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Running title: *Polystigma rubrum* stromata on *Prunus spinosa*

Short Communication

Factors affecting the local distribution of *Polystigma rubrum* stromata on *Prunus spinosa*

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15 Text Pages and Four Figures (6 appendices)

Abstract

Background and aims: *Polystigma rubrum* forms orange-red stromata on the surface of living leaves of *Prunus spinosa* and *P. domestica*. Records suggests that this fungus now has a much more limited distribution in Britain than recorded in the 19th and early 20th Century.

Methods: We studied the local distribution of the fungus in the Burren Hills of western Ireland where it remains very common.

Key results: Assessment of the local distribution of the fungus over two years found stromata to be occur more frequently on *P. spinosa* leaves in hedgerows than woodlands. On individual trees in areas of open limestone pavement, the frequency of stromata was ten times higher in 2016 than 2015, possibly related to interannual rainfall differences. On hedgerow trees subjected to winter flooding, stromata were much less abundant, whereas stromata were more abundant on leaves also infected by the gall mite *Eriophyes prunispinosae*. The identity of *Po. rubrum* was confirmed by ITS sequencing.

Conclusion: At a field location where *Po. rubrum* stromata are present in unusually high abundance, the distribution of stromata on trees in different habitats showed high levels of variation linked to both habitat and the presence of gall mites. Further work is required to determine whether variation in leaf surface and soil moisture are the key determinants of the observed distribution. Such investigations may reveal why *Po. rubrum*, once common in northern Europe is now restricted mainly to westerly, coastal locations.

Keywords: Ascomycete taxonomy; Endophyte; Fungal conservation; Leaf pathogen; Xylariales; Biotrophs; *Polystigma rubrum*

Introduction

Polystigma rubrum (Pers.) DC., colloquially known as the “red spot disease” or “blackthorn dotty”, is a distinctive parasitic ascomycete found on living leaves of *Prunus spinosa* and *Prunus domestica* (Cannon 1996). In southern Europe and the Middle East, the fungus is considered an important pathogen of plums but there is considerable variation in the susceptibility of different cultivars to disease (Mitre jr et al. 2015). Stromata of *Po. rubrum* are found from (Jul to Sep; Fig.1), and are restricted to leaf tissues (Grove 1884). Infection does not cause necrosis but rather bright orange localised discolouration and swelling (Fig.1).

Each stromatal patch is believed to be the result of a single infection. Distribution of the fungus often is highly uneven between trees, suggesting localised transmission. There is no evidence for systemic infection suggesting that reinfection each year is mediated via ascospores formed on dead fallen leaves (Blackman & Welsford 1912). The extent of any leaf discolouration caused by *Po. rubrum* may vary depending upon the age of the lesions, for example, young stromata can be a less intense yellow-brown colour, whilst older stromata have a striking orange or red pigment. The stromata are generally 5--20 mm in diameter, and have an irregular orbicular shape, producing hamate (hooked) conidia (possibly sterile or with spermatial function) from immersed conidiomata (Cannon 1996; Dayarathne et al. 2017). The sexual stage of the fungus is characterised by the same stromata turning black on the fallen overwintering leaves and producing immersed ascomata and ascospores, (Grove 1884) (A O. Chater, pers. comm.; Appendix1).

Polystimga rubrum is occasionally reported as a pathogen of domesticated plum trees (*Prunus domestica*; red leaf spot disease) in Mediterranean climates (Mitre jr et al. 2015), whilst other *Polystimga* spp. are also reported as pathogens of *Prunus dulcis* (almond; *Po. amygdalinum* [formerly *Po. ochraceum*, Iran; (Ghazanfari & Banihashemi 1976)) and *Prunus padus* / *Prunus ssiori* (bird cherry; *Po. fulvum*; (Suzuki et al. 2008)).

Polystimga rubrum is listed as a ‘vulnerable’ species in the provisional Red Data List of British Fungi (Evans et al. 2006). Data from FRDBI (Fungal Records Database of Britain and Ireland; http://www.fieldmycology.net/FRDBI) suggest that it was more widespread in the early 20th Century than currently. Recent record data suggesting that its range has been considerably reduced and between 1965 and 2014 it had been recorded at only seven sites across the British Isles, mostly coastal (Appendix. 2). However, recent publicity via RBG Kew's *Lost and Found* project has led to extensive records from 39 monads (1 km squares) in Great Britain from Anglesey, down the west Wales coast, and throughout Cornwall (Douglas 2018).

The abundance of *Po. rubrum* in the Burren, County Clare, Ireland, offered a unique opportunity to study the influence of environmental conditions on the localised distribution of this rare fungus. The aim of this study was to determine whether there was localised variation in the abundance of stromata of *Po. rubrum*, and to identify factors which might be responsible for this variation.

MATERIAL AND METHODS

Fieldwork was conducted near Carron, Co. Clare, Ireland in September 2015 and 2016. Abundance of stromata was quantified by placing 50 x 50 cm quadrat against the foliage of a *P. spinosa* tree and inspecting all leaves within this quadrat (n=100--200), in order to calculate the percentage of leaves bearing stromata. DNA barcode analysis was conducted as described in Appendix 5.

RESULTS AND DISCUSSION

Distribution of *Po. rubrum* infections

In contrast to its current rarity across most of the British Isles (Appendix. 2), where 1% leaves are only rarely encountered even in areas where the fungus has previously been located, *P. rubrum* was present on most surveyed *P. spinosa* trees in the Burren, with stromata abundant (8--80% of leaves) in both survey years in the study area (1 km^2 area, immediately east of Carran; N53.038, W9.068). There was no evidence that leaves infected with *Po. rubrum* were prematurely senescent although it has been reported that this contributes to the economic damage caused by other *Polystigma* spp. on *Prunus* hosts (Kranz 1962; Banihashemi 1990; Cannon 1996).

Rainfall in the Burren hills is high (ca. 2000 mm/yr; www.met.ie/climate/) and *P. spinosa* was commonly found not only in hedgerows and in the understorey of the hazel-dominated woodland areas but also as isolated trees on the large areas of limestone pavement habitats within this area of karstic geology. The distribution of *Po. rubrum* stromata was assessed on *P. spinosa* trees in three habitats, open limestone pavement (individual trees), within hazel dominated woodland and in hedgerows, with replicate quadrats placed against different trees ca. 10 m apart.

Since it believed that the ascospores which initiate infection originate from dead leaves at ground level (Grove 1884; Cannon 1996; Habibi & Banihashemi 2016), it was reasoned that leaves nearer the ground might be more heavily infected. Therefore, quadrat squares (50 x 50 cm) were place against *P. spinosa* vegetation at each location within these habitat types (n=3) and at three different heights (0--50cm, 50--100 cm and 100--150 cm above ground level).

However, no significant difference in the frequency of stromata with height was observed (Appendix 3). Thereafter quadrats were set at 100--150 cm above the ground.

The highest frequency of stromata in the 50 x 50 cm quadrats (79%) was found on limestone pavement habitats in 2016, though in 2015 only 7% of leaves bore stromata. Stromatal frequency for hedgerow trees showed the same pattern (35% in 2016 vs 21% in 2015; ANOVA P = 0.042; Fig. 2), whereas in woodland habitat stromatal frequency was similar in both years (7--8%).

Based on two years of survey data, *Po. rubrum* was more abundant in exposed habitats (open pavement, hedgerow) than in woodland. However, the high rate of infection observed in 2016 on trees growing in exposed locations on limestone pavement suggested a possible effect of climate. This difference may be due the higher rainfall in the winter of 2015-16 (1316 mm [Oct--Mch totals] vs 1090 mm 2014-15; Appendix 4) leading to higher rates of leaf infection the following spring. However, more detailed micrometeorological analysis or artificial inoculation (difficult since this fungus has not yet been successfully cultured axenically) to substantiate this suggestion.

Infection biology of *Po. rubrum*

The stromata of *Po. rubrum* overwinter on fallen leaves, and perithecia develop within these during the winter period, releasing ascospores in the spring (Grove 1884; Douglas 2018) (Appendix 1). Each separate stroma is thought to arise from a separate infection, and the fungus cannot spread beyond the spreading lesion once within the plant (Blackman & Welsford 1912). Both of these hypotheses suggest that higher frequencies of stromata are linked to elevated levels of ascospore release during leaf growth (Habibi & Banihashemi 2016). Ascospores of

Po. rubrum are similar in size (ca. 14 x 6 µm) to those of the *Venturia inaequalis* and epidemiological studies of this pathogen show that most lesions in orchards are derived from highly localised infection sources (Gadoury & MacHardy 1986). This is consistent with the high variation in stromatal frequency in the trees within the Carran area.

Ascospore release has not been studied in *Po. rubrum* but Ghazanfari and Banihashemi (1976) found that optimal ascospore release in *Po. amygdalinum* required a vernalisation period (>3 mths at 5 ˚C) and shallow burial (5 cm) in soil. They did not investigate the effect of different moisture regimes but for *V. inequalis*, ascospore release is closely linked to periods of rainfall (Aylor & Sutton 1992). Diaz et al. (2007) found that for *Blumeriella jaapii* (cherry leaf spot), also spread by ascospore release from infected overwintered leaves, that the phenology of bud burst was a key determinant of infection levels; a similar situation may pertain for *Po. rubrum*.

In order to test whether edaphic factors might affect abundance of *Po. rubrum* stromata, two hedgerows in an area of wet soil were surveyed as above. Two of these hedgerows are adjacent to a large transient lake (*turlough*) which expands in winter and floods the soil below the hedgerow (potentially washing away leaf litter), whilst the other two were along roads which are not flooded in winter (Fig. 3); the latter revealed a much higher abundance of stromata than the former (35% vs 5%; ANOVA P<0.001). This suggests that leaves infected with *Po. rubrum* which fall onto wetter soil or standing water are less likely to release ascospores the following spring.

Association of *Po. rubrum* infection with leaf galls

Leaf galls caused by the gall mite *Eriophyes prunispinosae* Nalepa, 1926 (syn. *Eriophyes similis* var. *pruni spinosae*; Acarida: Eriophyoidea; (O'Connor 2004; Ripka 2007; Chinery

2013)) were observed to be common on leaves of *P. spinosa*. *Eriophyes* mites infect and over-winter in leaf buds and are important vectors of plant viruses (Easterbrook 1979; Gispert et al. 1998). Examination of the co-occurrence of gall mites and *Po. rubrum* found that *Po. rubrum* stromata were more frequent in both 2015 and 2016 on leaves bearing *E. similis* galls (Fig 4; Chi-square P<0.0046). This suggests that the presence of *E. prunispinosae* on buds at leaf burst may facilitate infection by *Po. rubrum*, for instance by damaging the leaf surface or by modifying leaf development.

DNA barcoding of *Po. rubrum*

Confirmation of the identity of the *Po. rubrum* at the survey sites was undertaken by sequencing of the ITS region of the rRNA operon (Appendix 5). The sequence from the Burren voucher (MG768912) and from a sample from Cornwall, England (MG768911) were very similar (>96% identity) to the two other published sequences for this species: '*Polystigma* sp. Rub1' (KC996927) sequence from *P. domesticus* leaf tissue from Iran and a *Po. rubrum* sample on *Prunus cerasifera* from Russia (KY594023). Both fall into a clade adjacent to *Polystigma amygdalinum* (causal agent of red leaf blotch of almond [*Prunus dulcis*]) with 100% bootstrap support. *Polystigma* spp. were originally placed within the family Phyllachoraceae (order Phyllachorales) but recent phylogenetic analyses (Habibi et al. 2015; Mardones et al. 2017) showed this genus to be polyphyletic, with *Po. rubrum* and the other species infecting hosts in Rosaceae now moved to the family Polystigmataceae within order Xylariales (Dayarathne et al. 2017). One unknown sequence from Alaskan soil was also recovered in this clade (KC966927), which may represent *Po. fulvum*, since *Prunus padus* is highly invasive along riverbanks in Alaska (Roon et al. 2014).

Conclusions

Here we have shown that *Po. rubrum* stromata are very common in the high rainfall habitats of the Burren and that the localised distribution of these stromata varies according to the habitat of the host trees, which in turn may be linked to localised variation in the dynamics of ascospore formation on overwintered leaves below these trees. We also found a positive correlation between the occurrence of stromata and the presence of *E. prunispinosae* galls, and it may be the case that damage caused by these mites could predispose leaves to infection.

At a global level, *Po. rubrum* is predominantly reported from northwestern Europe and mainly from coastal locations (gbif.org/species/8917019). The fieldsite in Carran used for this study is ca. 10 km from the sea and subject to some salt from prevailing westerly winds. It is possible that such coastal climatic condition may predispose to leaf infection by *Po. rubrum*. It is interesting to note that the closely related species *Po. fulvum* found on *Prunus padus* (bird cherry) also exhibits a distinct coastal distribution in Scandinavia (gbif.org/species/9227084) (Appendix 6).

An additional possibility is that the coastal distribution is due to the relative absence of fossil fuel derived pollutants in these areas. The fact most UK records pre-date 1965 (Douglas 2018) is consistent with global data from GBIF, where most (82/159) records date from before 1917. Emissions of SO_2 from coal burning increased during the course of the Industrial Revolution, especially in the decades around 1900 (Mylona 1996) so it is possible that the general decline in abundance of this fungus and its present distribution are explained by its great sensitivity to SO_2 pollution.

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Figure captions

Figure 1 -- Orange-red stromata formed by *Polystigma rubra* on leaves of *Prunus spinosa* (A,B) and in the Burren these were present in abundance (C). Also present on some *P. spinosa* leaves were galls formed by the gall mite *Eriophyes prunispinosae* (B,D).

Figure 2 -- Percentage of *Prunus spinosa* leaves at the Carran fieldsite infected with *Polystigma rubrum in* open limestone pavement (P), hedgerow (H). Abundance of stromata was higher in 2016 than 2015 but only in pavement and hedgerow habitats (ANOVA). Combining data over two years, stromatal abundance was greater in hedgerows and pavement than woodlands (Kruskal-Wallis $P<0.001$; $n=6-12$ replicates); NS indicates not significant.

Figure 3 -- The percentage of *Prunus spinosa* leaves infected with *Polystigma rubrum* was lower in trees growing winter-flooded soil (WET) than in dry soil (ANOVA $P <0.001$; $n=12$ per treatment).

Figure 4 -- Frequency of occurrence of *Polystigma rubrum* galls on leaves also infected with the gall mite *Eriophyes prunispinosae* or not. More stromata were observed on galled leaves.

List of electronic appendices

Appendix 1 -- Spores formed by *Polystigma rubrum.* A,B) Asci containing ascospores in an ascostroma found on dead leaves beneath *P. spinosa* (Allt Wen, Aberystwyth [52.390,-4.095]; collected 18 Feb 2016. C,D) Conidia from *Prunus spinosa* at Ferwig, [52.113,-4.655]; collected 31 Aug 2010. Scalebars indicate 20 μm.

Appendix 2 -- Distribution map of *Polystigma rubrum* across the British Isles. Red indicates records dating before 1965 whilst yellow and green dots indicate records from 1965-2014 and 2015-present. Note that all recent records are from more westerly, coastal regions. Red arrow indicates location of present study (Carran, Co. Clare [N53.038, W9.068]). Data from FRDBI and Lost and Found Fungi project (http://fungi.myspecies.info/content/lost-found-fungi-project).

Appendix 3 -- Infection levels of *Prunus spinosa* leaves by *Polystigma rubrum* at different heights above the ground for 50x50 cm quadrats at different heights along a hedgerow (n=4; ANOVA P=0.7)

Appendix 4 -- Monthly rainfall totals for Carran (data collected by Mary and Patrick Cassidy).

Appendix 5 -- Maximum likelihood tree based on ITS1/2 sequences of *Polystigma rubrum* and related species. Sequences from the other clades in subclass Xylariomycetidae are used as outgroups (530 bp alignment). Salient bootstrap percentages (1000 replicates) are shown at nodes.

DNA was extracted from dried *P. rubrum* stromata using the methods of Edwards et al. (2013), with PCR amplification of the ITS region with the primers ITS1F and ITS4, as described by Edwards et al. (2013). Sequence management was conducted within the Geneious (v10.2.3) bioinformatics package, using MAFFT (Katoh et al. 2017) for sequence alignment (default settings). Phylogenetic reconstruction was conducted using PhyML (Guindon et al. 2010), implemented in Geneious and using the GTR substitution model.

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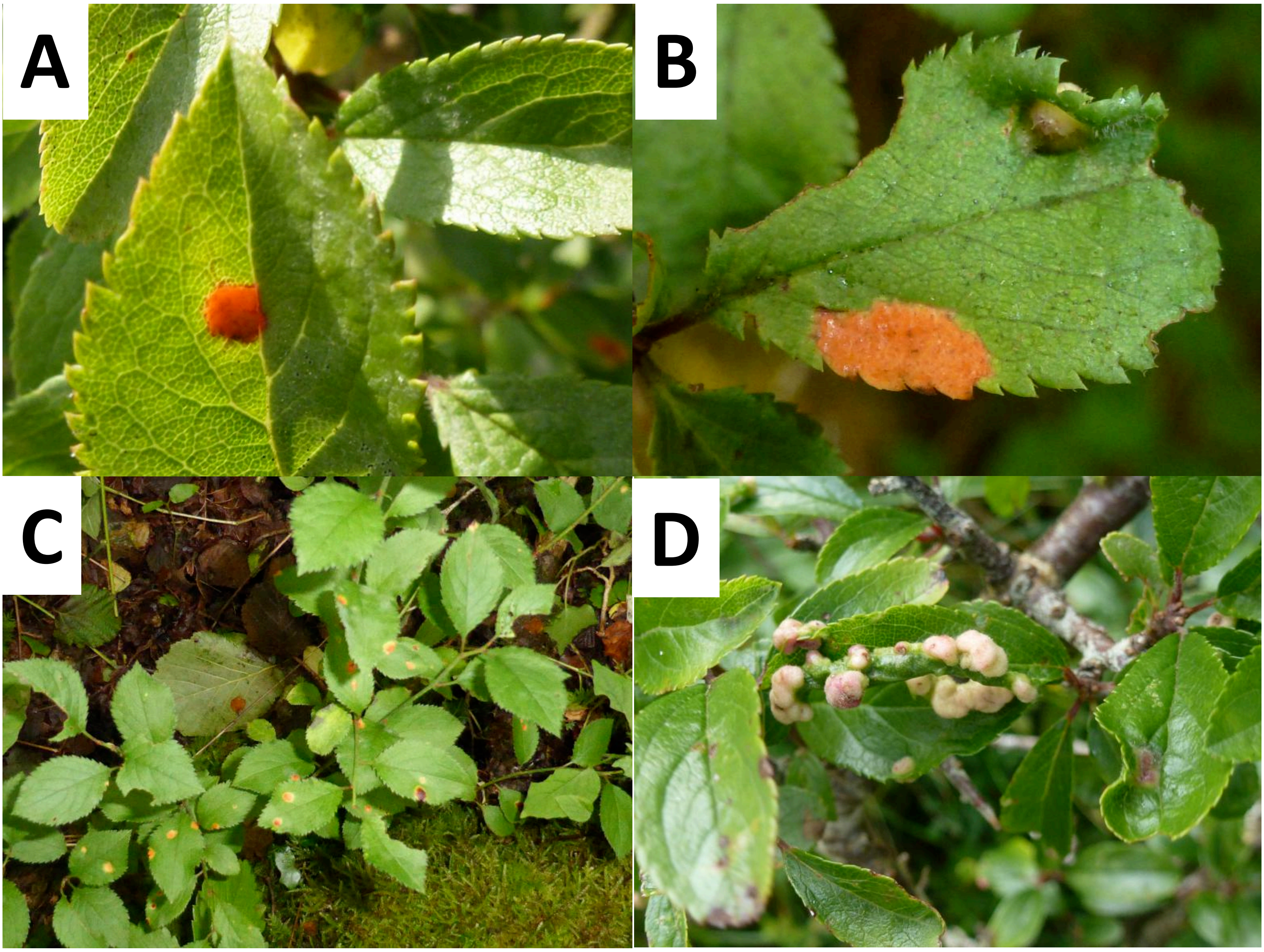
Appendix 6 -- Global distributions of (A) *P. rubrum* (gbif.org/species/8917019) and (B) *P. fulvum* (gbif.org/species/9227084) based on GBIF data.

Polystigma rubrum (Pers.) DC., 1815 in GBIF Secretariat (2017). GBIF Backbone Taxonomy. Checklist Dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-01-10.

Polystigma fulvum DC.:Fr. in GBIF Secretariat (2017). GBIF Backbone Taxonomy. Checklist Dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-01-10.



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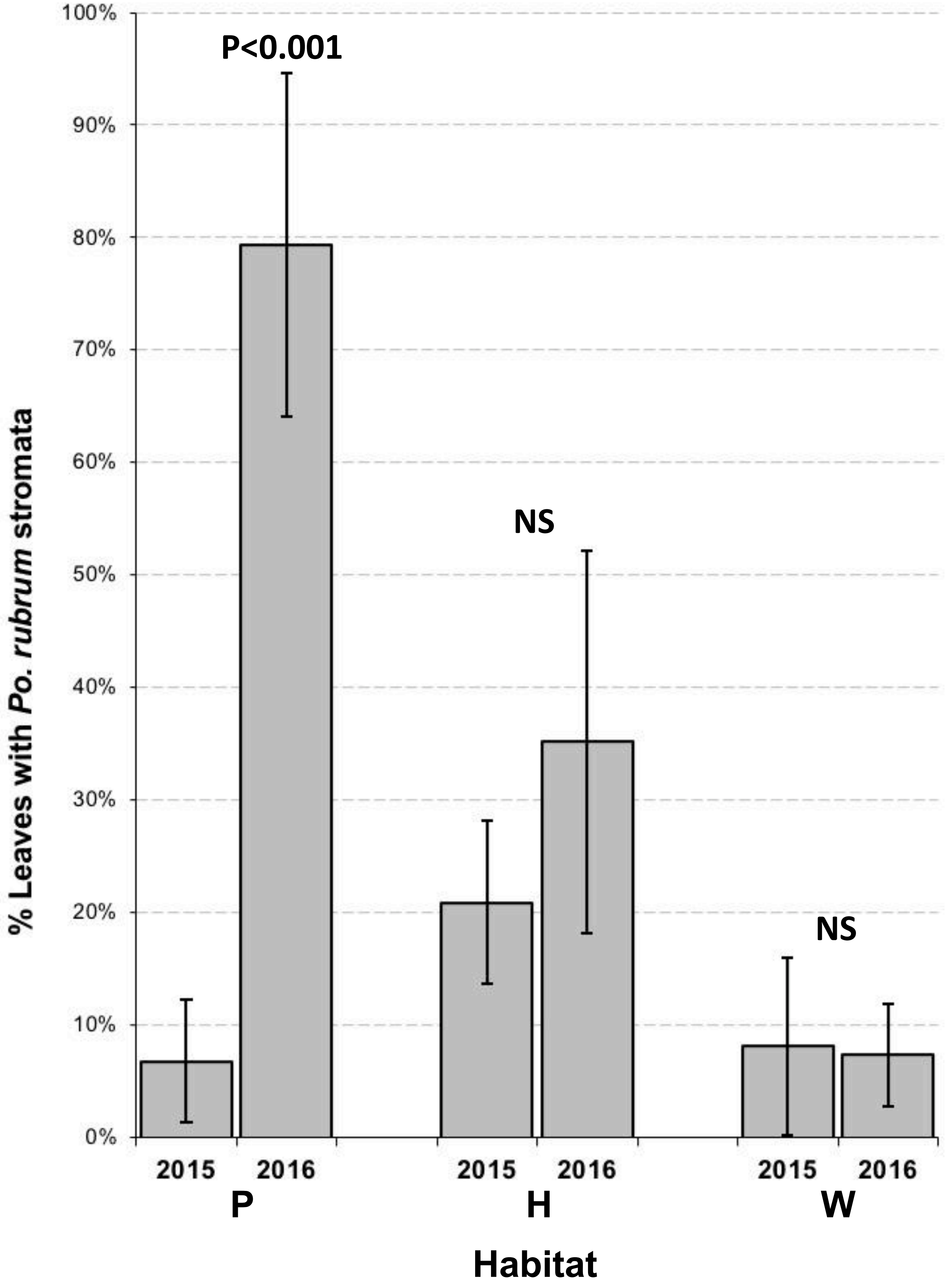
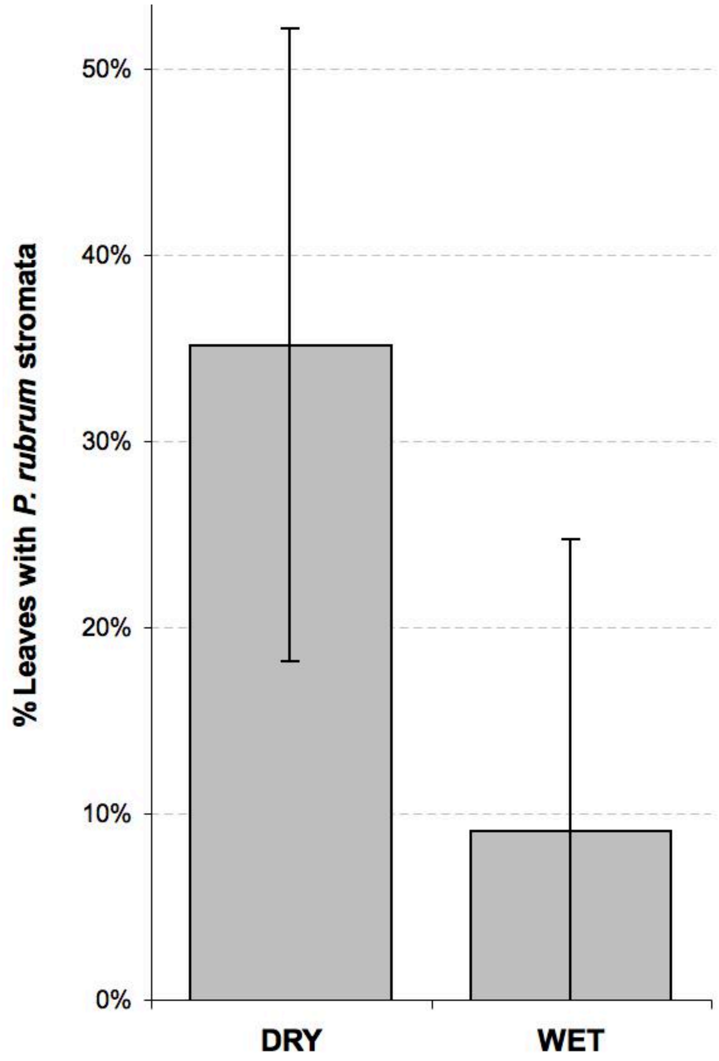


Fig. 2. Percentage of *Prunus spinosa* leaves at the Carran fieldsite infected with *Polystigma rubrum in* open limestone pavement (P), hedgerow (H) and woodland (W). Abundance of stromata was higher in 2016 than 2015 but only in pavement and hedgerow habitats (ANOVA). Combining data over two years, stromatal abundance was greater in hedgerows and pavement than woodlands (Kruskal-Wallis P<0.001; n=6-12 replicates); NS indicates not significant.



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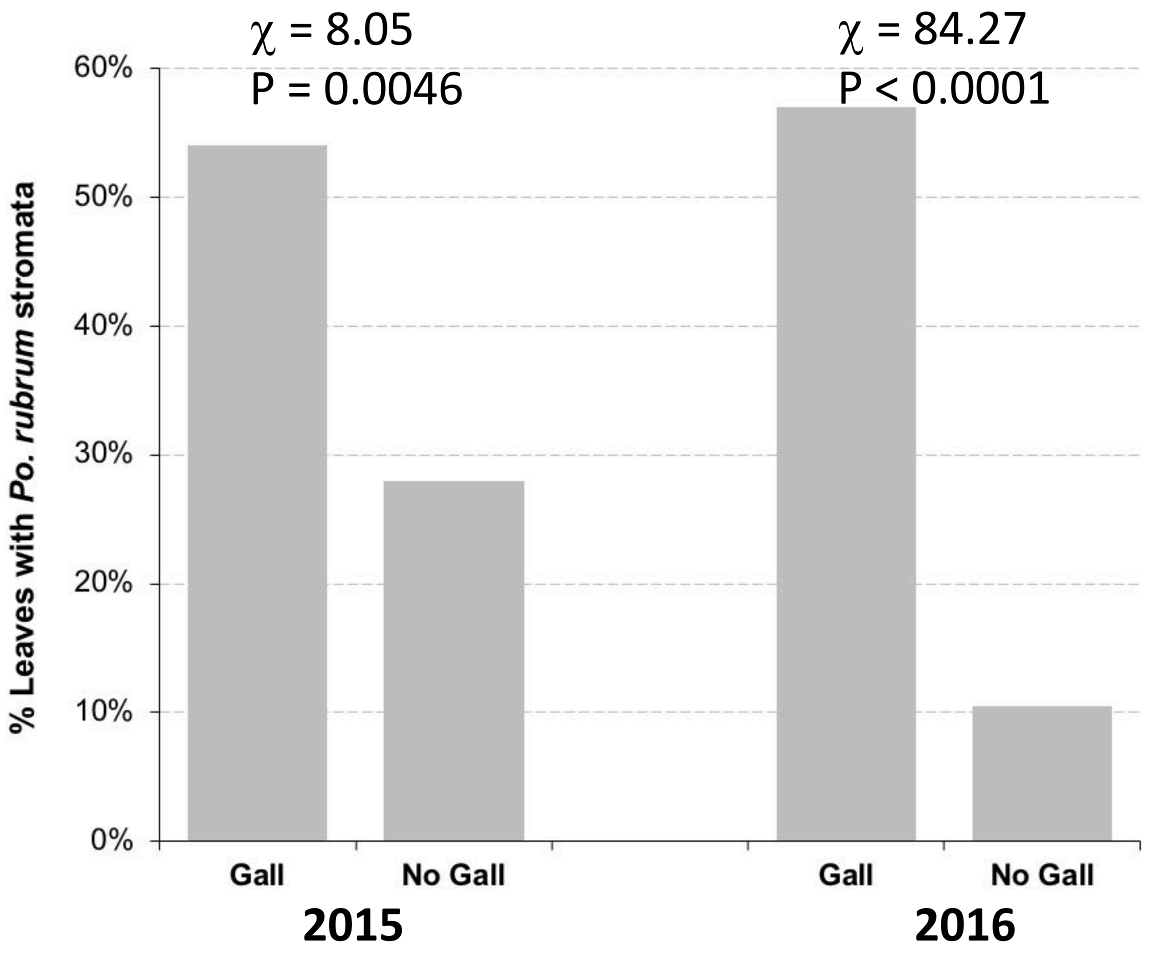

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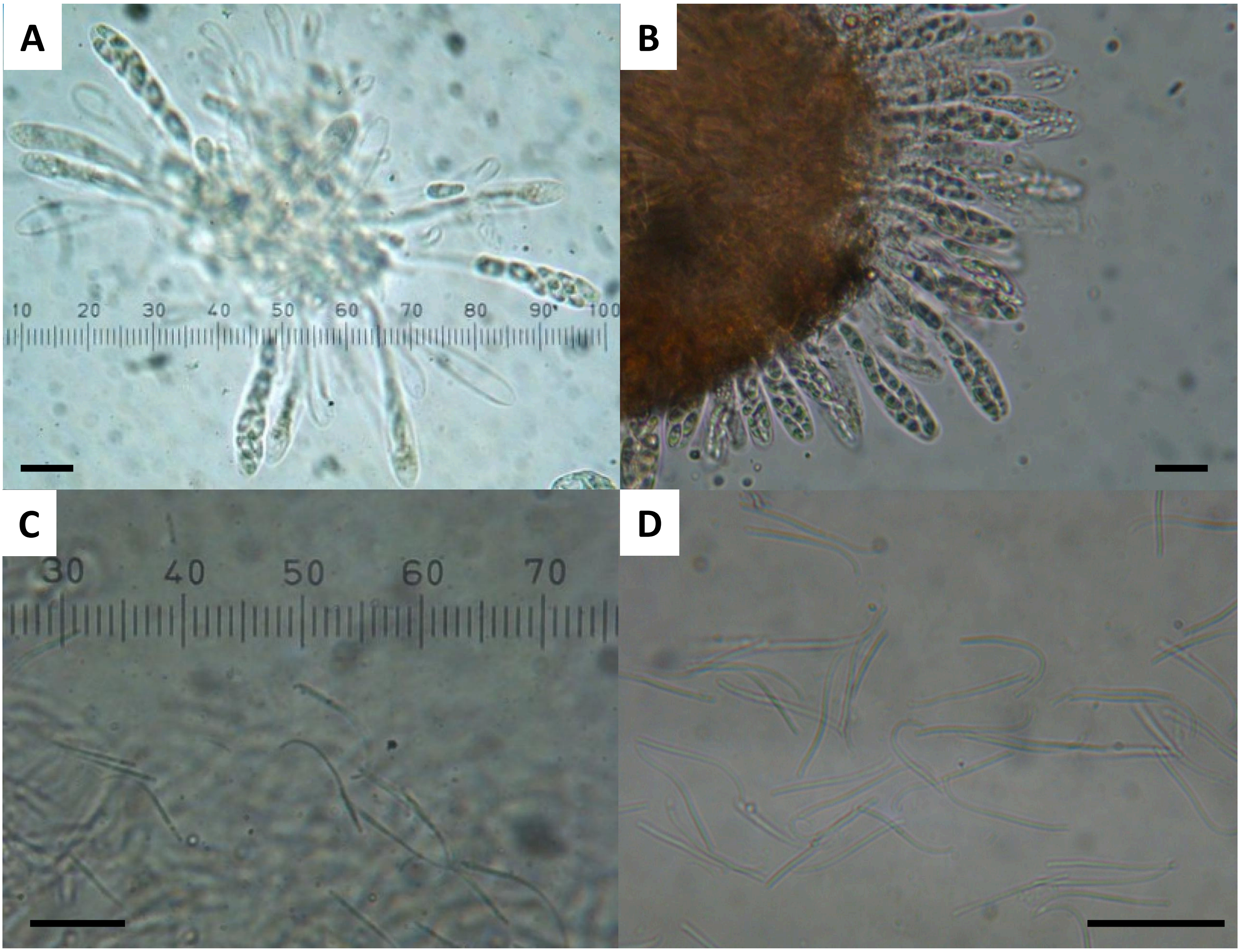

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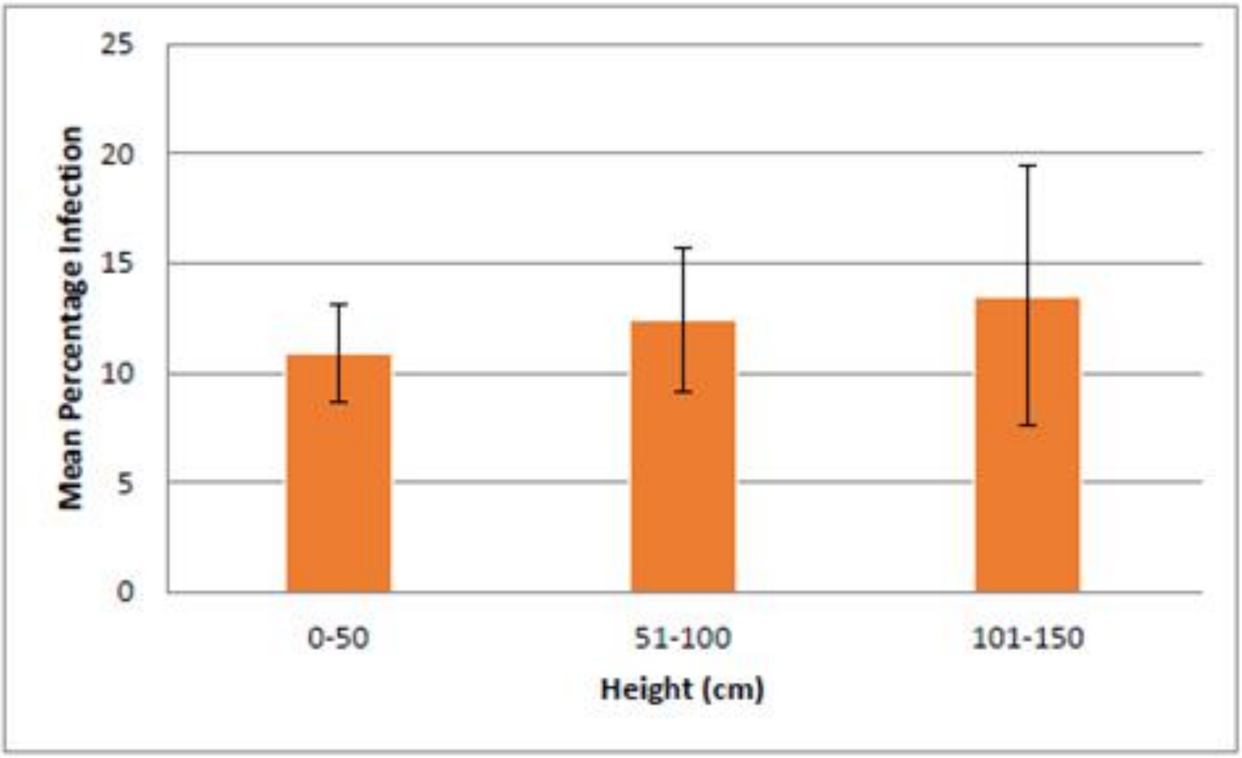

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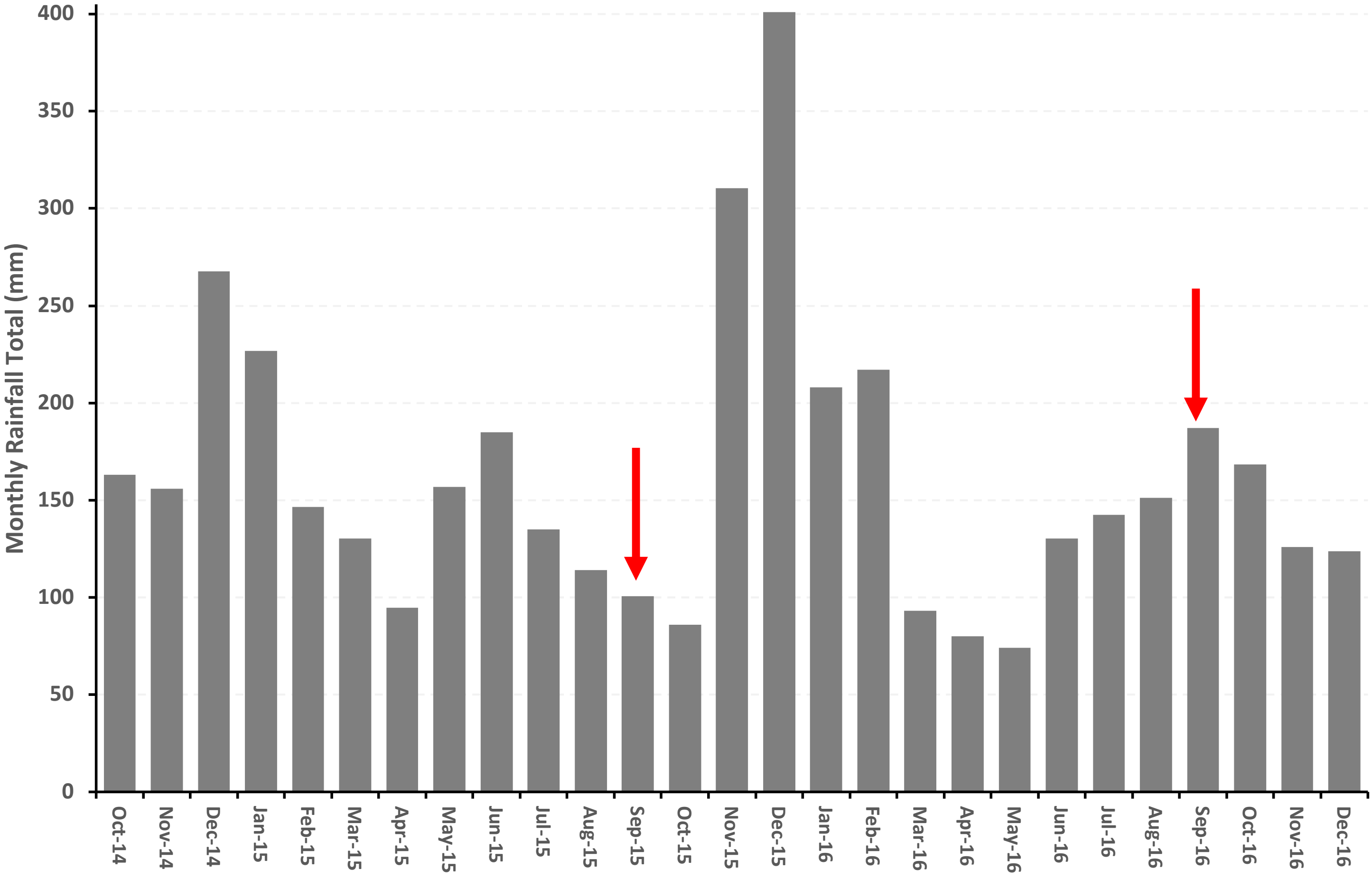
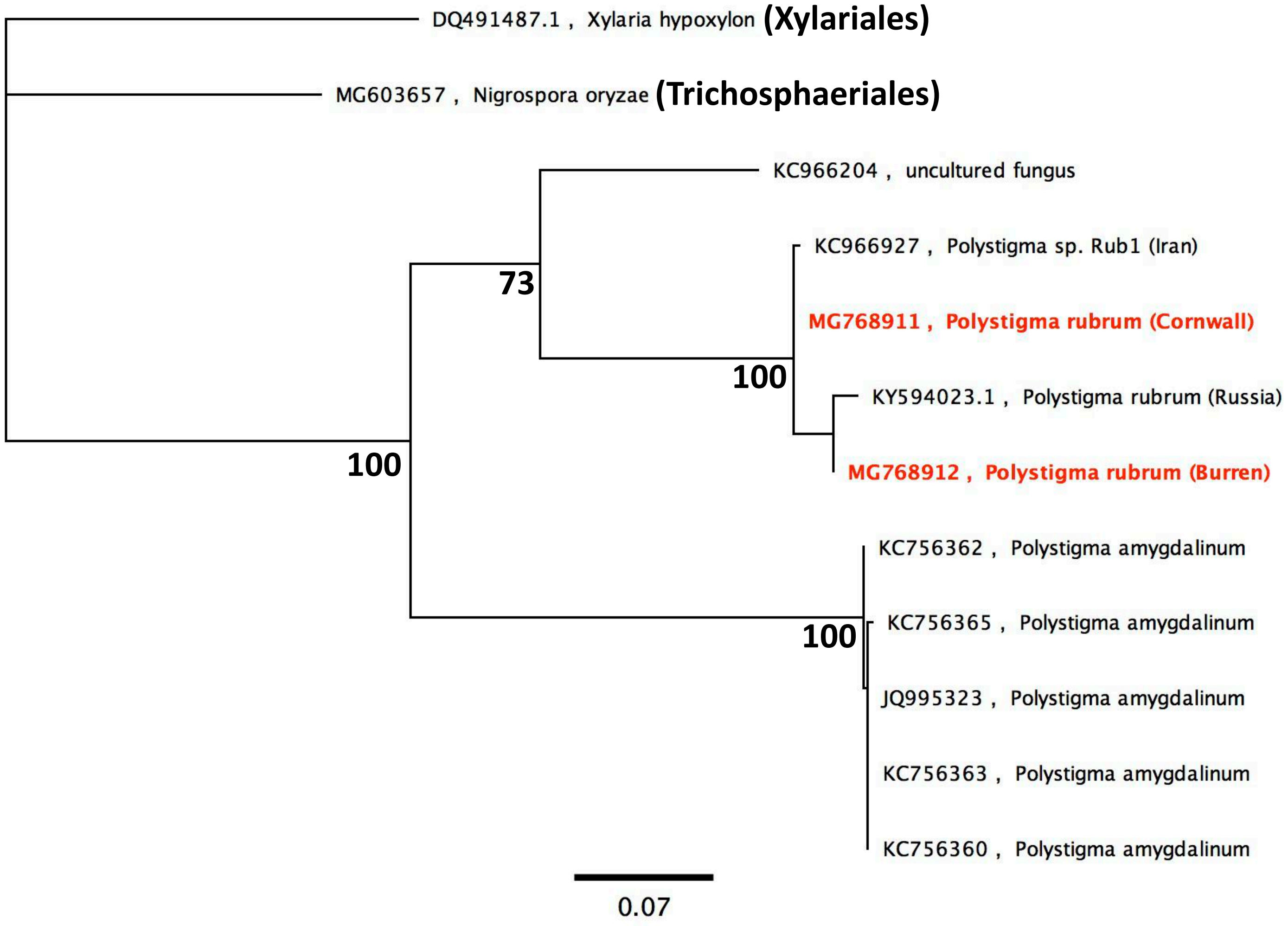


Appendix 4. Monthly rainfall totals for Carran (data collected by Mary and Patrick Cassidy).



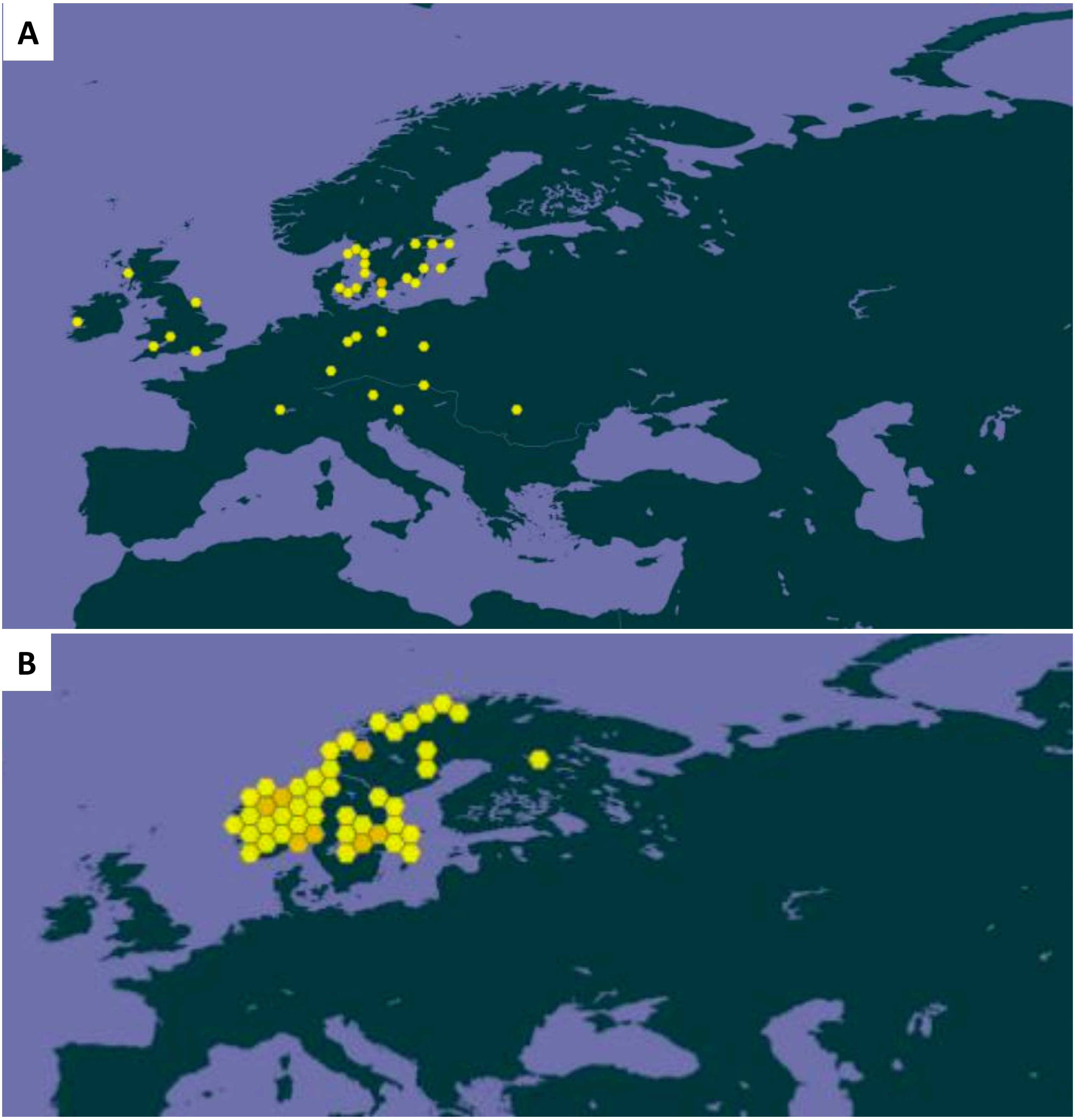
Appendix 5. Maximum likelihood tree based on ITS1/2 sequences of *Polystigma rubrum* and related species. Sequences from the other clades in subclass Xylariomycetidae are used as outgroups (530 bp alignment). Salient bootstrap percentages (1000 replicates) are shown at nodes.

DNA was extracted from dried *P. rubrum* stromata using the methods of Edwards et al. (2013), with PCR amplification of the ITS region with the primers ITS1F and ITS4, as described by Edwards et al. (2013). Sequence management was conducted within the Geneious (v10.2.3) bioinformatics package, using MAFFT (Katoh et al. 2017) for sequence alignment (default settings). Phylogenetic reconstruction was conducted using PhyML (Guindon et al. 2010), implemented in Geneious and using the GTR substitution model.

Edwards A, Douglas B, Anesio AM, Rassner SM, Irvine-Fynn TDL, Sattler B, Griffith GW (2013) A distinctive fungal community inhabiting cryoconite holes on glaciers in Svalbard. Fungal Ecol 6 (2):168-176.

Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59 (3):307-321.

Katoh K, Rozewicki J, Yamada KD (2017) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Briefings in Bioinformatics bbx108. doi: 10.1093/bib/bbx108



Appendix 6. Global distributions of (A) *P. rubrum* (gbif.org/species/8917019) and (B) *P. fulvum* (gbif.org/species/9227084) based on GBIF data.

Polystigma rubrum (Pers.) DC., 1815 in GBIF Secretariat (2017). GBIF Backbone Taxonomy. Checklist Dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-01-10.

Polystigma fulvum DC.:Fr. in GBIF Secretariat (2017). GBIF Backbone Taxonomy. Checklist Dataset https://doi.org/10.15468/39omei accessed via GBIF.org on 2018-01-10.