolbitis heteroclita* may invest more in rhizome-derived ramets than in frond-derived ramets. Since rhizome-derived ramets spread by centimeters whereas frond-derived ramets spread by tens of centimeters, this could represent a trade-off between growth and dispersal, part of a longer continuum from rhizome-derived ramets to frond-derived ramets to vagile bulbils to spores.

Further work could explore whether differences between ramet types vary between genotypes within species or between ramets in different positions within groups of connected ramets. Although this study used ramets collected tens of meters apart and so potentially from different clones, we did not test for clonal identity or whether different clones show different contrasts between ramet types. To make ramet types as comparable as possible in developmental stage, we used the youngest rhizome-derived ramets along rhizomes. Effects of integration might be different in older rhizome-derived ramets connected to a number of proximal and distal ramets. Another question is whether connections between different types of ramets persist for different lengths of time; there appear to be no data on the relative longevity of types of connections between ramets in clones.

A number of other fern species in the study region such as *Asplenium prolongatum, Camptosorus sibiricus*, and *Tectaria fauriei* also reproduce clonally both by rhizomes and by rooting at the tips of fronds (Li-Min Zhang and Hua-Zheng Lu, personal observations). There is now opportunity and reason to further test whether the differences between forms of clonal growth shown in *B. heteroclita* obtain more generally among species and to begin to test whether such differences reflect the constraints imposed by different organs or selection for multiple types of clonal offspring with contrasting functions. If the second appears true, this could lead the way to new work on life-history strategy in plants.

5. Conclusions

We conclude that clonal integration can greatly increase the survival and growth of both rhizome- and frond-derived ramets in natural populations of *B. heteroclita,* and that integration can have a larger effect on rhizome- than on frond-derived ramets in this species. This provides the first experimental evidence that effects of clonal integration can differ between ramet types within a species and the first test of the effect of clonal integration on the performance of ramets produced on leaves.

Acknowledgements

We thank Yuan-Xiang Lu and Yu-Xian Hu for assistance with field work, the Xishuangbanna Station for Tropical Forest Ecosystem Studies for use of the study site, the Xishuangbanna Tropical Botanical Garden Central Laboratory for use of equipment, and Jitka Klimesova, Zuzana Munzbergova, and three anonymous reviewers for comments. This work was supported by the Open Fund of the Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences; and by grants from the National Natural Science Foundation of China (No. 31872685, 31670452 and 31300459) and the CAS 135 Program (No. 2017XTBG-F03).

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