1   **Two Indigenous *Berberis* Species from Spain Were Confirmed as Alternate Hosts**

2   **of the Yellow Rust Fungus *Puccinia striiformis* f. sp*. tritici***

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15   **Abstract**

16   *Puccinia striiformis* f. sp. *tritici* (*Pst*), which causes yellow (or stripe) rust on wheat, is a macrocyclic

17   and heteroecious fungus. In this study, we investigated whether *Berberis vulgaris* subsp. *seroi*

18   and *B. vulgaris* subsp. *australis*, which are indigenous in Spain, may serve as alternate hosts for

19   *Pst*. Wheat leaves bearing telia of an isolate of *Pst* were harvested and used to inoculate plants

20   of both barberry subspecies. Pycnia were observed on the adaxial side of the leaves from 10 days

21   after inoculation (dai). Following successful fertilization, aecia were observed on the abaxial side

22   of the leaves from 16 dai. At 27 dai, barberry leaves bearing aecia were detached and used to

23   inoculate susceptible wheat seedlings of cultivar 'Morocco'. Uredinia were observed on wheat

24   seedlings from 12 days after aeciospore exposure. Eighty-three single lesions were recovered

25   from individual wheat leaves, of which 43 were genotyped using 19 *Pst* simple sequence repeat

26 markers (SSR). In total, 19 multilocus genotypes (MLGs) were identified among the 43 progeny

27 isolates. The SSR genotyping confirmed that all 43 isolates were derived from the parental isolate.

28 Seven heterozygous SSR markers showed segregation among the progenies, whereas none of

29 the 12 homozygous markers resulted in segregation. These results demonstrated that *B. vulgaris*

30 subspp. *seroi* and *australis* can serve as alternate hosts for *Pst*, which may result in novel

31 virulence combinations that can have a detrimental impact on wheat production. Although *Pst* has

32 not been detected on these barberry species in nature, this study highlights the importance of rust

33 surveillance in barberry areas where suitable conditions for completion of the sexual life cycle

34 may be present.

35
36 ***Keywords***: alternate host, barberry, segregation, sexual reproduction, stripe rust, wheat
37

38 Heteroecious cereal rust fungi within the genus *Puccinia* require botanically distant

39 primary and alternate hosts to complete the life-cycle involving both asexual and sexual

40 reproduction (Jin et al. 2010; Stubbs 1985). Sexual reproduction on the alternate host

41 possesses advantages of the generation of novel genotypes (and races), which may have

42 detrimental effects on the durability of rust resistance in wheat cultivars (Roelfs and Groth

43 1980; Zhao et al. 2016). *Puccinia striiformis* Westend. f. sp*. tritici* Erikss. (*Pst*), which

44 causes yellow (or stripe) rust on wheat, is considered a serious threat to a stable wheat

45 supply (Beddow et al. 2015; Singh et al. 2016). The search for the alternate host of *Pst*

46 date back to the 19th and 20th centuries, where teliospores were used to inoculate plant

47 species of the *Berberidacea* family. However, all attempts were unsuccessful (Stubbs

48 1985). The reasons behind the failure in finding the alternate host of *Pst* at that time may

49 be manifold, e.g., failure in establishing suitable conditions for infection, the use of *Pst*

50 races with low sexual capacity, and the use of non-susceptible plant species (Hovmøller

51   et al. 2016; Rodriguez-Algaba et al. 2014). Recently, Jin et al. (2010) discovered that

52   aeciospores from *B. chinensis* and *B. koreana* could infect *Poa pratensis* (Kentucky

53   bluegrass) and identified that *P. pseudostriiformis* (syn. *P. striiformis* f. sp. *poae*), as the

54   rust specie involved. Further experiments confirmed that teliospores of *Pst* were able to

55   infect *B. chinensis*, *B. koreana*, *B. holstii*, and *B. vulgaris* and that aeciospores from *B.*

56   *chinensis* infected wheat seedlings. Thus, the yellow rust fungus was confirmed

57   heteroecious and macrocyclic represented by five spore stages, i.e., uredinia, telia,

58   basidia, pycnia, and aecia. About 500 barberry species have been described within the

59   genus *Berberis* (Kim et al. 2004). To date, approximately 50 *Berberis* spp. have been

60   confirmed susceptible to *Pst* under natural or experimental conditions (Li et al. 2020;

61   Mehmood et al. 2020). Additionally, *Mahonia aquifolium* (Oregon grape) has also been

62   identified as alternate host for *Pst* under experimental conditions (Wang and Chen 2013).

63   Similarly, natural aecial infections collected in western China from *B. aggregata*, *B.*

64   *brachypoda*, *B. polyantha*, *B. shensiana,* and *B. soulieana* have been reported as *Pst*,

65   but only at low frequencies (Wang et al. 2016; Zhao et al. 2013). This may suggest that

66   proper conditions for sexual reproduction may exist in certain parts of Asia, where

67   barberry is ubiquitous and frequently growing close to wheat crops. However, the role of

68   *Berberis* species as alternate host of *Pst* in other parts of the world is still unclear (Berlin

69   et al. 2012; Wang et al. 2015). In Europe, *B. vulgaris* (also known as European or

70   common barberry) has historically been used as a hedge shrub to avoid livestock from

71   damaging cereal crops and for medicinal and ornamental purposes since it was

72   introduced from Asia in the Middle Ages (Barnes et al. 2020; Hermansen 1968; Roelfs

73   1982). Since the 17th century, eradication and legislation campaigns were established to

74   remove barberry species and particularly common barberry, aiming to reduce the novel

75   inoculum of *P. graminis* (the stem rust fungus infecting cereal and grasses) generated

76   after sexual reproduction (Hermansen 1968; Stakman 1923). Currently, common

77   barberry is widely distributed throughout Europe (iNaturalist 2021) due to the abolishment

78   of eradication programs and legislation restricting the planting of *B. vulgaris*. Recently,

79   Rodriguez-Algaba et al. (2020); (2014)reported that *Pst* isolates of exotic origin can

80   undergo sexual reproduction under experimental conditions using *B. vulgaris* plants

81   collected in Denmark and Sweden from natural areas. Although *Pst* has not yet been

82   found to reproduce sexually in Europe under natural conditions, the high prevalence of

83   common barberry could initiate the infection by *Pst* races of sexual origin (characterized

84   by high teliospore production) and that have been widely detected across Europe since

85   2011 (Ali et al. 2010; Ali et al. 2017; Hovmøller et al. 2016). Besides *B. vulgaris*,

86   indigenous barberry species, e.g., *B. catartica* in Croatia (Kosalec et al. 2009), *B. cretica*

87   in Greece, *B. aetnensis* in Italy (Ahrendt 1961), and *B. vulgaris* subsp*. seroi* (syn. *B.*

88   *garciae*) and *B. vulgaris* subsp*. australis* (syn. *B. hispanica*) in Spain (López González

89   1986) are present in Europe. In this study, we investigated whether *B. vulgaris* subsp.

90   *seroi* and *B. vulgaris* subsp. *australis* may serve as alternate hosts for *Pst*. Better

91   knowledge of the role of indigenous barberry species in rust epidemiology in Europe is

92   crucial as these could potentially contribute to increase genetic diversity in rust

93   populations and thereby pose a threat to European wheat production.

94   Seeds of *B. vulgaris* subspp. *seroi* and *australis* were collected from Huesca and

95   Albacete provinces in Spain, respectively, where these barberry subspecies grow

96   naturally. *Berberis vulgaris* subsp. *seroi* is found at altitudes from 470 to 2,000 m above

97   mean sea level (AMSL), occasionally growing close to cereal crops. *Berberis vulgaris*

98   subsp. *australis* grows at altitudes from 1,000 to 2,500 m AMSL, but it is infrequently

99   found in close proximity to cereal crops (López González 1986). Species identification of

100   barberry plants were confirmed based on specific morphological characters, e.g., color of

101   one-year-old stems, size of racemes, color of matured fruits, and type and morphology of

102   leaves (López González 1986). After harvesting, seeds were dried at room temperature.

103   Ten seeds of each *B. vulgaris* subspecies were placed in petri dishes containing a peat-

104   based substrate and stratified at 5°C for approximately three months to promote seed

105   germination. After germination, seedlings were transferred to 10 cm square pots

106   containing an organic peat-based substrate with slow release of plant nutrients and grown

107   in rust quarantine spore-proof greenhouse cabins. Subsequently, barberry plants were

108   grown for approximately one year until specific morphological characters used for specie

109   identification were clearly visible.

110   The sexual life cycle of *Pst* was completed according to the methodology described in

111   Rodriguez-Algaba et al. (2014), with slight modifications. Yellow rust infected wheat

112   leaves bearing uredinia and telia were harvested from an experimental field nursery

113   previously inoculated with *Pst* isolate DK219_19, originally collected in Denmark (in year

114   2019) from wheat cultivar 'Ambition' and characterized by a high production of teliospores

115   on seedlings and adult plants. The wheat leaves were kept dried at 5°C until further use.

116   Germination of telia was initiated by soaking the dried leaves bearing telia in distilled

117   water at room temperature (22-25°C) for two days, followed by incubation on 2.5% water

118   agar containing 50 mg/l chloramphenicol at 12°C for two days in darkness. Microscopic

119   observations confirmed that basidiospores were produced after two days of incubation.

120   Inverted agar plates containing germinating teliospores were placed on top of open-ended

121   transparent plastic cylinders each containing barberry plants of a single barberry

122   subspecies and incubated at 12°C for 4 days in darkness, 100% relative humidity (RH).

123   Pycnia were observed on the adaxial leaf surface from 10 days after inoculation (dai) (Fig.

124   1a and 1e). Infected plants were regularly sprayed with mist water to facilitate fertilization

125   of pycnia. Aecia were observed on the abaxial leaf surface from 16 dai (Fig. 1b and 1f).

126   The recovery of aeciospores was carried out by exposing susceptible wheat seedlings of

127   cultivar 'Morocco' below barberry leaves bearing multiple aecial clusters at 27 dai. The

128   wheat seedlings were incubated at 10°C for 24 h in darkness, 100% RH, and transferred

129   to spore-proof cabins under the conditions described in Rodriguez-Algaba et al. (2014).

130   Subsequently, new wheat seedlings were placed below the infected barberry leaves and

131   incubated as described above. Uredinia and telia were observed on wheat seedlings from

132   12 and 28 days after aeciospore exposure, respectively (Fig. 1c and 1g; 1d and 1h).

133   Variability in telia production was observed among the progeny isolates (data not shown).

134   From 14 to 22 days after aeciospore exposure, 83 clearly separated single lesions

135   bearing uredinia (59 derived from *B. vulgaris* subsp. *seroi* and 24 from *B. vulgaris* subsp.

136   *australis*, respectively) were collected from wheat leaves. A total of 43 single lesions (19

137   derived from *B. vulgaris* subsp. *seroi* and 24 from *B. vulgaris* subsp. *australis*) were

138   selected for genotypic analysis using 19 *Pst* simple sequence repeat (SSR) markers

139   according to Rodriguez-Algaba et al. (2014) (Table 1). Telia representing the parental

140   isolate was included as control in the SSR marker analysis. New alleles were not

141   observed among the progeny isolates, in comparison to the parental isolate, confirming

142   that all progeny isolates were indeed descendants of the parental isolate. The parental

143   isolate was homozygous at 12 loci and heterozygous at 7 loci, which revealed segregation

144   among the progenies resulting in 19 novel multilocus genotypes (MLGs) (Table 1, Fig. 2).

145   The identification of the MLGs was carried out using the Poppr genetic analysis software

146   for sexual populations (Kamvar et al. 2014). Progeny isolate DK219_19_F1 (MLG number

147   8) shared MLG with the parental isolate. Other progeny isolates also shared MLG with

148   one another, suggesting that some progeny isolates originated from the same fertilization

149   event at the pycnial stage and thus from spores produced within a single aecial cup (a.k.a.

150   aecium) (Table 1, Fig. 2) (Rodriguez-Algaba et al. 2017). Particularly, progenies derived

151   from *B. vulgaris* subsp. *australis* showed fewer MLGs compared to *B. vulgaris* subsp.

152   *seroi*, which may be explained by the relatively few aecia present on the leaves of the

153   former being used for aeciospore recovery on wheat seedlings.

154

155   This study demonstrated that *B. vulgaris* subsp. *seroi* and *B. vulgaris* subsp. *australis*

156   are susceptible to *Pst* and may serve as alternate hosts for this important wheat rust

157   pathogen. Although there are no indications of sexual recombination in the contemporary

158   European *Pst* population (Hovmøller et al. 2016), the results stress the importance of rust

159   surveillance in areas where barberry and wheat coexist and appropriate environmental

160   and physiological conditions for completion of the sexual cycle of *Pst* are present. Further

161   studies aiming to investigate the role of alternate hosts (barberry species), primary host

162   (wheat) and auxiliary hosts (wild cereal and grasses) would help to better understand the

163   epidemiology of *Pst* in Europe. In conclusion, sexual reproduction occurring on

164   indigenous *Berberis* subspecies, such as the ones present in Spain, could result in novel

165   and unique virulence combinations (Rodriguez-Algaba et al. 2020; Zhao et al. 2016) that

166   may have negative consequences for wheat production in Europe and beyond.

167

168   **Funding**

169   This research was supported by the European Commission, Research and Innovation,

170   Horizon 2020-Sustainable Food Security (Grant number 773311-2, RUSTWATCH) and

171   the Bill and Melinda Gates Foundation (Delivering Genetic Gain in Wheat, DGGW).

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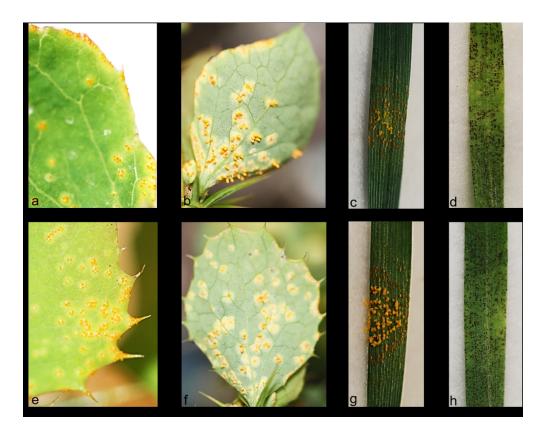202   J. Microbiol. Biotechnol. 25:145–150.

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242   virulence variation in the pathogen isolates from natural infection of barberry plants in China.
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Isolate(s)	Barberry subspecie	No. of segregated SSR loci	RJO4	
			a1	a2
DK219_19 (Parental isolate)	-	-	199	199
DK219_19_F1	seroi	0	199	199
DK219_19_F2, F9	seroi	4	199	199
DK219_19_F3	seroi	3	199	199
DK219_19_F4	seroi	4	199	199
DK219_19_F5	seroi	3	199	199
DK219_19_F6	seroi	4	199	199
DK219_19_F7	seroi	3	199	199
DK219_19_F8	seroi	3	199	199
DK219_19_F10	seroi	1	199	199
DK219_19_F11	seroi	2	199	199
DK219_19_F12, F18, F20	seroi	3	199	199
DK219_19_F13	seroi	5	199	199
DK219_19_F14	seroi	1	199	199
DK219_19_F16	seroi	3	199	199
DK219_19_F17	seroi	2	199	199
DK219_19_F19	seroi	3	199	199
DK219_19_F51, F52, F53, F54, F55, F56, F57, F58, F59, F60, F61, F67, F68, F70	australis	1	199	199
DK219_19_F74, F75	australis	4	199	199
DK219_19_F76, F77, F78, F79, F80, F81, F82, F83	australis	3	199	199

RJO24		RJN12		RJN8		RJN13		RJN3		RJN11		RJO27		RJN6		RJO21		RJN10		RJO18		WU6		RJO20	
a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	287
284	293	196	196	316	316	147	147	336	336	180	180	242	242	318	318	170	170	221	221	331	331	210	210	284	287
284	284	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	224	224	331	331	210	210	284	287
293	293	196	196	316	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	284
284	284	196	196	307	307	147	147	336	336	176	180	242	242	318	318	170	170	221	221	331	331	210	210	284	287
284	284	196	196	316	316	147	147	336	336	180	180	242	242	318	318	170	170	221	224	331	331	210	210	287	287
284	293	196	196	307	316	147	147	336	336	180	180	242	242	318	318	170	170	221	224	331	331	210	210	287	287
284	284	196	196	316	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	287	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	224	224	331	331	210	210	284	287
284	284	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	284
284	284	196	196	307	307	147	147	336	336	176	176	242	242	318	318	170	170	221	224	331	331	210	210	284	287
284	293	196	196	307	316	147	147	336	336	176	176	242	242	318	318	170	170	221	221	331	331	210	210	287	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	287
284	284	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	224	224	331	331	210	210	284	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	221	331	331	210	210	284	287
293	293	196	196	316	316	147	147	336	336	176	180	242	242	318	318	170	170	221	224	331	331	210	210	284	284
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	221	331	331	210	210	284	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	221	221	331	331	210	210	287	287
284	293	196	196	307	316	147	147	336	336	176	180	242	242	318	318	170	170	224	224	331	331	210	210	287	287

RJO24		RJN12		RJN8		RJN13		RJN3		RJN11		RJO27		RJN6		RJO21		RJN10		RJO18		WU6		RJO20	
a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2	a1	a2

RJN2		RJN4		RJN9		RJN5		WU12	
a1	a2	a1	a2	a1	a2	a1	a2	a1	a2
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
187	187	253	255	332	332	226	226	332	332
181	181	253	255	332	332	226	226	332	332
181	181	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
187	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
187	187	253	253	332	332	226	226	332	332
181	187	255	255	332	332	226	226	332	332
181	187	253	253	332	332	226	226	332	332
187	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
181	187	253	255	332	332	226	226	332	332
187	187	255	255	332	332	226	226	332	332
187	187	253	255	332	332	226	226	332	332

Pycnia and aecia developed on Berberis vulgaris subspp. seroi (top) and australis (bottom) and uredinia and telia observed on wheat seedlings, a,e: Pycnia observed on the adaxial side of the leaf at 10 days after inoculation (dai), b,f: Aecia observed on the abaxial side of the leaf at 20 dai, c,g: Uredinia observed on wheat seedlings of cultivar 'Morocco' at 14 days after aeciospore recovery, d,h: Telia observed on wheat seedlings of cultivar 'Morocco' at 28 days after aeciospore recovery.

233x184mm (150 x 150 DPI)

Minimum distance spanning tree of the parental isolate (in yellow) and the 19 multilocus genotypes (MLG) detected among the 43 progeny isolates. Sizes of nodes are proportional to the number of progeny isolates detected on each MLG, which are indicated by different colors.

198x190mm (150 x 150 DPI)