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

Roberts, Hattie Rose; Pidcock, Sara Elizabeth; Redhead, Sky C.; Richards, Emily; O'Shaughnessy, Kevin; Douglas, Brian; Griffith, Gareth

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tel: +44 1970 62 2400
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1 **Running title:** *Polystigma rubrum stromata* on *Prunus spinosa*

2

3 **Short Communication**

4

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

6

7 Roberts, Hattie R¹, Sara E Pidcock¹, Sky C Redhead¹, Emily Richards¹, Kevin O'Shaughnessy¹,
8 Brian Douglas² and Gareth W Griffith^{1*}

9

10 ¹ Institute of Biological, Environmental and Rural Sciences, Cledwyn Building, Aberystwyth
11 University, Penglais, Aberystwyth, Ceredigion Wales GB-SY23 3DD

12 ² Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey, England GB-TW9 3DS

13 * **Corresponding author:** gwg@aber.ac.uk; 0044-1970-622325

14 15 Text Pages and Four Figures (6 appendices)

15

16

17 **Abstract**

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

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

23 Key results: Assessment of the local distribution of the fungus over two years found stromata
24 to be occur more frequently on *P. spinosa* leaves in hedgerows than woodlands. On individual
25 trees in areas of open limestone pavement, the frequency of stromata was ten times higher in
26 2016 than 2015, possibly related to interannual rainfall differences. On hedgerow trees
27 subjected to winter flooding, stromata were much less abundant, whereas stromata were more
28 abundant on leaves also infected by the gall mite *Eriophyes prunispinosae*. The identity of *Po.*
29 *rubrum* was confirmed by ITS sequencing.

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

36

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

39

40

41 **Introduction**

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

49

50 Each stromatal patch is believed to be the result of a single infection. Distribution of the
51 fungus often is highly uneven between trees, suggesting localised transmission. There is no
52 evidence for systemic infection suggesting that reinfection each year is mediated via
53 ascospores formed on dead fallen leaves (Blackman & Welsford 1912). The extent of any
54 leaf discolouration caused by *Po. rubrum* may vary depending upon the age of the lesions, for
55 example, young stromata can be a less intense yellow-brown colour, whilst older stromata
56 have a striking orange or red pigment. The stromata are generally 5--20 mm in diameter, and
57 have an irregular orbicular shape, producing hamate (hooked) conidia (possibly sterile or
58 with spermatial function) from immersed conidiomata (Cannon 1996; Dayarathne et al.
59 2017). The sexual stage of the fungus is characterised by the same stromata turning black on
60 the fallen overwintering leaves and producing immersed ascomata and ascospores, (Grove
61 1884) (A O. Chater, pers. comm.; Appendix1).

62

63 *Polystigma rubrum* is occasionally reported as a pathogen of domesticated plum trees (*Prunus*
64 *domestica*; red leaf spot disease) in Mediterranean climates (Mitre jr et al. 2015), whilst other
65 *Polystigma* spp. are also reported as pathogens of *Prunus dulcis* (almond; *Po. amygdalinum*

66 [formerly *Po. ochraceum*, Iran; (Ghazanfari & Banihashemi 1976)) and *Prunus padus* / *Prunus*
67 *ssiori* (bird cherry; *Po. fulvum*; (Suzuki et al. 2008)).

68

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

77

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

83

84

MATERIAL AND METHODS

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

90

RESULTS AND DISCUSSION

91

92 **Distribution of *Po. rubrum* infections**

93 In contrast to its current rarity across most of the British Isles ([Appendix. 2](#)), where 1% leaves
94 are only rarely encountered even in areas where the fungus has previously been located, *P.*
95 *rubrum* was present on most surveyed *P. spinosa* trees in the Burren, with stromata abundant
96 (8--80% of leaves) in both survey years in the study area (1 km² area, immediately east of
97 Carran; N53.038, W9.068). There was no evidence that leaves infected with *Po. rubrum* were
98 prematurely senescent although it has been reported that this contributes to the economic
99 damage caused by other *Polystigma* spp. on *Prunus* hosts (Kranz 1962; Banihashemi 1990;
100 Cannon 1996).

101

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

109

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

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

117

118 The highest frequency of stromata in the 50 x 50 cm quadrats (79%) was found on limestone
119 pavement habitats in 2016, though in 2015 only 7% of leaves bore stromata. Stromatal
120 frequency for hedgerow trees showed the same pattern (35% in 2016 vs 21% in 2015; ANOVA
121 $P = 0.042$; Fig. 2), whereas in woodland habitat stromatal frequency was similar in both years
122 (7--8%).

123

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

132

133 **Infection biology of *Po. rubrum***

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

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

144

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

152

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

161

162 **Association of *Po. rubrum* infection with leaf galls**

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

165 2013)) were observed to be common on leaves of *P. spinosa*. *Eriophyes* mites infect and over-
166 winter in leaf buds and are important vectors of plant viruses (Easterbrook 1979; Gispert et al.
167 1998). Examination of the co-occurrence of gall mites and *Po. rubrum* found that *Po. rubrum*
168 stromata were more frequent in both 2015 and 2016 on leaves bearing *E. similis* galls (Fig 4;
169 Chi-square $P < 0.0046$). This suggests that the presence of *E. prunispinosae* on buds at leaf burst
170 may facilitate infection by *Po. rubrum*, for instance by damaging the leaf surface or by
171 modifying leaf development.

172

173 **DNA barcoding of *Po. rubrum***

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

188

189 **Conclusions**

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

196

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

204

205 An additional possibility is that the coastal distribution is due to the relative absence of fossil
206 fuel derived pollutants in these areas. The fact most UK records pre-date 1965 (Douglas 2018)
207 is consistent with global data from GBIF, where most (82/159) records date from before 1917.
208 Emissions of SO₂ from coal burning increased during the course of the Industrial Revolution,
209 especially in the decades around 1900 (Mylona 1996) so it is possible that the general decline
210 in abundance of this fungus and its present distribution are explained by its great sensitivity to
211 SO₂ pollution.

212

213

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215 photomicrographs of conidia and asci; Ray Woods and Margaret Howells for their guidance
216 during experimental design and data collection; Anthony Morris for assistance with data
217 collection; Mary and Patrick Cassidy of Carran for provision of local weather data. BD is
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220

221

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283

284

285 **Figure captions**

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

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

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

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

299

300 **List of electronic appendices**

301 **Appendix 1** -- Spores formed by *Polystigma rubrum*. A,B) Asci containing ascospores in an
302 ascostroma found on dead leaves beneath *P. spinosa* (Allt Wen, Aberystwyth [52.390,-
303 4.095]; collected 18 Feb 2016. C,D) Conidia from *Prunus spinosa* at Ferwig, [52.113,-4.655];
304 collected 31 Aug 2010. Scalebars indicate 20 μm .

305 **Appendix 2** -- Distribution map of *Polystigma rubrum* across the British Isles. Red indicates
306 records dating before 1965 whilst yellow and green dots indicate records from 1965-2014 and
307 2015-present. Note that all recent records are from more westerly, coastal regions. Red arrow
308 indicates location of present study (Carran, Co. Clare [N53.038, W9.068]). Data from FRDBI

309 and Lost and Found Fungi project ([http://fungi.myspecies.info/content/lost-found-fungi-](http://fungi.myspecies.info/content/lost-found-fungi-project)
310 project).

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

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

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

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

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334 **Appendix 6** -- Global distributions of (A) *P. rubrum* (gbif.org/species/8917019) and (B) *P.*
335 *fulvum* (gbif.org/species/9227084) based on GBIF data.
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339 Dataset <https://doi.org/10.15468/39omei> accessed via GBIF.org on 2018-01-10.
340
341
342

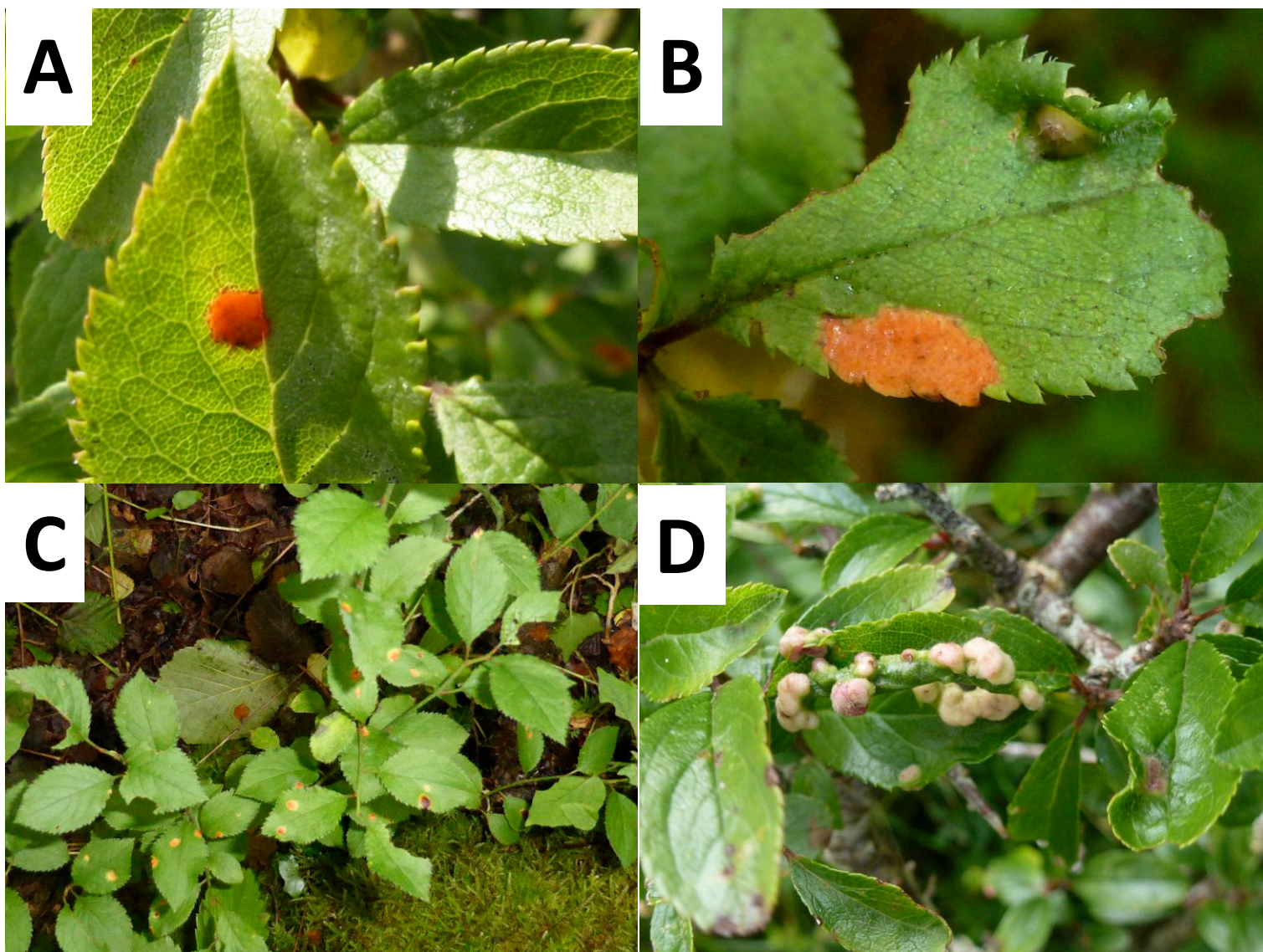


Fig. 1. Orange-red stromata formed by *Polystigma rubra* on leaves of *Prunus spinosa* (A,B) and in the Burren these were present in abundance (C). Also present on some *P. spinosa* leaves were galls formed by the gall mite *Eriophyes prunispinosae* (B,D).

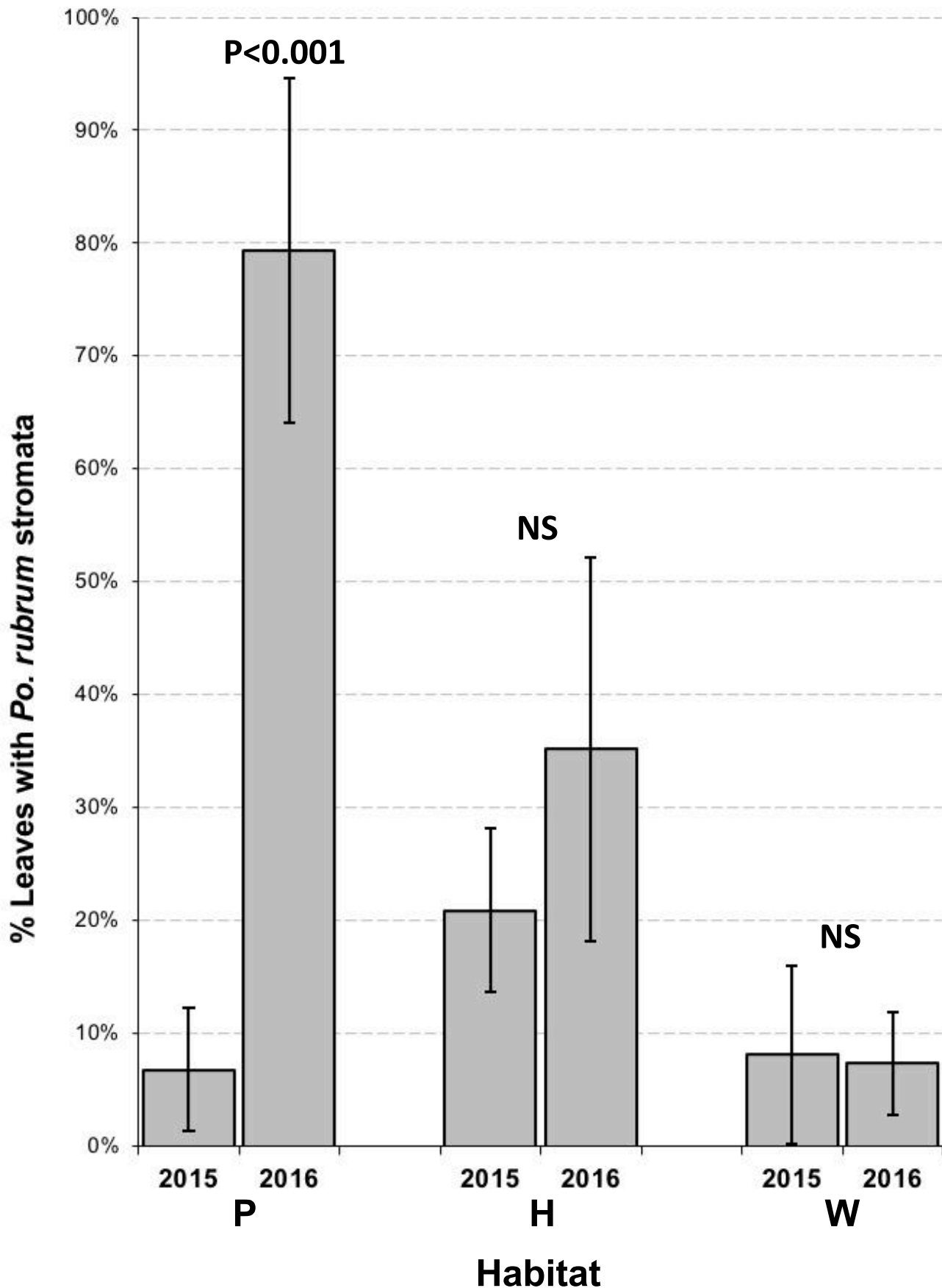


Fig. 2. Percentage of *Prunus spinosa* leaves at the Carran fieldsite infected with *Polystigma rubrum* in open limestone pavement (P), hedgerow (H) and woodland (W). Abundance of stromata was higher in 2016 than 2015 but only in pavement and hedgerow habitats (ANOVA). Combining data over two years,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

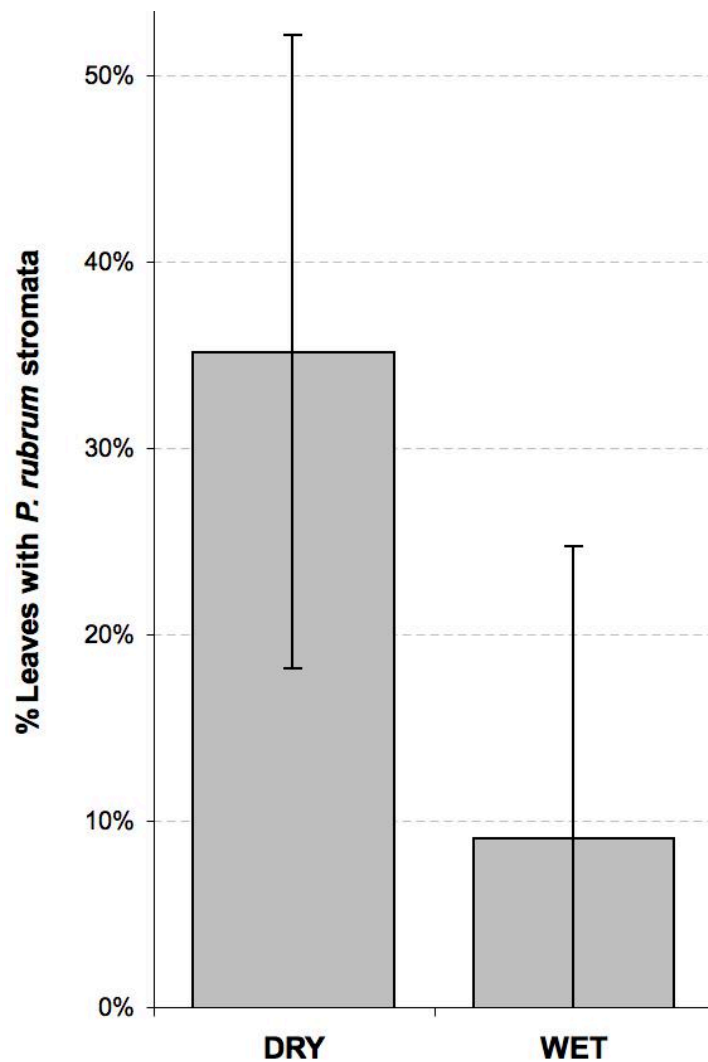


Fig. 3. The percentage of *Prunus spinosa* leaves infected with *Polystigma rubrum* was lower in trees growing winter-flooded soil (WET) than in dry soil (ANOVA $P < 0.001$; $n=12$ per treatment).

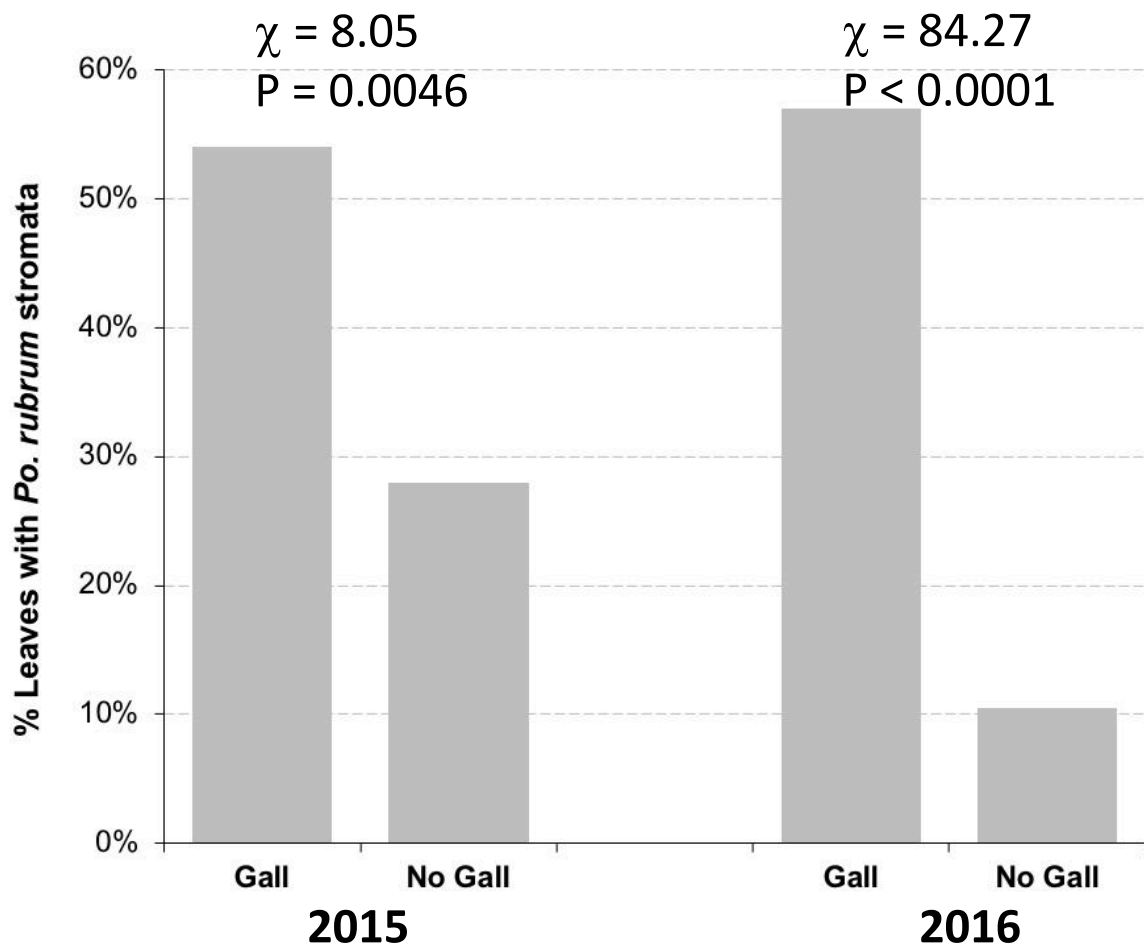
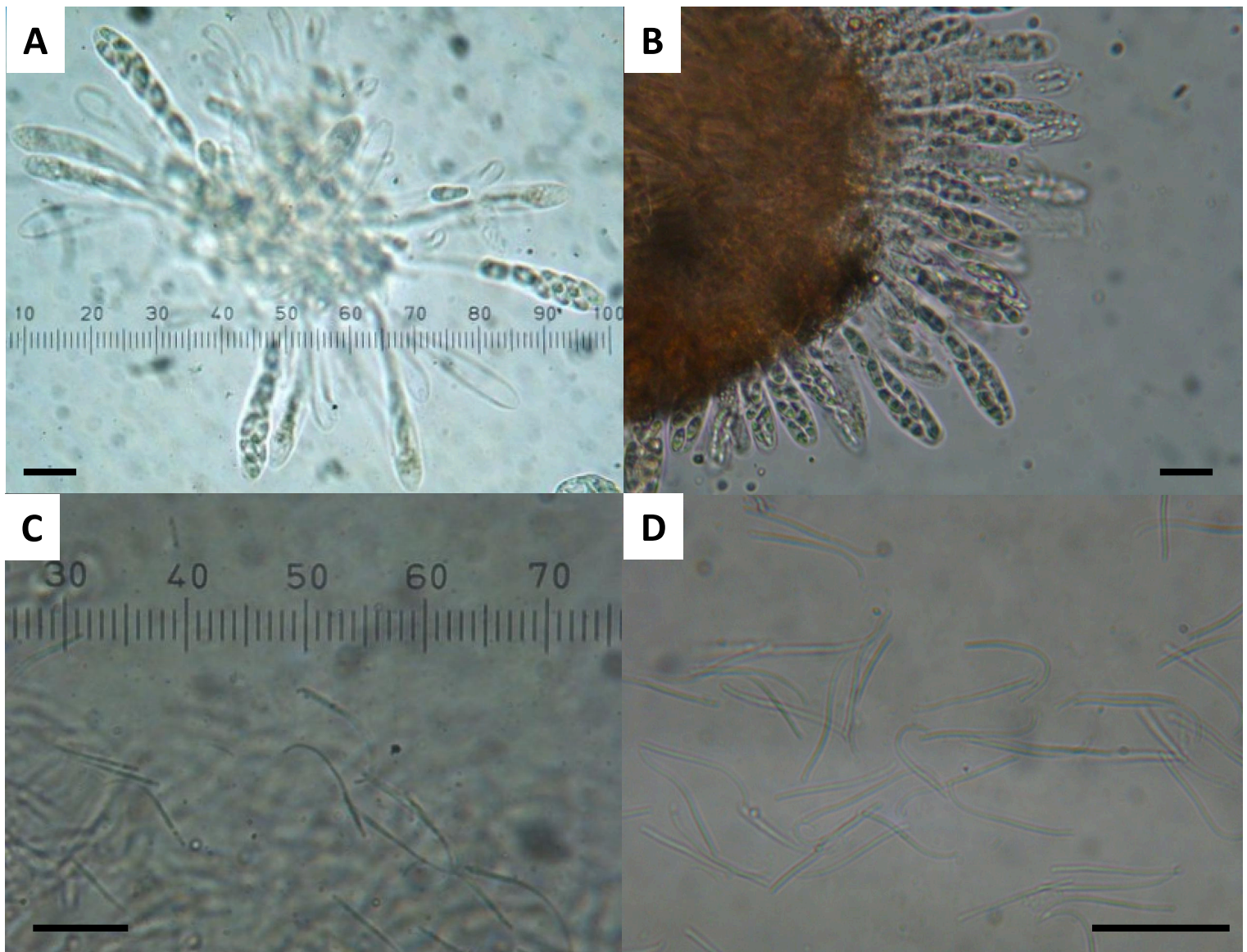


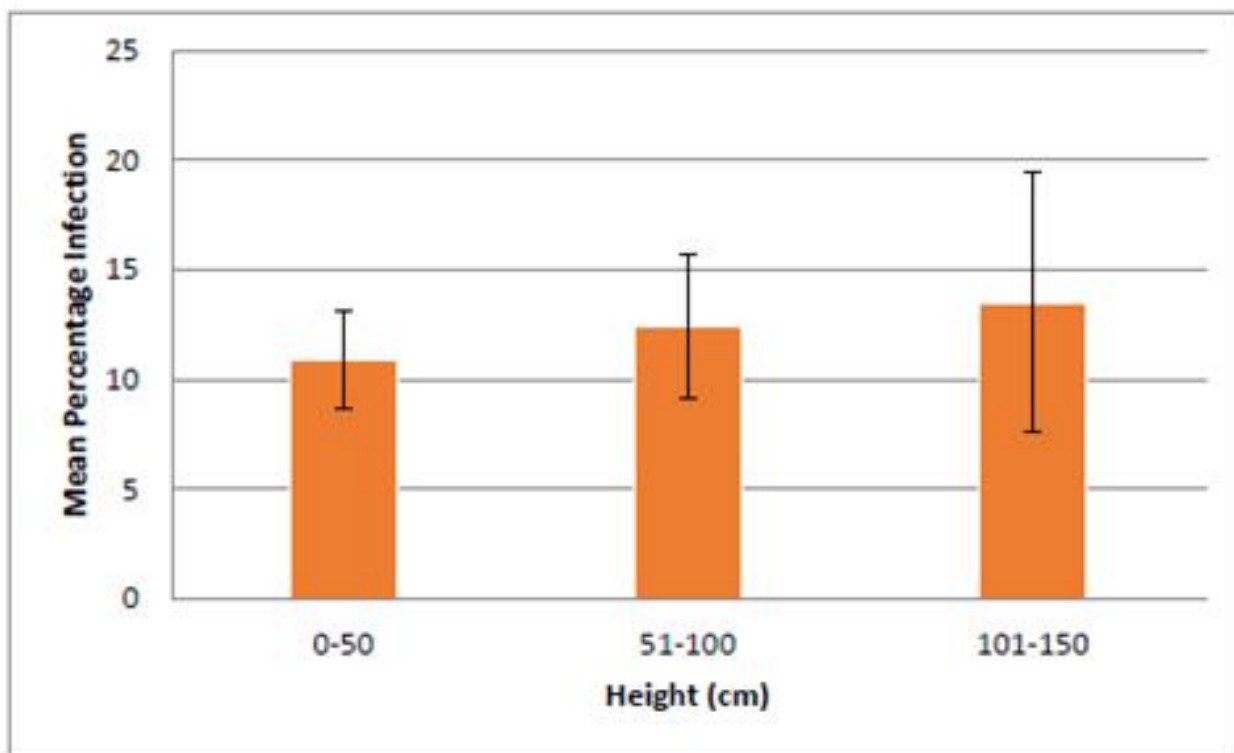
Fig. 4. Frequency of occurrence of *Polystigma rubrum* galls on leaves also infected with the gall mite *Eriophyes prunispinosae* or not. More stromata were observed on galled leaves.



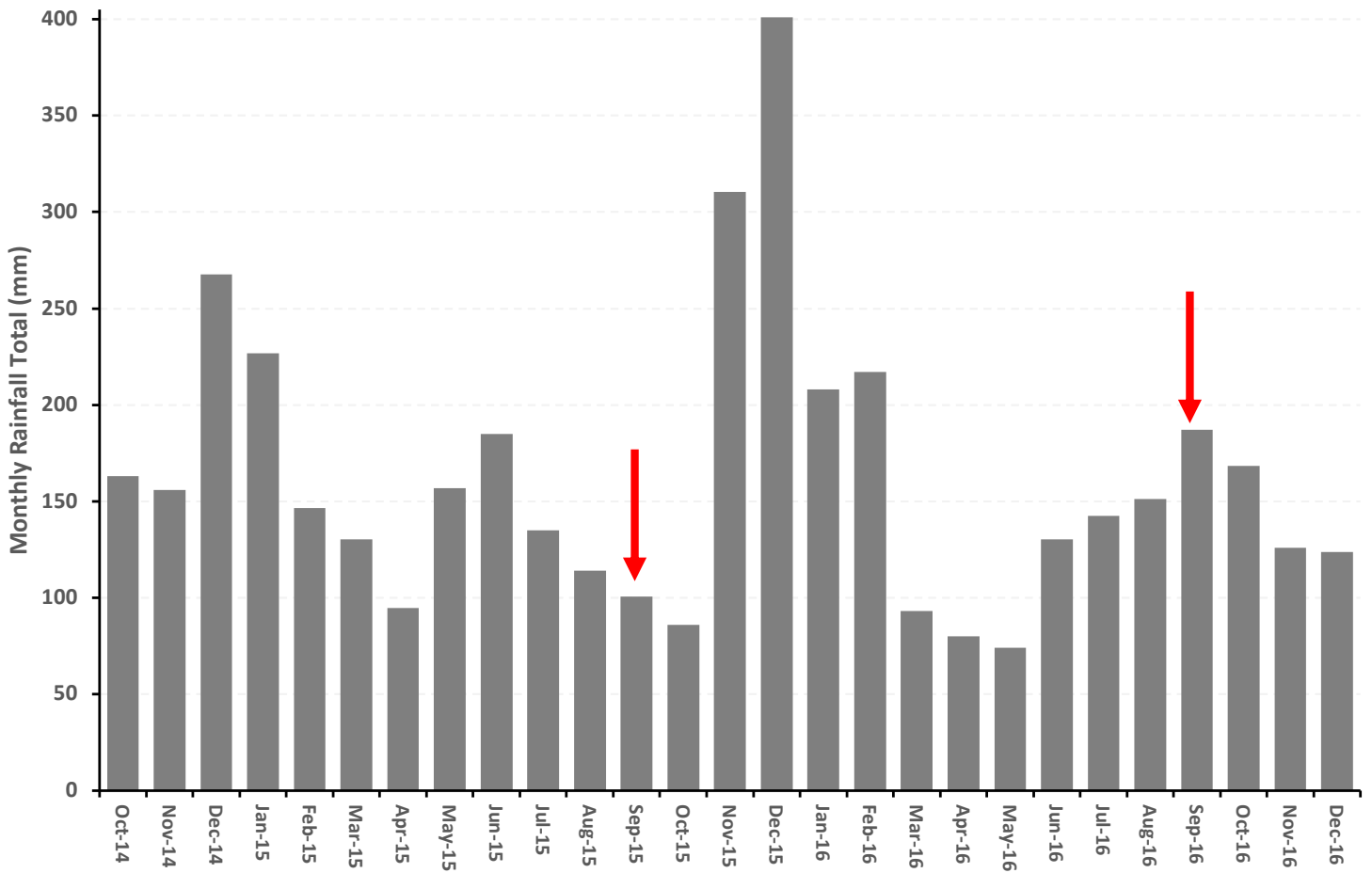
Appendix 1. Spores formed by *Polystigma rubrum*. A,B) Asci containing ascospores in an ascostroma found on dead leaves beneath *P. spinosa* (Allt Wen, Aberystwyth [52.390,-4.095]; collected 18 Feb 2016. C,D) Conidia from *Prunus spinosa* at Ferwig, [52.113,-4.655]; collected 31 Aug 2010. Scalebars indicate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



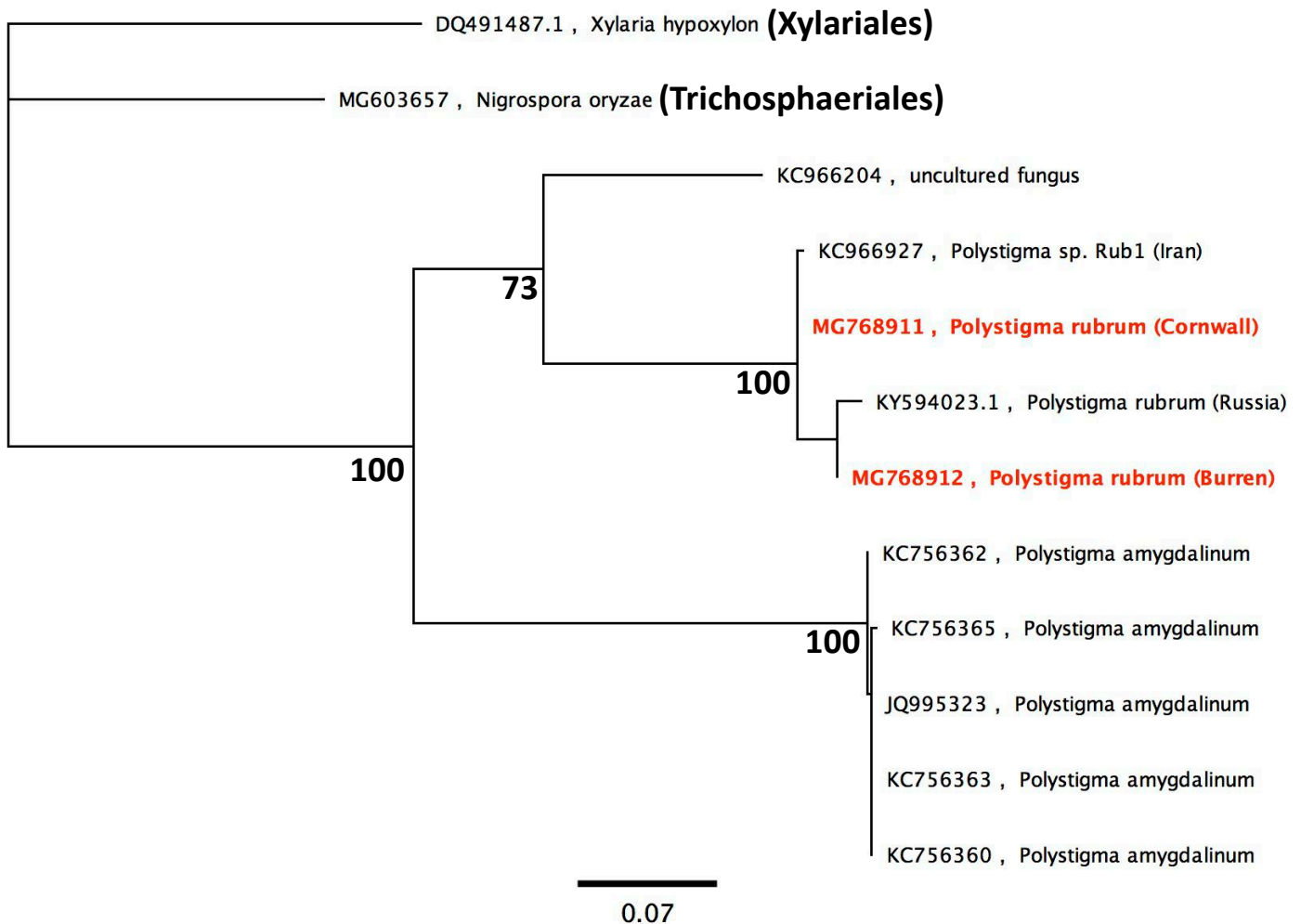
Appendix 2. Distribution map of *Polystigma rubrum* across the British Isles. Red indicates records dating before 1965 whilst yellow and green dots indicate records from 1965-2014 and 2015-present. Note that all recent records are from more westerly, coastal regions. Red arrow indicates location of present 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 (Kato 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.

Kato 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.