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Investigations into Biological Control for Common Reed and Flowering Rush

Patrick Häfliger, Carol Ellison, and Hariet L. Hinz

August 2019

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Investigations into Biological Control for Common Reed and Flowering Rush

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Abstract

The noctuid moths *Archanara geminipuncta* and *A. neurica* were selected as the most promising candidates for biological control of common reed. Complete development was possible on the native North American subspecies *P. australis americanus*. However, open-field oviposition tests showed a strong preference of female moths for both European and introduced *P. australis*. An egg overwintering experiment also showed that neither of the two moth species will survive at latitudes which correspond to regions where the subspecies *P. australis berlandieri* is occurring. The authors contributed to a petition for field release, which will be submitted by North American partners during 2018.

The semi-aquatic weevil *Bagous nodulosus* is one of the most promising potential agents for biological control of flowering rush. The authors established a rearing colony and began sequential no-choice oviposition tests, which confirmed the narrow host range of the weevil. In a preliminary impact experiment, a reduction of 33% above-ground biomass was found due to adult feeding. The authors also began work with the agromyzid fly *Phytoliriomyza ornata* and the white smut *Doassansia niesslii*. The teleomorphic state of this pathogen is able to infect flowering rush under water, which will be advantageous for controlling completely submerged populations of the plant.

Contents

Abstract ii

Figures and Tables iv

Preface v

1 Biological Control of Common Reed, *Phragmites australis* 1

1.1 Background 1

1.2 Rearing and shipments of *A. geminipuncta* and *A. neurica* 3

1.3 Egg overwintering experiment with *A. geminipuncta* and *A. neurica* 4

2 Investigation into Biological Control of Flowering Rush, *Butomus umbellatus* 6

2.1 Introduction 6

2.2 Surveys and collections 7

2.3 *Bagous nodulosus* Gyllenhal (Coleoptera, Curculionidae) 8

2.3.1 Biology 8

2.3.2 Rearing 9

2.3.3 Host-specificity tests 10

2.3.4 Impact experiment 13

2.4 *Bagous validus* Rosenhauer (Coleoptera, Curculionidae) 15

2.4.1 Field collections and rearing attempts 15

2.4.2 Observations at field sites 16

2.4.3 Discussion 17

2.5 *Phytoliriomyza ornata* (Meigen) (Diptera, Agromyzidae) 17

2.6 *Hydrellia concolor* (Stenhammer) (Diptera, Ephydridae) 19

2.7 *Doassansia niesslii* De Toni (Basidiomycota) 20

2.7.1 Field work 21

2.7.2 General isolation and inoculation methodology 22

2.7.3 Butomus umbellatus biotype susceptibility to D. niesslii ex Bremen, Germany 23

2.7.4 Host-specificity testing 24

2.7.5 Discussions and conclusions 24

References 26

Report Documentation Page

Figures and Tables

Figures

Figure 1. Location of flowering rush sites visited between 2013 and 2017 (green dots for 2016 and 2017); one dot can represent up to three sites in close proximity. 8

Figure 2. Bagous nodulosus underwater on a flowering rush leaf (photo: Tim Haye). 9

Figure 3. Boxplots for differences in above-ground dry weight of *Butomus umbellatus* exposed or not exposed (control) to feeding by adult *Bagous nodulosus*. 14

Figure 4. *Bagous validus* first instar larva (a), *B. nodulosus* first instar larva (b), and *B. validus* adult female on leaf (c). 16

Figure 5. *Bagous validus* site in eastern Slovakia; flooded in spring (left) and ploughed in fall (right). 16

Figure 6. First generation pupa (a), overwintering pupa (b), pupa in mine (c), and adult of *P. ornata* (d). 17

Figure 7. Butomus umbellatus plant damaged by larval mining of Phytoliriomyza ornata. 19

Figure 8. Empty pupa (a) and adult (b) of *Hydrellia concolor*. 20

Figure 9. Population of *Butomus umbellatus* growing at the outer edge of a reed stand (*Phragmites australis*), Staines, Surrey, UK. 21

Figure 10. *Butomus umbellatus* from Staines, UK, infected with *Doassansia niesslii* from Bremen, Germany, five weeks after inoculation (left); uninfected control (right). 24

Tables

Table 1. Results of sequential oviposition tests with *Bagous nodulosus* carried out between 2014 and 2017(species marked in red will require additional replicates to complete host-specificity testing). 11

Table 2. Results of adult feeding impact experiment with *Bagous nodulosus* in 2017. Means given are ± SE for eight replicates each. 14

Table 3. Results of inoculations of *Butomus umbellatus* with *Doassansia niesslii* in 2016 and 2017. 23

Preface

This report was prepared by Drs. Patrick Häfliger, Carol Ellison and Hariet Hinz, Centre for Agriculture and Bioscience International (CABI), Delémont Switzerland, and Egham, UK, for the U.S. Army Engineer Research and Development Center - Environmental Laboratory (ERDC-EL) under Contract Number W9132T-16-1-0001. The technical monitor was Mr. Jeremy Crossland, Headquarters, U.S. Army Corps of Engineers (HQUSACE), Natural Resources Management

At the time of publication, Dr. Alfred F. Cofrancesco, CEERD-EZT, was the technical director for Environmental Engineering and Sciences-Civil Works; Dr. Linda Nelson, CEERD-EZT, was program manager for the Aquatic Plant Control Research Program. The Acting Deputy Director for the EL was Dr. W. Andy Martin and the Acting Director was Dr. Jack E. Davis.

The work reported herein was performed by CABI Switzerland (Hariet Hinz and Patrick Häfliger) and CABI UK (Carol Ellison). The following students assisted in the field and in the laboratory: Ms. F. Sorge, Ms. J. Baniszewski, Ms. J. Klötzli, Ms. L. Kernen, Ms. L. Mann, Mr. G. Zorzetto, Ms. I. Hasanovic, and Ms. D. Bouraoui. Dr. I. Toševski (Institute for Plant Protection and Environment, Zemun, Serbia) performed the molecular analysis of the weevil, *Bagous nodulosus,* and was also involved in field work in Serbia.

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In 2016 and 2017, the projects were financed by the New York State Department of Transportation through Cornell University, the Washington Department of Agriculture, the Washington Department of Ecology, the USDA Forest Service, the Montana Department of Natural Resources and Conservation and the U.S. Army Corps of Engineers in the USA, and the British Columbia Ministry of Forests, Lands and Natural Resource Operations in Canada.

COL Ivan P. Beckman was Commander of ERDC, and Dr. David W. Pittman was the Director.

1 Biological Control of Common Reed, *Phragmites australis*

1.1 Background

In 1998, a project was started at Centre for Agriculture and Bioscience International (CABI) in Switzerland to evaluate the potential for biological control of common reed in North America. During a two-year survey, 15 sites in Central Europe were sampled for endophagous herbivores of *P. australis*. In a first step, eight moth species and one chloropid fly were prioritized for further investigations as potential biological control agents (Häfliger et al. 2001). Currently, work is focused on the two noctuid moths with the highest impact on common reed, *A. geminipuncta* (Haworth) and *A. neurica Hübner*.

Common reed, *Phragmites australis* (Cav.) Trin. ex Steudel, is a cosmopolitan, perennial, clonal grass that can form large, nearly monospecific stands in wetlands and along rivers and lakesides. Due to its high genetic and morphological variability, *P. australis* is able to grow in a wide range of habitats with different climates (van der Toorn 1972). In Europe, reed beds are inhabited by a rich insect community and are valuable and endangered ecosystems (Tscharntke 1999; Tewksbury et al. 2002). In North America and Australia, however, *P. australis* is considered invasive and a threat to biodiversity (Wapshere 1990; Marks et al. 1994; Tewksbury et al. 2002).

Only in the last century did *P. australis* start to spread in North America. Before that, it had been present for at least 3,500 years without being invasive (Orson et al. 1987). The dramatic increase of common reed populations in the second half of the 20th century has often been attributed to land use changes and eutrophication (Marks et al. 1994). However, the alternative hypothesis of the introduction of an invasive European genotype was verified by genetic studies of Saltonstall (2002). We now know that there are several native haplotypes in North America, but particularly in the East and Midwest they are usually rare and out-competed by the invasive European haplotype M (subsequently referred to as introduced reed). The native North American populations of common reed have been recognized as a distinct subspecies, *P. australis* subsp. *americanus* by Saltonstall et al.

(2004) (subsequently referred to as native reed). In addition, another lineage, *P. australis* subsp. *berlandieri* (E. Fourn.) Saltonst. and Hauber, was described from the U.S. Gulf Coast, (Saltonstall and Hauber 2007) (subsequently referred to as haplotype I). Lambertini et al. (2012) assumes haplotype I to be a hybrid between *P. mauritianus* and an African/ Mediterranean population of *P. australis*. Their data indicate haplotype I to be an "ancient introduction." However, it is commonly considered as native (Gucker 2008; Ward and Jacono 2009).

For the development of biological control, the presence of native subspecies means that herbivores are required that can selectively reduce the invasiveness of the introduced European type without adversely affecting the native North American subspecies. Host-specificity tests carried out at CABI and at the University of Rhode Island demonstrated that both *Archanara* species have a very narrow host range. Larvae were only able to complete development on plants in the genus *Phragmites*. However, larval development tests carried out in 2004 showed similar development rates on the native *P. australis* subsp. *americanus* and European *P. australis* (Häfliger et al. 2005).

Open-field oviposition tests carried out between 2011 and 2015 showed a strong preference of both species for invasive reed. Less than 7% of eggs were laid on the native *P. australis americanus* (Blossey et al. 2018).

In addition, an overwintering experiment carried out in a common garden in 2006/2007 suggested that any eggs laid on native reed would suffer higher mortality due to differences in phenology of the two *P. australis* subspecies (Häfliger and Foresti 2008). Eggs laid on native reed will likely fall off together with leaf sheaths before or during winter, and thus, be exposed to climatic conditions, predators, and pathogens. Eggs overwintering unprotected on the soil had a 42% higher mortality (P = 0.008) compared to eggs protected under leaf sheaths (Häfliger and Foresti 2008). The mortality of unprotected eggs is expected to be even higher under field conditions. Especially on reed inundated by water, eggs might get washed away, or changing moisture regimes may increase fungal attack.

Should the noctuids be released in North America, their impact on native reed is expected to be negligible due to the low number of eggs that are laid on native reed and higher egg mortality due to plant phenology. Because larvae have very limited dispersal abilities, there is also no

significant non target attack to be expected where native reed is growing close to, or intermixed with, invasive reed. The authors are currently contributing to the petition for field release of *A. geminipuncta* and *A. neurica*, which is being prepared by Bernd Blossey (Cornell University) and Richard Casagrande (University of Rhode Island).

1.2 Rearing and shipments of *A. geminipuncta* and *A. neurica*

METHODS: The moth rearing was carried out as in years before. Freshly hatched larvae of *A. neurica* and *A. geminipuncta* were transferred individually with a paint brush into cut stems of *Phragmites australis* (one larva per stem). A maximum of 12 stems were inserted in moist horticulture sponges, welded in plastic foil, and placed in plastic cylinders (diameter 10 cm, height 37 cm), then covered with a gauze lid. Cylinders were checked daily, and as soon as a larva had left its shoot, it was transferred onto a new shoot section. Thus, three to five stems were needed per larva until pupation. Pupae were removed from stems, sexed, and about five pupae were placed together on a layer of vermiculite in a plastic cup (diameter 5.5–-6.5 cm, height 8 cm). A wet cotton pad was added to avoid desiccation of pupae. After four weeks, emergence of adult moths was checked daily. One to three pairs of newly emerged moths were held for mating and oviposition in wooden cages (40 x 40 x 65 cm) under outdoor conditions. Six shoot bases of reed with dry but intact leaf sheaths were provided as oviposition sites, and moist paper towels were provided as a source of moisture. Shoot bases were replaced once after three days and the cages were emptied after females had died (usually after 7–10 days). Leaf sheaths were subsequently checked for eggs, which were kept for hibernation in Petri dishes (5 mm in diameter; maximum 300 eggs per dish) placed in a styrofoam box and stored in a wooden hut at ambient temperatures (minimum average at night -10°C, maximum average at day 30°C).

RESULTS: Rearing of *A. neurica* resulted in 2500 eggs in 2016, and over 3000 eggs in 2017. However, we have noted increasing problems with *A. geminipuncta*. In 2016, nearly all larvae died before pupation, and we obtained only 300 eggs. Thanks to 20 field-collected pupae, we were able to increase this number to 680. An additional 700 eggs were obtained in 2017.

In February 2016, a total of 2000 eggs were shipped to URI for further host-specificity tests. In June 2017, we shipped for the first time 60 pupae of both species to allow egg production in quarantine. The same number of

pupae were also sent to Lethbridge (Agriculture and Agri-Food Canada), where pheromones of both moth species were analyzed for potential use in monitoring establishment, should the moths be released.

1.3 Egg overwintering experiment with *A. geminipuncta* and *A. neurica*

Despite the expected safety of native *P. australis americanus* towards release of both *Archanara* species, there are some concerns about a further subspecies, *P. australis berlandieri*, which occurs along the Gulf Coast. Since the distribution of both moth species in Europe is restricted to areas north of 35° latitude, they might not reach *P. australis berlandieri* stands, if released in North America. In an egg overwintering experiment setup in fall 2016, the hypothesis was tested that eggs would not survive the winter under temperature conditions of the latitude of Fort Pierce (Florida).

Methods: In August 2016, eggs of *A. neurica* and *A. geminipuncta* were placed in groups of ten into Petri-dishes. Eggs were exposed in incubators (MIR-254 cooled incubator, Panasonic, Etten Leur, The Netherlands), set to the photoperiod and fluctuating average day and night temperatures of Fort Pierce, Florida (maximum 32°C, minimum 11°C), and to average constant conditions in their core Central European range (15°C to mid-October, then 2°C until March 2017). We reprogrammed day and night temperatures and day length to follow changing conditions in Fort Pierce once a month until December, and then weekly until we terminated the experiment in April 2017. The incubator simulating Fort Pierce climate received 30 Petri-dishes with *A. neurica* and 15 Petri-dishes with *A. geminipuncta* eggs. The incubator simulating Central European climate received 23 Petri-dishes with *A. neurica* and 5 Petri-dishes with *A. geminipuncta* eggs. We placed half of the Petri-dishes in the incubator simulating Fort Pierce climate in a Styrofoam box together with a moist paper towel that was re-moistened once a week to provide additional moisture should desiccation be a problem.

We further tested the effect of a brief cold period on egg hatch rates. We limited this to a greatly reduced number of Petri-dishes due to lack of source material. In January 2017, we removed 8 Petri-dishes with *A. neurica* eggs from the Fort Pierce incubator and exposed them to 2°C (conditions in the Central European incubator) for 4, 8, 16, and 32 days (two Petri-dishes per cold period duration). We did the same with four

dishes with *A. geminipuncta* eggs, but only for two Petri-dishes each for 8 and 32 days. The Petri-dishes were then returned to the Fort Piece climate incubator. We assessed larval emergence weekly starting in mid-January 2017.

Results: No larvae hatched from eggs overwintered in growth chambers simulating Fort Pierce, Florida climate conditions and adding a 4–32 day cold stratification period did not change results. Only eggs kept at temperatures resembling Central European conditions (15°C August-mid-October, 2°C mid-October to February) remained viable and larval hatch (approx. 40%) was observed. A similar test carried out at the University of Rhode Island during winter 2017/2018 confirmed our results.

Discussion: The results clearly show that neither of the two moth species will be able to establish in Gulf Coast conditions, where *P. australis berlandieri* occurs. Even eggs kept in Styrofoam boxes with adequate humidity did not hatch when kept under Gulf Coast conditions, excluding the possibility that purely desiccation of eggs limited larval hatch. We therefore conclude that risk to the native subspecies *P. australis berlandieri* will be negligible, should the moths be released in North America.

2 Investigation into Biological Control of Flowering Rush, *Butomus umbellatus*

2.1 Introduction

Flowering rush (*Butomus umbellatus* L.) is a perennial aquatic plant that grows along lake shores and in slow-moving bodies of water, irrigation ditches, and wetlands in temperate Europe and Asia. In several European countries, the plant is considered rare and endangered (Stöhr et al. 2006; Raabe et al. 2011). Fluctuating water levels favor the plant. It usually grows as an emergent with upright foliage in up to 60 to 80 cm deep water (Hroudová 1989). In North America, where *B. umbellatus* was introduced as an ornamental more than 100 years ago, the common emergent form is found in up to 3 m deep water. Submerged populations with flexible leaves suspended in the water column can be found in up to 6 m deep water (Jacobs et al. 2011). Flowering rush is now considered an aggressive invader of freshwater systems, and is becoming an increasing problem in the midwestern and western states of the USA and western Canada.

Two ploidy levels are known for *B. umbellatus*: diploids (2n = 26) and triploids (2n = 39). Plants of the two ploidy levels differ in various ways. Diploids produce abundant fertile seeds, whereas triploids produce far fewer and sterile seeds (Krahulcová and Jarolímová 1993). In Europe, low seed fertility in triploids is compensated for by production of bulbils (vegetative reproductive structures) in flower heads and increased production of lateral rhizome buds (Hroudová and Zákravský 1993), while in North America, bulbils in flower heads have only been found in diploids (Kliber and Eckert 2005). Despite heavy investment in seed production by diploids, little or no evidence of sexual recruitment was found in North America, suggesting predominantly clonal reproduction via bulbils (Fernando and Cass 1997; Kliber and Eckert 2005; Lui et al. 2005). In contrast, North American triploids invest heavily in a large, carbohydrate-rich rhizome and appear to only propagate by rhizome fragmentation (Thompson and Eckert 2004; Brown and Eckert 2005). Rhizome fragments, broken at fine constrictions by minor disturbances such as moving water, waves and passing boats or waterfowl, disperse on water currents, sometimes over long distances (Jacobs et al. 2011). Sparsely vegetated or un-vegetated silty substrate, where water is shallow and currents have slowed, are ideal for establishment (Jacobs et al. 2011).

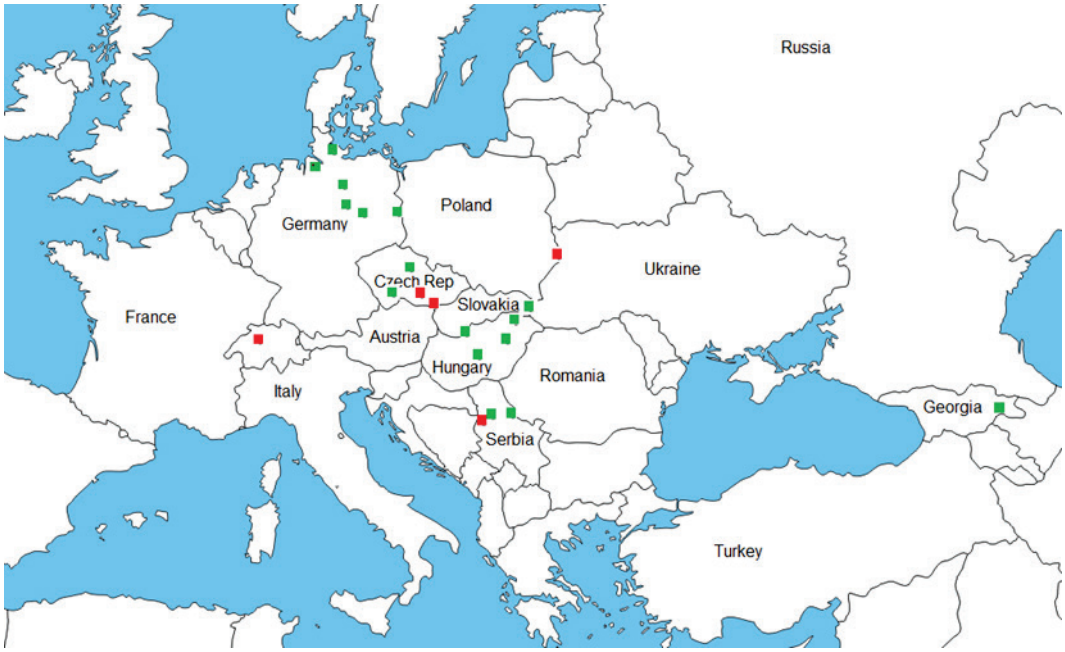
Since no effective long-term control methods are currently available, a biological control project was started in spring 2013 on the initiative of Jennifer Andreas (Integrated Weed Control Project, Washington State University), and CABI in Switzerland was subcontracted to conduct surveys for potential insect agents. After a literature survey, four herbivores were prioritized as the most promising potential biological control agents; the weevils *Bagous nodulosus* and *B. validus*, the agromyzid fly *Phytoliriomyza ornata* and the ephydrid fly *Hydrellia concolor*. All are reported as monophagous on flowering rush. Because few insect species appear to be available with biological control potential, we involved plant pathologist Carol Ellison, from CABI's Centre in the UK, to start working on the white smut *Doassansia niesslii* in 2016.

2.2 Surveys and collections

A total of 28 sites were visited in 2016 and 2017 in Germany, Czech Republic, Slovakia, Hungary, Serbia and Georgia (Figure 4). During these visits, new sites with the weevils *B. nodulosus* and *B. validus* were found. The same is true for the white smut *Doassansia niesslii*, which allowed first studies of the biology of this pathogen and first host-specificity tests. In both years, about 240 *B. nodulosus* were collected for host-specificity tests and rearing trials. In fall 2017, over 100 pupae of the agromyzid fly *P. ornata* were collected in northern Germany to establish a rearing colony. In addition, the ephydrid fly *Hydrellia concolor* was recorded for the first time for Germany.

As in previous years, leaf samples were collected from several new sites (including one in Kazakhstan collected by our colleague Sonja Stutz) and sent to Dr John Gaskin (USDA-ARS Sidney, Montana) for molecular analysis.

Figure 1. Location of flowering rush sites visited between 2013 and 2017 (green dots for 2016 and 2017); one dot can represent up to three sites in close proximity.



2.3 *Bagous nodulosus* Gyllenhal (Coleoptera, Curculionidae)

2.3.1 Biology

The semi-aquatic weevil *B.s nodulosus* remains the most promising in-sect candidate for biological control of flowering rush. No other herbivore found so far during dissections of field-collected plants causes more damage to leaves and rhizomes of *Butomus umbellatus*. Entrance and exit holes found frequently on dissected leaves are indicators that larvae do not remain mining internally in a leaf, but may leave it and re-enter, probably lower down, or even at the leaf base. Observations made during an impact experiment in 2017 (see section 4.4) showed that larvae must be able to swim and move to other plants. The species is reported to be univoltine, with oviposition and larval development time restricted to June/July (Gosik, 2006). However, we found developing larvae in field-collected samples from May to September. Thus, damage due to larval feeding is not limited to a certain time period, but occurs over almost the entire growing season of flowering rush. During dissections of field-collected plants we regularly found larval parasitoid larvae and cocoons. The adult weevils spend most of their time underwater, can reach at least two years old, and overwinter on plant debris underwater.

Figure 2. Bagous nodulosus underwater on a flowering rush leaf (photo: Tim Haye).

2.3.2 Rearing

Keeping adult weevils on potted plants covered with gauze bags and placing them in pools or in artificial ponds works very well. Winter survival of up to 80% was observed, and some weevils can reach at least two years old. However, the issue with high larval mortality in our rearing colony has not yet been solved. In the past four years, we have tried many different set-ups with varying water levels, avoiding overly high water temperatures, using different plant sizes, transferring larvae, exposing plants to ovipositing females, etc. However, we did not find a method that significantly increased the development success. Although we managed to nearly double the number of adults developing in our rearing colony in 2017 (52 compared to 30 in 2016), the success rate of 3.9% of transferred larvae is still not satisfactory. There is a tendency towards increased success when larger water containers are used. The above-mentioned observation that larvae apparently change plants during their development could be another explanation for low larval development success: larvae could become stuck in the gauze bags and die before finding a suitable leaf to continue development. This is also supported by the fact that up to one hundred weevils were collected in our artificial pond where plants were not covered by gauze bags. These weevils can be considered as an unplanned rearing success. We are considering investigating this in more detail in 2018.

2.3.3 Host-specificity tests

METHODS: As in previous years, we continued sequential no-choice oviposition tests using cut leaves. Females from three origins (Germany, Serbia and Slovakia) were used and we tried to set up at least two valid replicates of each population for each test plant species between May and July. To ensure that only egg-laying females were used for this test, females were kept individually for two days in plastic cups (diameter 5.5–6.5 cm, height 8 cm) with 1 cm of water and two cut leaves of flowering rush. Only females that laid at least one egg within two days were used. Cut leaves of test plants were individually exposed to ovipositing females for two days in plastic cylinders (volume 1.3 litres) half-filled with water. Females were then placed back onto cut leaves of flowering rush to verify that they were still laying eggs. Tests were only considered valid if the female laid at least one egg on the control (flowering rush) within two days after the test. Females that were still laying eggs were subsequently exposed to another set of test plants. Eggs found during the tests were used to supplement our rearing colony.

Results: Using this method, a total of 41 test plant species, 27 native to North America, were exposed to *B. nodulosus* females in 2014–2017. The weevils laid a slightly higher number of eggs on North American compared with European *Butomus umbellatus*. A few feeding marks were found on some of the test plants, but only one of them, *Baldellia ranunculoides*, a European species, was accepted for oviposition, and for one replicate only (Table 1). Females laid on average 1.4 eggs per replicate on controls. Slovak weevils laid on average twice as many eggs as weevils collected in Germany (2.1 eggs vs. 1 egg). To complete host-specificity testing with *B. nodulosus* in 2018, we will need to source 1–2 additional test plant species (*Najas guadalupensis* and *Iris advena*) that will probably be added to the test plant list, and increase the number of replicates for eight species. In general, we are trying to establish six replicates per species, two from each weevil origin.

Table 1. Results of sequential oviposition tests with *Bagous nodulosus* carried out between 2014 and 2017(species marked in red will require additional replicates to complete host-specificity testing).

Plant species[a]	No. replicates set up	No. valid replicates	No. eggs per replicate	Feeding[b]
Order Alismatales				
Family Butomaceae				
Butomus umbellatus EU triploid[c]	2783		1.44	+++
Butomus umbellatus EU diploid[c]	7		1.43	+++
Butomus umbellatus US	86		1.84	+++
Family Alismataceae				
Alisma plantago-aquatica	13	7	0	+
Alisma subcordatum[a]	10	6	0	
Alisma triviale[a]	19	6	0	+
Baldellia ranunculoides	14	9	0.78	+
Damasonium californicum[a]	13	5	0	
Echinodorus berteroi[a]	24	13	0	+
Echinodorus cordifolius[a]	31	11	0	+
Sagittaria cuneata[a]	14	7	0	+
Sagittaria graminea[a]	19	6	0	+
Sagittaria latifolia[a]	29	10	0	+
Sagittaria platyphylla[a]	11	9	0	+
Sagittaria rigida[a]	11	6	0	-
Order Hydrocharitales				
Family Hydrocharitaceae				
Blyxa aubertii	3	2	0	++
Elodea bifoliata[a]	6	6	0	-
Elodea canadensis[a]	20	8	0	+
Elodea densa	7	4	0	+
Elodea nuttallii[a]	11	8	0	-
Hydrilla verticillata	19	10	0	+
Hydrocharis morsus-ranae	12	6	0	+
Limnobium spongia[a]	11	6	0	+
Vallisneria americana[a]	12	8	0	++
Order Nymphaeales				
Family Ceratophyllaceae				
Ceratophyllum demersum[a]	23	6	0	+
Family Nymphaceae				
Nuphar lutea[a]	16	6	0	-
Nymphaea odorata	12	6	0	-
Order Haloragales				-
Family Haloragaceae				
Myriophyllum spicatum	16	9	0	+

Plant species[a]	No. replicates set up	No. valid replicates	No. eggs per replicate	Feeding[b]
Order Najadales				
Family Potamogenaceae				
Potamogeton amplifolius[a]	2	1	0	+
Potamogeton natans	11	5	0	+
Potamogeton lucens	11	6	0	+
Potamogeton richardsonii[a]	11	7	0	-
Stuckenia pectinata[a]	12	9	0	-
Order Liliales				
Family Pontederiaceae				
Heteranthera dubia[a]	9	5	0	-
Family Iridaceae				
Iris pseudacorus	19	10	0	-
Order Cyperales				
Family Cyperaceae				
Carex obnupta[a]	16	6	0	-
Schoenoplectus acutus[a]	18	9	0	-
Schoenoplectus tabernaemontani[a]	17	4	0	+
Family Poaceae				
Glyceria borealis[a]	26	11	0	+
Oryza sativa	12	7	0	-
Phalaris arundinacea	5	4	0	
Zizania aquatica[a]	14	8	0	-
Order Myrtales				
Family Lythraceae				
Lythrum salicaria	8	6	0	-
Order Polygonales				
Family Polygonaceae				
Polygonum amphibium[a]	11	7	0	+

[a] Plant species native to North America.
[b] - = no feeding, + = minor feeding on single leaves, ++ = some feeding on few leaves, +++ = major feeding on most leaves.
[c] Ploidy to be confirmed.

Discussion: Host-specificity tests carried out so far have confirmed the extremely narrow host range of *B. nodulosus*. Females laid only seven eggs on the European *Baldellia ranunculoides* in one replicate under no-choice conditions. However, the thin petioles, leaves and stems of this plant would not support larval development of the weevil.

The adult feeding we observed on test species was in general not much more than probing. However, we are planning to conduct single-choice tests (i.e., exposing the test species simultaneously with flowering rush) for a few species like *Vallisneria americana* and *Blixa aubertii* that showed more extensive feeding.

2.3.4 Impact experiment

Owing to problems with high larval mortality during *B. nodulosus* rearing, we have not yet been able to evaluate impact of larval feeding on the host plant. We therefore focused on quantifying the impact of adult feeding in 2017.

Methods: In order to measure impact due to adult feeding, we set up an experiment in early June 2017 in a pool (2 m × 4 m × 0.8 m) filled to a depth of 50 cm with water. Five pairs of *B. nodulosus* were released onto each of eight potted *Butomus* plants covered with gauze bags. Eight additional covered plants were set up as controls (no weevils). We used wider-gauge gauze than usual (1-mm mesh width), because the gauze we usually use tends to become blocked by algae and to negatively influence plant growth owing to reduced light levels. For each of the 16 plants, we measured number of leaves and length of the longest leaf and made sure that plant size did not differ between treatments at the time of experimental set-up.

After six weeks, number of detectable weevils, number of leaves, length of leaves, and above- and below-ground dried biomass were recorded for each pot. All plants were dissected and any larvae or mines recorded. Before measuring dry weight, plant samples were dried in a drying oven at 80°C for 48 hours.

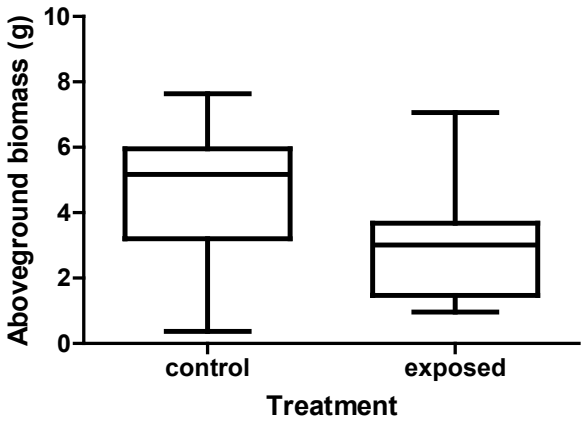
Results: Although above-ground biomass of plants exposed to weevils was on average 33% lower than biomass of controls and total leaf length was reduced by 38%, these differences were not statistically significant ($P > 0.05$). This result was influenced by two outliers (there were two extremely small plants in both treatments, which increased variation). Excluding the two outliers, this result made differences in leaf length, leaf number and above-ground biomass significant.

Table 2. Results of adult feeding impact experiment with *Bagous nodulosus* in 2017. Means given are ± SE for eight replicates each.

	Sum of leaf length (cm)	No. of leaves	Above-ground biomass (g)	Below-ground biomass (g)	Total no. of larvae found
Control[a]	1012 ± 211	12.0 ± 2.6	4.6 ± 0.8	4.6 ± 1.1	3
Exposed[b]	620 ± 120	9.4 ± 2.1	3.1 ± 0.7	4.2 ± 1.1	10
Statistics[c]	P = 0.141	P = 0.448	P = 0.160	P = 0.797	

[a] no weevils released
[b] five pairs of *B. nodolosus* released
[c] independent samples t-test

Figure 3. Boxplots for differences in above-ground dry weight of *Butomus umbellatus* exposed or not exposed (control) to feeding by adult *Bagous nodulosus*.



Discussion: We found on average only 1.25 larvae per plant while dissecting plants exposed to weevils. Therefore, as expected, most "impact" found was from adult feeding. We believe that differences between plants with and without weevils could have been statistically significant if the experiment had been set up earlier in the season, since adults were already active at the end of March/beginning of April. This will be done in 2018. We will also try to improve larval development on experimental plants, which should increase measurable impact.

As a side effect of this experiment, we learned more about the larval behaviour of *B. nodulosus*. The fact that we found three larvae on controls showed that they must be able to swim and move from plant to plant. The mesh size of the gauze used was narrow enough to prevent adults escaping, but wide enough for larvae to pass through. This observation

could potentially help to explain our difficulties in obtaining successful larval development on rearing plants (see section 2.3.2).

2.4 *Bagous validus* Rosenhauer (Coleoptera, Curculionidae)

This weevil is recorded as monophagous on flowering rush, co-occurring with *B. nodulosus* (Dieckmann, 1983; Caldara and O'Brien, 1998). Its distribution is reported from eastern and southern Europe to the Middle East. However, there are only a few site records in the literature, and not much is known about its biology. After several unsuccessful attempts, we finally found adult weevils at a site in southern Slovakia in 2015, at two sites in eastern Slovakia in 2016, and at one site in Serbia in 2017. Although we were able to rear one individual from egg to adult on a piece of *Butomus umbellatus* in the laboratory, we never found larvae in the field or on potted plants. This raised doubts about the true host plant of *B. validus*.

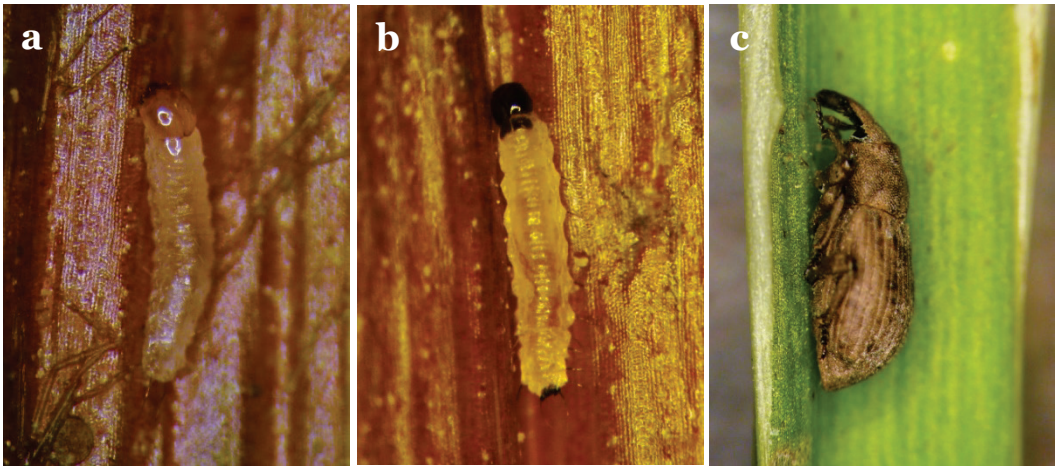
2.4.1 Field collections and rearing attempts

METHODS: In order to learn more about the biology of *B. validus*, we visited the three known sites in Slovakia between 2016 and 2017 three times in May, three times in June, and once each in July, August, September and October. In May and June, 2016 and 2017, we collected a total of 110 adults at the three sites in Slovakia for rearing trials and studies on the biology of the species at CABI. In addition, we checked other plant species in the vicinity for weevils. At each visit, we also collected *Butomus* plants for dissection in the lab.

In 2017, we tried two different rearing techniques for *B. validus* that had worked for *B. nodulosus* (1) we transferred 19 freshly hatched larvae onto seven potted plants covered with gauze bags, and (2) we set up 23 adult pairs on eight potted plants covered with gauze bags. After larval transfers or release of adults, plants were kept under three different water conditions (1) in trays filled with 5 cm of water, (2) in trays filled with 17 cm of water, and (3) in a pool filled with 50 cm of water.

In autumn 2017, we set up about 48 adults for overwintering on seven potted plants covered with gauze bags. Two plants were placed submerged in a pool filled with 50 cm of water, and five unsubmerged on a framed plastic foil filled with 5–10 cm of water.

Figure 4. *Bagous validus* first instar larva (a), *B. nodulosus* first instar larva (b), and *B. validus* adult female on leaf (c).



2.4.2 Observations at field sites

The only site where *B. validus* was found more common than *B. nodulosus* is a field with a slight depression that is only temporarily flooded in spring (Figure 4). Although this field seems to be ploughed regularly (observed in July 2016 and in September 2017), the populations of *Butomus umbellatus* and *B. validus* remain surprisingly stable. At this site, adult weevils were found both underwater on submerged plants in early May and at the base of leaves on drained plants at the end of May and June. The other sites where we found *B. validus* are channels that contain water all year round, but where water levels might drop slightly towards the end of the summer. In these channels, we found *B. validus* adults mostly on higher parts of the leaves, generally on plants close to the water's edge.

Figure 5. *Bagous validus* site in eastern Slovakia; flooded in spring (left) and ploughed in fall (right).

2.4.3 Discussion

Although we collected over 100 adults on flowering rush in two years, we were not able to establish a rearing colony and we still have no idea where the larvae develop. The fact that we only obtained a low number of eggs from field-collected females and that we found no indication of larval development of *B. validus* on *Butomus umbellatus* in the field could be an indication that this weevil has an alternate host. However, we have not yet been able to prove this hypothesis. We did not find adult *B. validus* on any plant other than *Butomus*, and no species with leaves or stems wide enough to support larval development was present at any of the three sites.

Since we were not able to find larvae of B. validus on flowering rush during three consecutive years of study, we do not believe that it is warranted to invest additional time on this species is warranted.

2.5 *Phytoliriomyza ornata* (Meigen) (Diptera, Agromyzidae)

Another herbivore only known from flowering rush in Europe is the agromyzid fly *Phytoliriomyza ornata* (Spencer, 1976; McLean, 1988). The fly seems to have two generations per year. Pupae of the first generation are transparent, while pupae of the overwintering generation are black (Figure 6a, 6b). We found larvae and pupae of *P. ornata* during dissections of plants at many of our sites. Eggs are laid in the leaf epidermis and hatching larvae feed downwards to the leaf base in an inconspicuous mine. Mature third instar larvae feed from the leaf base up again for 20–40 cm, where the pupa is formed below an emergence window (Figure 6c).

Figure 6. First generation pupa (a), overwintering pupa (b), pupa in mine (c), and adult of *P. ornata* (d).

Methods: In fall 2016, about 30 pupae dissected from leaves collected in Germany and Slovakia were set up for overwintering in Petri-dishes. These were stored in a styrofoam box in a wooden shelter at ambient temperatures (minimum average at night -10°C, maximum average at day 30°C). In mid-April 2017, 17 adults emerged and were set up in the laboratory on six potted *Butomus umbellatus* plants covered with a plastic cylinder with one female and 1–2 males in each. Flies were moved to a new plant after 3–4 days. Plants were transferred outside to the CABI garden after about ten days. After 2–4 weeks, all plants were dissected for larvae or pupae.

Flowering rush plants were collected during field trips in September and October 2017 in northern Germany and Slovakia and dissected for overwintering pupae of *P. ornata*.

RESULTS: Up to 11 pupae successfully developed on plants that were exposed to single ovipositing females, and several plants started wilting after 2–3 weeks (see Figure 7). Thus, contrary to our earlier assumption, *P. ornata* can have a strong impact on *Butomus* and should be further investigated as a potential agent.

About 120 pupae found during dissections of plants collected in autumn 2017 were set up for overwintering. Provided emergence is as successful as in 2017, we should obtain a sufficient number of flies to set up a rearing colony in spring 2018 and to start developing methods for host-specificity testing.

Over 40% of overwintering pupae found on field-collected plants were parasitized. Emerging parasitoids will be sent to specialists for identification.

Interestingly, the larvae that developed on plants infested in April 2017 in the laboratory developed directly into black, overwintering pupae and not into transparent pupae from which adults would usually emerge in summer. Apparently, the conditions during oviposition or early larval development determine the type of pupa (transparent and emerging the same year, or black and overwintering).

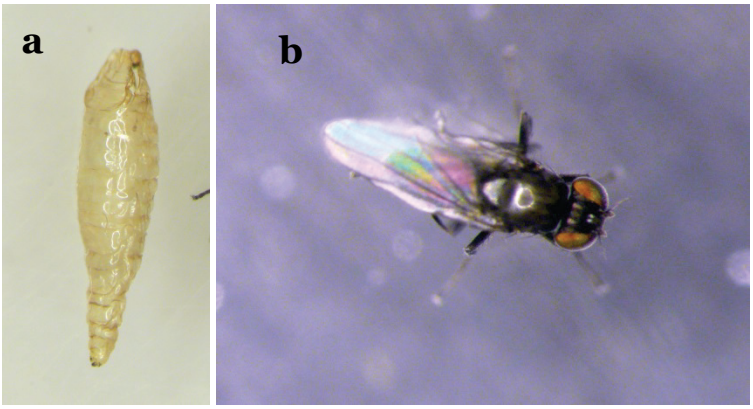
Figure 7. Butomus umbellatus plant damaged by larval mining of Phytoliriomyza ornata.

2.6 *Hydrellia concolor* (Stenhammer) (Diptera, Ephydridae)

The ephydrid fly *Hydrellia concolor* is a second fly species recorded to develop monophagously on *B. umbellatus* (Séguy 1934; Hering 1957; Hollmann-Schirrmacher and Zatwarnicki 1999). Until 2016, we had found it at five sites in the Czech Republic, Slovakia, Hungary, and in Switzerland (Stuke and Häfliger 2017). In 2017, we also found it at two sites in Germany, a first record for the country.

Apart from its host plant, nothing is known about the biology of this species. The fact that we found only empty pupae on plants dissected in fall excludes the pupal stage as the overwintering stage. A congeneric species, *Hydrellia pakistanae*, has been used to control *Hydrilla verticillata* in North America (Wheeler and Center 2001). However, of all potential agents described here, larval feeding by *Hydrellia concolor* seems to cause the lowest impact on flowering rush, so we have not invested much time on this species so far.

Figure 8. Empty pupa (a) and adult (b) of *Hydrellia concolor*.



2.7 *Doassansia niesslii* De Toni (Basidiomycota)

The white smut *Doassansia niesslii* is a leaf pathogen of *B. umbellatus* and was identified as a potential biological control agent for flowering rush in 2015. This damaging pathogen had been collected from near Bremen, in northern Germany during the arthropod surveys, and sent to CABI in the UK for identification. It has only been recorded to infect flowering rush, and there are records from former Czechoslovakia, Poland, Russia, and Sweden, including Germany (Farr and Rossman undated). White smuts are hemi-biotrophic fungal pathogens; their life cycle can only be completed on the host plant, but there is a necrotrophic phase of the life cycle that can grow in culture (on agar). White smuts generally prefer wet environments and overwinter as resting spores in the plant tissue; they can be very damaging. An example of the successful control of an invasive plant with a white smut is mistflower (*Ageratina riparia*) in Hawaii and New Zealand (Fröhlich et al. 2000).

An isolate of the white smut, collected most probably on triploid plants at Reedeich, near Bremen, Germany in 2016 was used in all the studies described here. Research in 2016 identified two spore states in the life cycle; the teleomorph and the anamorph. The teleomorphic state is a resting spore, forming completely within the leaf tissue (mesophyll) and requiring a period of dormancy (over winter) before germination can occur. The resting spores are only liberated by rupture of old and decaying litter. Under laboratory conditions, the teliospores were able to infect leaves growing underwater. This indicates that the white smut should be able to infect completely submerged *B. umbellatus*. In the North American invasive range, flowering rush grows fully submerged in many habitats. In addition, we observed plant die-back four weeks after infection, indicating

potential strong impact of the pathogen. The anamorphic state forms as pycnidia just under the epidermis and releases spores outside the plant through leaf stomata. These spores germinate immediately and infect new leaf material, causing severe damage throughout the growing season. It is as yet unknown whether these spores are able to infect plants growing completely submerged, but work is underway to investigate this.

2.7.1 Field work

A second isolate of the white smut from Elsnig, Saxony (near Leipzig), Germany, was collected in October 2017 and will be tested once the teliospores have completed their dormancy in spring 2018. Plants of *B. umbellatus* from this site are established and growing at CABI in the UK.

Figure 9. Population of *Butomus umbellatus* growing at the outer edge of a reed stand (*Phragmites australis*), Staines, Surrey, UK.

Plants of *B. umbellatus* from two sites in the UK (Staines and Camberley in Surrey, southern England) (Figure 9) have been collected for molecular analysis at United States Department of Agriculture-Agricultural Research Service (USDA-ARS), to help identify the origin of the North American invasion of flowering rush. Plants from Georgia, Caucasus, were sent to CABI's centre in the UK by our colleague Stefan Töpfer in June 2017. Unfortunately, no white smut was found, but plants have been established for potential future testing of isolates. Depending on funding, additional isolates of the white smut will be sought from other areas in the plant's Eurasian native range in 2018.

2.7.2 General isolation and inoculation methodology

Good, reliable infection of susceptible plants with the white smut can be obtained using freshly produced sporidia in culture and isolated from the anamorphic state. Unfortunately, the infectivity of the sporidia decreases over time, with them becoming non-infective after approximately four weeks of growing on agar (infectivity of the sporidia is currently being investigated) and they did not maintain their infectivity if subbed-on to fresh agar plates. Therefore, recently isolated sporidia need to be produced for each plant inoculation.

Sections of leaves (approximately 4 cm long) showing significant *D. niesslii* symptoms of infection, but prior to leaf senescence, were cut from plants inoculated 6–10 weeks previously. Sections were surface sterilized by wiping with a piece of tissue soaked in ethanol. With the aid of a dissecting microscope, a slit was made along the leaf, piercing to about half the thickness of the tissue, using a sterilized scalpel blade, in an area where pycnidia could be seen just under the epidermis. The pycnidia were exposed by peeling back the upper leaf surface and they were then extracted using a hypodermic needle. The pycnidia were placed on potato carrot agar (PCA) containing antibiotics and kept at 19°C. At least six agar plates, each with approximately six pycnidia placed on them, were prepared for each plant inoculation. This is because not all pycnidia produced sporidia, and there were inevitably some contaminated plates. After a week, small (1 mm), white, slimy sporidial colonies could be seen developing from many (~25%) of the pycnidia.

After four weeks, the colonies (approximately 10 mm in diameter) were picked-off and dispersed in 0.1% agar ('sloppy agar') containing 0.05% v/v Tween 80 (a surfactant to help sporidia disperse). The agar helped inoculum adhere to the vertical leaves of *B. umbellatus*, rather than run-off onto the soil. The sporidial suspension was brush inoculated (using a camel hair paint brush) onto the leaves of plants and placed in a dew chamber set at 15°C for 24 hours to allow for infection. Plants were then maintained in a greenhouse chamber with supplemented lighting and a minimum night temperature of 17°C and maximum day temperature of 25°C.

2.7.3 *Butomus umbellatus* biotype susceptibility to *D. niesslii* ex Bremen, Germany

Methods: Plants were inoculated with sporidia as described in section 2.7.2 above. At least four plants of each genotype were inoculated with the sporidial suspension, and this has been (or will be), repeated for each genotype. Additional plants of each population were brushed with agar carrier only, and placed in a separate dew chamber for 24 hours as controls.

Table 3. Results of inoculations of *Butomus umbellatus* with *Doassansia niesslii* in 2016 and 2017.

Population	Ploidy level	North American AFLP genotype	Susceptibility to D. niessliib
Grollander Deich, Bremen, Germany (site 1)	---		moderately susceptible
Bremen, Germany (site 2)	---		susceptible
Reedeich, Bremen, Germany (site 4)	---		strongly susceptible
Water Garden Plants, UK supplier	---		resistant
Slovakia	triploid[a]		resistant
Vojany, Slovakia	diploid[a]		resistant
Bouchie Lake, Canada	triploid	2	strongly susceptible
Montana, USA	triploid	1	resistant
South Dakota, USA	triploid	1	resistant
Horticultural supplier, Switzerland	---		resistant
Wisconsin, USA	triploid	1	resistant
Staines, Surrey, UK	---		strongly susceptible

[a] Still to be confirmed

[b] resistant = no chlorosis and no spores; moderately susceptible = scattering of chlorosis on inoculated leaves, spores develop within infected area, no complete dieback of leaves; susceptible = 50-75% of inoculated leaf area infected develop chlorosis, spore production apparent in chlorotic areas, leaves die back where infected; strongly susceptible = all inoculated parts of the leaves develop chlorosis, prolific spore production apparent throughout leaves and leaves die back completely.

Results: Table 3 provides a summary of the results of all the *B. umbellatus* inoculations that have been undertaken with the white smut so far. The results from the Wisconsin population provide additional evidence that North American genotype 1 is resistant to the isolate of *D. niesslii* collected from Reedeich, Bremen, Germany. The genotype from a horticultural supplier in Switzerland is also resistant to the pathogen, as was the genotype from the UK garden plant supplier.

It is likely that the native plant genotype from Staines, UK is close to the one from northern Germany, since it is equally susceptible to the white smut isolate. There is no record of *D. niesslii* from the UK (a place with a rich history of plant-disease data collection by field mycologists), although it may once have been present, centuries ago or perhaps before the last Ice Age. None of the controls (agar only) became infected.

Figure 10. *Butomus umbellatus* from Staines, UK, infected with *Doassansia niesslii* from Bremen, Germany, five weeks after inoculation (left); uninfected control (right).

2.7.4 Host-specificity testing

Methods: *Alisma plantago-aquatica* and the native North American *Sagittaria graminea* and *Carex obnupta* were screened for their susceptibility to the white smut isolate from Bremen, Germany. Plants were inoculated with sporidia as described in section 2.7.2 above. At least four plants of each test plant species were inoculated with the sporidial suspension; this has been repeated for each species. A susceptible biotype of *B. umbellatus* (Bouchie Lake, Canada) was included as a control, to prove that the spores were infective.

Results: All three plant species were immune to infection by the pathogen (no symptoms observed). The controls were fully susceptible.

2.7.5 Discussions and conclusions

A reliable culturing and inoculation methodology has been developed for *D. niesslii* infection of *B. umbellatus*. More research is required, however,

on how to retain infectivity of sporidial cultures and to confirm their ability to infect submerged plants.

Doassansia niesslii is a damaging potential agent of *B. umbellatus*, but a single isolate will not be sufficient to infect all genotypes present in North America, owing to the intraspecies specificity it demonstrates. Critically, additional isolates of *D. niesslii* need to be collected from a wide geographical range, starting with countries where it has been recorded (namely Germany, former Czechoslovakia, Poland, Russia and Sweden). A new isolate from Saxony, Germany will be screened as soon as the spores are viable.

Butomus umbellatus belongs to a single-species genus and fungal pathogens belonging to the genus *Doassansia* are recorded in the literature to have a very narrow host range; *D. niesslii* is only recorded from *B. umbellatus*. Hence, it is highly unlikely that this pathogen will infect any other plant species.

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14. ABSTRACT

The noctuid moths *Archanara geminipuncta* and *A. neurica* were selected as the most promising candidates for biological control of common reed. Complete development was possible on the native North American subspecies *P. australis americanus*. However, open-field oviposition tests showed a strong preference of female moths for both European and introduced *P. australis*. An egg overwintering experiment also showed that neither of the two moth species will survive at latitudes which correspond to regions where the subspecies *P. australis berlandieri* is occurring. The authors contributed to a petition for field release, which will be submitted by North American partners during 2018.

The semi-aquatic weevil *Bagous nodulosus* is one of the most promising potential agents for biological control of flowering rush. The authors established a rearing colony and began sequential no-choice oviposition tests, which confirmed the narrow host range of the weevil. In a preliminary impact experiment, a reduction of 33% above-ground biomass was found due to adult feeding. The authors also began work with the agromyzid fly *Phytoliriomyza ornata* and the white smut *Doassansia niesslii*. The teleomorphic state of this pathogen is able to infect flowering rush under water, which will be advantageous for controlling completely submerged populations of the plant.

15. SUBJECT TERMS
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