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Sulla Powdery Mildew: Phylogeny and Host Range

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Abstract: Sulla is a biannual forage legume cultivated throughout the Mediterranean Basin. It can be severely damaged by powdery mildew, but there is little understanding on its causal species or its host range. The taxonomic characterization of *Erysiphe* species is mainly based on the examination of chasmothecia morphology, or on the molecular analysis of ITS1 and ITS2 sequences. However, a description of chasmothecia morphology or ITS sequences is not always available to clearly assign a given isolate to an existing or novel *Erysiphe* species. In an attempt to clarify the identity of the powdery mildew infecting sulla crop we studied the morphology and ITS of nine populations collected over years and countries and compared them with available ITS sequences. Phylogenetic analysis showed that the powdery mildews collected on sulla clustered together with isolates collected on *Coronilla varia* in an independent clade between the *E. guarinonii*/*E. trifoliorum* and *E. palczewskii* clades, strongly suggesting that all these sequences correspond to the same *Erysiphe* species. Measurements of conidia and conidial foot cells of the sulla-infecting powdery mildews partially overlap those of other *Erysiphe* species, although they clearly differentiate from them, supporting the assumption that powdery mildews from sulla belong to a distinct species as observed from the phylogenetic analysis. As far as we know, our study is the first to report the molecular characterization of powdery mildew isolated on plants from the *Hedysarum* clade. Cross-inoculations confirmed a high specialization of the powdery mildew of sulla, with the sulla's isolates infecting only the *S. coronaria* accessions, and none of the accessions from the other legume genera studied. All studied *S. coronaria* accessions were heavily infected by the sulla isolate but not by any other isolate used. All this points to *E. hedysari* as the causal agent of the *S. coronaria* powdery mildew.

Keywords: sulla; *Hedysarum*; powdery mildew; resistance; breeding; ITS

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1. Introduction

Sulla (Sulla coronaria [(L.) B.H. Choi and H. Ohashi)] (syn. *Hedysarum coronarium* L.) is a semiperennial legume cultivated throughout the Mediterranean Basin as a biannual forage crop for grazing and/or hay or silage production [1]. It is well adapted to marginal and drought-prone environments, producing a good-quality, high-protein forage crop with moderate levels of condensed tannins, beneficial to ruminant production [1,2]. It also has potential as green manure [3]. Although sulla has been domesticated rather recently, it is grown all over the Mediterranean Basin, and has been introduced into other areas, notably New Zealand and Australia [4,5]. It was reported as the second largest forage after alfalfa by the 1990s in Southern Italy, grown over 300,000 ha [6]; however, little additional records on the actual cultivated area are available since this crop is mainly grazed. Wide morpho-physiological and adaptive diversity is available in the existing germplasm [1,7–10] which is being exploited in breeding programs.

Powdery mildew, often cited as "oidium", is an important airborne fungal disease in many crops, including legumes [11–13], affecting all green parts of plants. The first symptoms are small, diffuse spots on leaflets and stipules that grow and become white to pale grey powdery areas that later coalesce and completely cover plant surfaces. Powdery mildew can be important also in sulla, but detailed information on its causal species is

missing in most reports [9,14,15]. In fact, powdery mildews (Erysiphales) on legumes represent a taxonomically complicated group of diseases where species differentiation is mainly based on chasmothecia morphology, fruiting bodies that unfortunately are not always formed, hampering species determination. The only detailed taxonomic study reports *Erysiphe hedysari* (U. Braun) U. Braun and S. Takam. on *Hedysarum* spp. [16,17] infecting *Anthyllis* and *Hedysarum*. A number of powdery mildew species were previously cited on various *Hedysarum* sp. including *Microsphaera trifolii* (Grev.) U. Braun [18], *Podosphaera leutricha* [14], *Microsphaera diffusa* Cooke. et Peck [19] or *Microsphaera hedysari* Braun [20]. Former *Erysiphe* and *Microsphaera* are now treated as sections of *Erysiphe* emend. U. Braun and S. Takam. [16]. In addition, molecular characterization of the isolates infecting *S. coronaria* through examination of their ITS sequences is lacking.

A better understanding of the identity and host range of the possible powdery mildews infecting the cultivated *S. coronaria* is, therefore, needed to assist powdery mildew management. To this aim, we collected several powdery mildews infecting *S. coronaria* and characterized them at the cellular and molecular level through comparison of their ITS sequences. We also established their host range and compared them to other legume-infecting powdery mildews, showing that powdery mildew on *S. coronaria* is caused by distinct and highly specialized *Erysiphe* species.

2. Materials and Methods

2.1. Plant and Fungal Material

Nine populations of powdery mildew collected on *Sulla coronaria* in different years and regions were used in this study (Table 1). Three of these samples (Co08, Co09 and Co11) were collected from *S. coronaria* field nurseries in Córdoba over different years, in 2008, 2009 and 2011. Four isolates were collected from natural populations of *S. coronaria* in different locations of Cádiz province during 2008 to 2017, two of them (Ub10 and Ub17) in Ubrique, one (Je11) in Jerez de la Frontera and the last one (Ca09) in Tarifa counties. The last two populations were collected in 2017 in Oeiras, Portugal (Po17) and in 2011 in Hammemet, Tunisia (Tu11), respectively. All these samples were used for molecular studies. Four of them (Co11, Tu11, Je11 and Ub17) were also used for morphological studies together with powdery mildew populations collected on naturally infected pea and common bean growing near the collected *S. coronaria* plants (Table 1).

2.2. Morphology Assessments

For morphological measurement, well-formed powdery mildew colonies were scraped out of symptomatic leaves and transferred onto a microscope slide in a 10 µL drop of 0.1% trypan blue in lactoglycerol before visualization under bright-field microscopy (Leica DMLB, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) at ×40 and ×100 magnification [11,21]. Chasmothecia were never detected on the powdery mildew samples, not even on old material nor after maintaining infected leaves under a controlled environment at several temperatures between 20 and 25 °C until the end of the powdery mildew cycle. Morphological characters were, therefore, limited to the assessment of the vegetative growth stage and it included the length and width of conidia, and conidiophore foot cells. Measurements were based on the observation of 50 conidia and foot cells per sample. These measurements (Table 2) were compared with a morphological description of related legume-infecting powdery mildew species [22–26].

Table 1. List of powdery mildew samples recollected from *Sulla coronaria* and other related *Erysiphe* species used for phylogenetic inferences, host range estimation and/or morphological studies.

Host	Powdery Mildew	Sample	Origin	Genbank
Sulla coronaria		Co08	Córdoba, Spain	ON729986
S. coronaria		Co09	Córdoba, Spain	ON729988
S. coronaria		Co11	Córdoba, Spain	ON729985
S. coronaria		Ca09	Tarifa, Spain	ON729987
S. coronaria		Ub10	Ubrique, Spain	ON729991
S. coronaria		Je11	Jerez de la Frontera, Spain	
S. coronaria		Tu11	Hammemet, Tunisia	ON729989
S. coronaria		Ub17	Ubrique, Spain	ON729990
S. coronaria		Po17	Oerias, Portugal	ON729992
Pisum sativum	*E. pisi*	Ps11	Córdoba, Spain	
Phaseolus vulgaris	*E. diffusa*	Pv11	Oerias, Portugal	
Lathyrus sativus	*E. trifoliorum* ex. *L. sativus*		Córdoba, Spain	
Medicago truncatula	*E. trifoliorum* ex. *M. truncatula*		Córdoba, Spain	
Vicia articulata	*E. trifoliorum* ex. *V. articulata*		Córdoba, Spain	
Albizia julibrissin	*Erysiphe* cf. *trifoliorum*	MUMH0133	Japan	LC010085
Alhagi sp.	*E. bremeri*	AK113	Guilan, Iran	AB104463
Astragalus glycyphyllus	*E. astragali*	MUMH2585	Ukraine	LC010052
Astragalus sp.	*E. astragali*		Guilan, Iran	AB104515
Baptisia australis	*E. guarinonii*	HAL2337	Germany	MT524083
Caragana arborescens	*E. palczewskii*	MUMH2581	Ukraine	LC010048
C. rosea	*E. longissima*	HMJAU91780	China	MH371103
C. rosea	*E. longissima*	HMJAU91781	China	MH371105
Coronilla varia	*Pseudoidium* sp.	MUMH2587	Ukraine	LC010054
Desmodium incanum	*E. diffusa*	MUMH3121	Corrientes, Argentina	LC010060
D. laxum	*E. glycines*	MUMH396	Shiga, Japan	LC009948
Euonymus maackii	*E. euonymi*	HMJAU91794	China	MK182295
E. japonica	*E. euonymicola*	MUMH133	Japan	AB250228
E. japonica	*E. euonymicola*	MUMH2470	Argentina	AB250229
Glycine max	*E. diffusa*	MUMH791	Oita, Japan	AB078800
G. max	*E. glycines*	MUMH1462	Mie, Japan	AB078807
Hardenbergia sp.	*P. hardenbergia*	VPRI19879	Australia	LC010094
Hypericum ascyron	*E. hypericin*	F29454	Jinan, Korea	MF099408
Labrunum alpinum	*E. guarinonii*	MUMH1425	Switzerland	LC009983
Lathyrus latifolium	*E. pisi*		Belden, USA	AF011306
L. pratensis	*E. ludens*	OE2016PMCS45	Balerno, UK	KY661116
Lens culinaris	*E. trifolii*	LGH06	Pullman, USA	FJ378883
Lupinus sp.	*E. intermedia*	OE2015PMCS297	Chelmsford, UK	KY660904
Maytenus boaria	*E. oehrensii*	MUMH2492	Bariloche, Argentina	LC010022
M. boaria	*E. oehrensii*	MUMH1936	Bariloche, Argentina	LC010008
Medicago littoralis	*E. trifoliorum*	MUMH7038	Baku, Azerbaijan	LC270860
Oenothera amoena	*E. howeana*	MUMH2572	The Netherlands	LC010043
O. biennis	*E. howeana*	UC1512301	Redlands, USA	AF011301
Oxalis corniculatus	*E. russellii*	MUMH0105	Mie, Japan	LC009922
Phaseolus vulgaris	*Erysiphe* sp.	EB2004	Londrina, Brazil	AY739109
Pisum sativum	*E. pisi*	P1	India	KX455922
Robinia pseudoacacia	*E. palczewskii*	ZKEP001	China	KX578824
Senna septemtrionalis	*Erysiphe* sp.	3D	Buenavista, Mexico	JQ730709
Tecoma capensis	*Erysiphe* sp.	DAG08-36	Yuma, USA	GU117107
T. capensis	*Erysiphe* sp.	08-3618S	Yuma, USA	GU987124
Trifolium arvense	*E. trifoliorum*	MUMH0701	Budapest, Hungria	LC009955
Vicia hirsute	*E. baeumleri*	MUMH0240	Shiga, Japan	LC009933
V. angustifolius	*E. viciae-unijugae*	MUMH0817	Yamanashi, Japan	LC009962
V. unijuga	*E. viciae-unijugae*	TPU-153	Japan	LC010086
Xanthoxalis sp.	*E. russellii*	MUMH2593	Ukraine	LC010056

Table 2. Morphological characterization of powdery mildew samples from *S. coronaria* and comparison with samples collected on nearby *Pisum sativum* and *Phaseolus vulgaris* plants and with related *Erysiphe* species.

	Erysiphe* sp. ex. *Sulla coronaria	***E. pisi* Ps11**	***E. diffusa* Pv11**	***E. palcewskii* [23]**	***E. longissimi* [26]**	***E. trifoliorum* [24]**	***E. pisi* [25]**	***E. diffusa* [22]**
conidia								
Length (µm)	22.5–50	27.5–47.5	25–50	20–37.5	19.2–48.2	25.5–39.5	30–44	28–36
Width (µm)	10–22.5	12.5–20	15–22.5	8.75–17.5	12.3–17.2	12–18	18–20	12.5–16
Ratio L/W	1–5	1.4–3.8	1.1–3.3	1.54–3.14	1.4–2.9	1.4–3.3	1.5–2.4	1.8–2.9
Foot cell								
Length (µm)	15–52.5	17.5–42.5	15–30	15–40	24.5–54.2	13–45	35–70	25–33
Width (µm)	5–12.5	5–12.5	7.5–10	nd	6–9.1	7–9.5	8–12.5	7.7–9
Ratio L/W	1.2–10.5	1.4–8.5	1.5–4	nd	2.69–9.03	1.4–6.4	2.8–8.75	2.8–4.3

2.3. DNA Extraction and ITS Sequencing

The mycelium of each *S. coronaria* powdery mildew samples was obtained by scraping the corresponding symptomatic leaves. The obtained mycelium and conidia mixture were then collected in a 2 mL Eppendorf tube, flash frozen and maintained in −80 °C until DNA extraction. Genomic DNA was extracted following a previously reported protocol [27]. Molecular characterization of each *S. coronaria* powdery mildew sample was performed by analysis of the internal transcribed spacers (ITS). ITS sequences were obtained by nested PCR amplification with primers ITS-5/P3 and ITS1/P3. Each 50 µL reaction mixture contained 50 ng of template DNA, 2 units of BioTaq DNA polymerase (Bioline, London, UK), 1× PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, and 0.3 µM of each primer. The PCR amplifications were performed on a MyCycler (Biorad, Hercules, CA, USA) thermocycler as follows: 94 °C for 2 min, 40 cycles at 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2.5 min followed by a final step at 72 °C for 10 min. All amplifications were purified with the PCR cleanup kit of QIAgen and cloned into pGEMT vector (Promega, Madison, WI, USA). Two positive clones per amplicon were sequenced by STABVida (Setubal, Portugal) using the pGEMT vector-specific primers SP6 and T7. To characterize the ITS sequence, local pairwise comparison was performed to establish the level of similarity existing between them (Table 3). Pairwise analyses were performed with the Water pairwise algorithm implemented on the EMBOSS webserver [28]. Each sequence was also analyzed by BLAST onto the Genbank *nr* database to compare them with already-sequenced ITS with the online NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed and BLAST searches performed on the 10 November 2021). All sequences have been submitted to Genbank and assigned the reference numbers ON729985 to ON729992 (Table 1).

Table 3. Comparison of ITS sequence from *S. coronaria*-infecting powdery mildew samples. Values in the lower diagonals are % similarity as estimated by pairwise local comparison.

	Co08	**Co09**	**Co11**	**Ca08**	**Tu11**	**Ub17**	**Ub10**	**Po17**
Co08	100							
Co09	99.4	100						
Co11	99.2	99.7	100					
Ca09	98.9	99.1	99.1	100				
Tu11	98.9	99.2	99.2	98.8	100			
Ub17	99.2	99.7	99.7	99.2	99.2	100		
Ub10	99	99.7	99.6	99	99.1	99.7	100	
Po17	99.4	99.9	99.9	99.3	99.4	99.9	99.7	100

2.4. Phylogenetic Study

For the reconstruction of the phylogenetic relationship of the *S. coronaria* powdery mildew samples, the ITS sequences of these samples were aligned together with the ITS sequences of related *Erysiphe* species (Table 1). ITS sequences were aligned with the MAFFT algorithm [29] implemented in the NGphylogeny webserver (http://www.NGphylogeny.fr;

accessed on 10 November 2021) [30] with default setting. The alignment was then manually corrected to resolve poorly aligned regions. To establish the phylogenetic relationship of *S. coronaria* powdery mildew samples, four different methods were used including neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI). To remove gaps and trim alignment ends, alignment was further curated with the BMGE (for NJ and MP) [31] or Gblocks (ML and BI) [32] algorithms implemented in the NGPhylogeny webserver before phylogenetic analysis. The curated alignments were then analyzed with jModelTest 2.1.7 [33] to identify the optimum substitution model for NJ, ML and BI. NJ and MP trees were estimated with MEGA10 software [34] with 1000 bootstraps after partial gap deletions. Prior to NJ reconstruction, the distance matrix was obtained using the TN93 substitution model [35] with gamma distribution of 4 categories and $\alpha = 0.36$. ML and BI trees were obtained based on 1000 bootstrap replicates following the GTR substitution model with gamma distribution of 4 categories and $\alpha = 0.39$. The ML tree was obtained with PhyML 3.1 [36]. ML tree topology was optimized based on 5 random trees with the subtree pruning and regrafting (SPR) method. BI tree was obtained with MrBayes 3.2.7a [37]. Two chains were run simultaneously for 1×10^6 generations. Results from each independent run were compared to ensure convergence of the two runs onto a single stationary distribution of trees. The first 250,000 generations were discarded as burn-in, after which 2000 trees were sampled from each replicate run to determine the optimal consensus tree and calculate the posterior probabilities of the clades.

2.5. Host Range

Five accessions of *Sulla coronaria*, together with 5 to 16 accessions from species of the related genera *Pisum*, *Lathyrus*, *Vicia*, *Cicer*, *Medicago*, *Trigonella* and *Phaseolus* (Table 4 and Supplementary Table S1), were cross-inoculated with powdery mildew isolates collected on sulla (*S. coronaria*), pea (*P. sativum; E. pisi*), grasspea (*L. sativus; E. trifoliorum*), barrel medic (*M. truncatula; E. trifoliorum*) and one-flowered vetch (*V. articulata; E. trifoliorum*). Seeds from all accessions were planted in 0.5 L plastic pots containing a 1:1 sand–peat mixture, under controlled conditions (20 °C, 12 h light/12 h dark photoperiod). Each powdery mildew isolate was purified by single sporing and maintained in isolation at the Institute for Sustainable Agriculture, CSIC, Córdoba, on leaving plants of its respective crop on which it was collected, until use. Three independent inoculation experiments were performed with each isolate. Inoculations were performed on detached leaves from two-week-old seedlings (three to five seedlings per accession, per inoculation experiment) [38]. Inoculations with each isolate were performed on consecutive days to avoid isolate cross-contamination. Inoculations were performed in a settling tower [21,39]. Inoculum density was maintained at approximately 15 and 20 conidia/mm^2. Petri dishes containing inoculated leaflets were placed in a growth chamber (20 °C, 12 h light/12 h dark photoperiod). Seven or eight days after inoculation, disease severity was assessed as the percentage of leaflet covered by the mycelium.

Table 4. Comparison of host range of the *S. coronaria* isolate with other isolates infecting closely related legume genera including *E. pisi* and *E. trifoliorum* isolates. *(- = DS < 1%; x = 1% < DS < 25%; xx = 26% < DS < 50%; xxx = DS > 51%; ns = not studied).

			Response* to Powdery Mildew Isolate				
Crop	**Host specie**	**Number of accessions studied**	***Erysiphe* sp. ex *S. coronaria***	***E. pisi* ex *P. sativum***	***E. trifoliorum* ex *L. sativus***	***E. trifoliorum* ex *M. truncatula***	***E. trifoliorum* ex *V. articulata***
Sulla	***Sulla coranaria***	**5**	**xx/xxx**	-	-	-	-
Pea and related sp.	*Pisum sativum*	6	-	xx/xxx	xx/xxx	-/x	x
	Pisum fulvum	2	-	-	xxx	-/x	-
Lentil	*Lens culinaris*	5	-	-	xx/xxx	x	xxx
Grasspea and related sp.	*Lathyrus sativus*	5	-	x/xx	xxx	-	-
	Lathyrus cicera	6	-	x/xxx	x/xxx	-	-
	Lathyrus ochrus	3	-	-/x	xxx	-	xx
	Lathyrus clymenum	2	-	-/x	xxx	-	ns
Vetches	*Vicia articulata*	1	-	-	xx	-	xxx
	Vicia monantha	2	-	x	ns	-	xxx
	Vicia villosa	1	-	ns	-	-	xx
	Vicia sativa	5	-	-	-/x	-	-/x
	Vicia pannonica	1	-	-	x	-	-
	Vicia tetrasperma	1	-	-	-	-	-
Faba bean	*Vicia faba*	5	-	-	-	-	-
Fenugreek	*Trigonella foenun-graecum*	6	-	-	-/xx	-/xx	-
Annual medics	*Medicago truncatula*	1	-	ns	x	xxx	ns
	Medicago polymorpha	1	-	-	x	xxx	ns
Alfalfa	*Medicago sativa*	4	-	-	-	-	ns
Chickpea and related sp.	*Cicer arietinum*	5	-	-	-	-	-
	Cicer reticulatum	2	-	-	-	-	ns
Common bean and related sp.	*Phaseolus vulgaris*	9	-	-	-	-	-
	Phaselus filiformis	2	-	-	-	-	ns
	Phaseolus leptostachyus	2	-	-	-	-	ns

3. Results

3.1. Mycelial Morphology

All samples collected on *S. coronaria* displayed typical powdery mildew symptoms. The mycelia of all samples were mainly epiphyllous in white effuse patches often covering the entire adaxial and abaxial leaf surfaces and stems. Hyphae were branched, septate, hyaline and thin-walled. In addition, infecting colonies developed lobed appressoria singly or in opposite pairs (Figure 1A). Conidiophores were composed of the conidial foot cell followed by 1 to 2 additional cell-forming terminal conidia singly (Figure 1B,C). Foot cells were erect and straight.

Figure 1. Micrographs of powdery mildew isolated on *Sulla coronaria*. Pictures show typical lobed appressoria (**A**) conidial foot cell composed of 1 or 2 intermediate cell(s) before the forming conidia (**B**,**C**) and mature and germinating conidia (**D**). All pictures were taken under bright-field microscope at ×40 magnification after staining with 0.1% trypan blue solution. Bar represents 10 µm.

Small variations in conidia and foot cell measurement were detected between *S. coronaria*-infecting powdery mildew samples, although they were not statistically different (Figures 1D and 2). Conidia and foot cell measurement was largely overlapping the variation detected for powdery mildew collected on nearby pea (*E. pisi)* and common bean (*E. diffusa)* plants, although they could be clearly differentiated from them (Figure 3). Altogether, conidia varied from 22.5 to 50 µm in length and from 10 to 22.5 µm in width while foot cell measurements varied from 15 to 52.5 µm in length and 5 to 12.5 µm in width (Table 2). All these features would support the assignation of these *S. coronaria*-infecting powdery mildew to a distinct *Erysiphe* species.

3.2. ITS Amplification

PCR amplification was successful for all samples except one (Je11) that turned out as being parasitized by some *Ampelomyces* sp. For all the other *S. coronaria*-infecting powdery mildew samples, a single ITS sequence was amplified and sequenced. The different *S. coronaria*-infecting powdery mildew sequences were almost identical, sharing between 98.8 and 99.9% of identity (Table 3). Blast analysis revealed strong homology with many *Erysiphe* species ITS sequences. The closest sequence shared 99.2% identity with the ITS from the *S. coronaria*-infecting samples. This sequence corresponded to a *Pseudoidium* sp. collected on *Coronilla varia*, another Fabaceae closely related to *S. coronaria.* The second and all subsequent matches corresponded to sequences of *E. trifoliorum*, *E. palczewskii*, *E. longissima* and *E. guarinonii* with homology ranging from 98 to 98.2% identity. Although the level of similarity between these sequences was very high, it was significantly lower than between the sequences of the different *S. coronaria*-infecting powdery mildews.

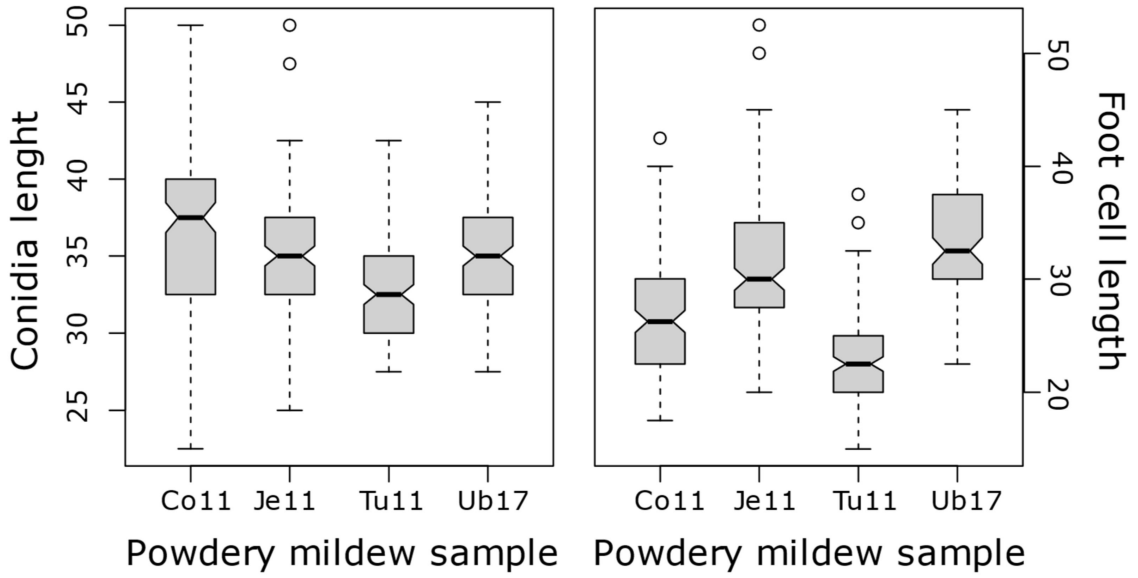

Figure 2. Box plots showing variations in conidial (left box) and foot cell (right box) lengths among 4 samples of powdery mildew collected on *S. coronaria*. The boxes and middle lines represent the middle 50 percentiles and medians, respectively. The whiskers represent upper and lower limits and circle represent outliers.

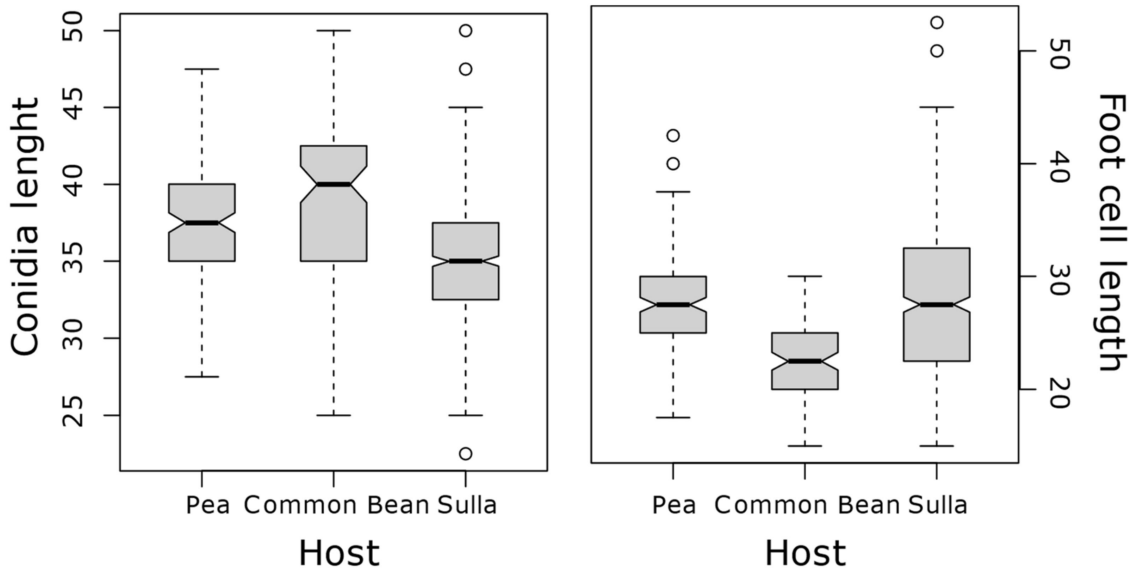

Figure 3. Box plots showing variations in conidial and foot cell lengths of powdery mildew samples collected on sulla (*S. coronaria*), pea and common bean. The boxes and middle lines represent the middle 50 percentiles and medians, respectively. The whiskers represent upper and lower limits and circle represents outliers.

3.3. Phylogenetic Relationship

To decipher the identity of the powdery mildew infecting *S. coronaria*, we established the phylogenetic relationship existing between the ITS sequences of the different *S. coronaria*-infecting samples and with other *Erysiphe* species (Table 1). The four phylogenetic approaches performed (NJ, MP, ML and BI) led to very similar, if not identical, phylogenetic trees. To simplify, only the ML and BI trees are shown in Figure 4. The phylogenetic tree separates with strong support the different *Erysiphe* isolates according to their species or host plant with three exceptions. As expected, members of *E. baeumleri*, *E. hyperici*, *E. intermedia* and *E. redens* clustered together with the *E. trifoliorum* isolates forming the

E. trifoliorum complex. Similarly, species of *E. palczewskii* infecting *Caragana arborecens* or *Robinia* sp. clustered together with the isolates from *E. longissima* that infect another related species, *Caragana rosae,* forming the *E. palczewskii* complex. Interestingly, all powdery mildew collected on *S. coronaria* clustered on a well-differentiated branch together with the isolate collected on *Coronilla varia*. In all phylogenetic trees, the cluster of the *S. coronaria*-infecting powdery mildew was located between the cluster of the *E. trifoliorum* complex and *E. guarinonii* and that of the *E. palczewskii* complex, but it was clearly differentiated from them which would support that they correspond to a distinct *Erysiphe* species.

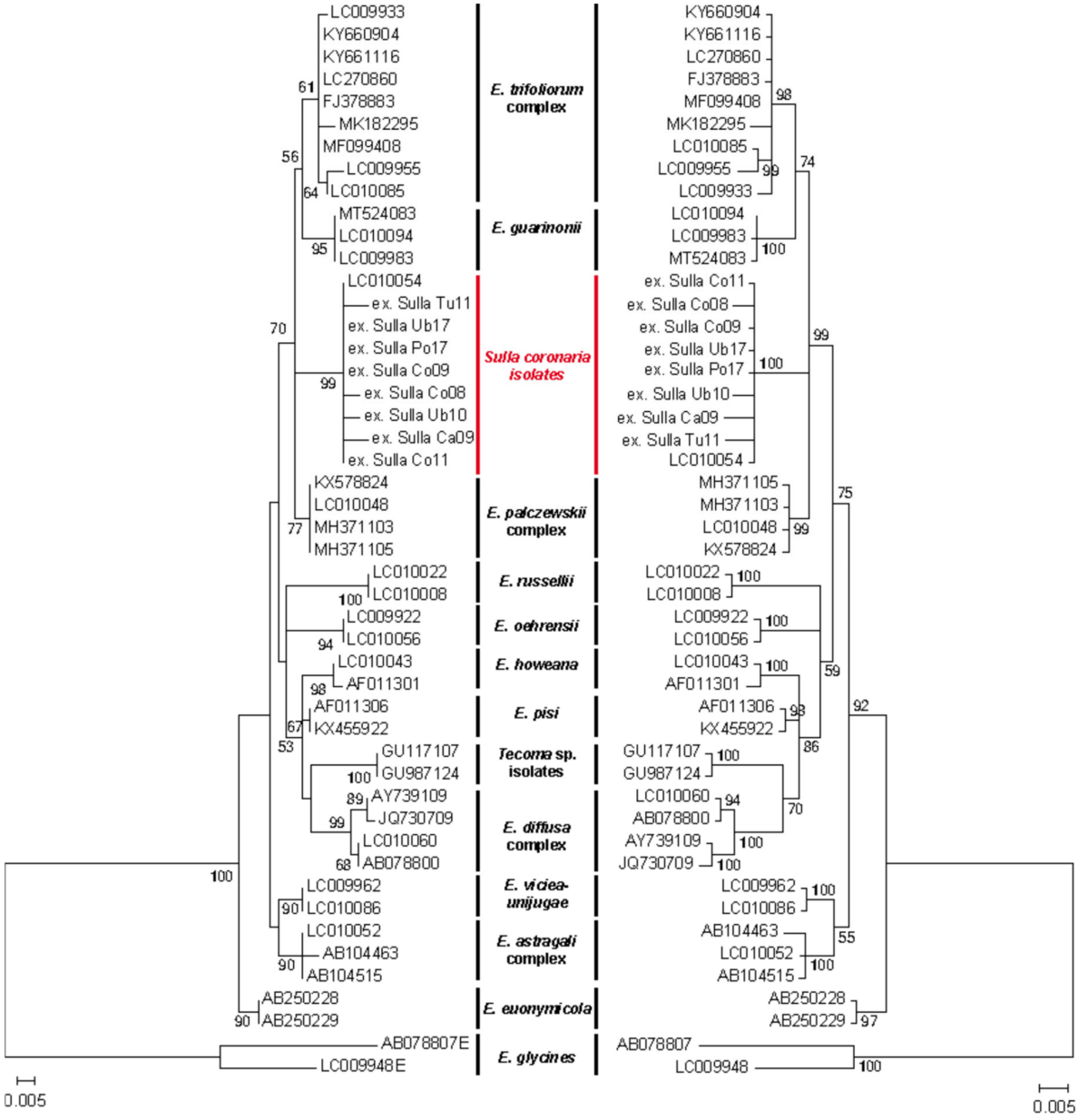

Figure 4. Phylogenetic relationship of legume-infecting *Erysiphe* spp. The figure represents the phylogenetic reconstruction of legume-infecting powdery mildews based on the analysis of ITS sequences with the maximum likelihood or Bayesian approaches. Numbers on node represent the bootstrap value or the Bayesian posterior probability value (BPP) expressed as percentages. Only bootstrap or BPP values >50% are shown. For both trees, scale bars below the trees represent numbers of substitution per sites. Sequences included in the trees are listed in Table 1.

3.4. Host Range

All five *S. coronaria* accessions were heavily infected by the sulla isolate (DS > 25%) (Table 4 and Supplementary Table S1) but not by any of the other isolates used. Conversely, the sulla isolate only infected the *S. coronaria* accessions, but not any of the other accessions from the other legume genera studied.

Such a level of specificity was not observed for the other powdery mildew isolates/legume genera studied. The pea (*P. sativum*) accessions were equally susceptible to pea (*E. pisi*) and *Lathyrus* (*E. trifoliorum*) isolates, some accessions being also slightly infected by *E. trifoliorum* isolates collected on *M. truncatula* and *V. articulata*. Wild pea (*P. fulvum*) accessions were not infected by the pea isolate, but were highly infected by the *Lathyrus* one. Lentil accessions were not infected by the pea isolate, but highly infected by *Lathyrus* and *Vicia* isolates, and also slightly by the *Medicago* one. All *Lathyrus* sp. accessions were heavily infected by the *Lathyrus* isolate but not by the *Medicago* one. The response to *E. pisi* varied, with high susceptibility for some *L. cicera* and *L. sativus* accessions, and rather low susceptibility for *L. ochrus* and *L. clymenum* accessions. *L. ocrhus* accessions could be severely infected by the *Vicia* isolate, by contrast to *L. cicera* and *L. sativus* accessions that were resistant.

Accessions of the various *Vicia* species genus were all resistant to *Sulla* and *Medicago* isolates, but could be heavily infected by the *Vicia* one and slightly infected by pea or *Lathyrus* isolates. The four alfalfa (*M. sativa*) accessions were resistant to all isolates whereas the two annual medics (*M. truncatula* and *M. polymorpha*) were heavily infected by the *Medicago* isolate and slightly by the *Lathyrus* one. The six fenugreek (*T. foenum-graecum*) accessions studied were highly resistant to *Sulla*, *Pisum* and *Vicia* isolates, whereas a few of them could be infected by *Lathyrus* or *Medicago* isolates. All accessions of *Cicer* spp. and *Phaseolus* spp. studied were immune to the different isolates tested.

4. Discussions

Powdery mildew is a widespread disease incited by numerous fungal species of the *Erysiphaceae* family. The taxonomy and host range of these fungal species remain, in many cases, uncertain. This is particularly true for the powdery mildews infecting legumes. In these host species, the disease is mainly caused by fungus belonging to the *Erysiphe* genera. The large morphological variability existing within and between *Erysiphe* species makes difficult taxonomic designation. The taxonomic characterization of *Erysiphe* species is mainly based on the examination of chasmothecia morphology, which is not always formed, or on the molecular analysis of ITS1 and ITS2 sequences. The combination of both approaches largely improves *Erysiphe* taxonomy, allowing clarification of the situation of many *Erysiphe* species [16,40]. However, description of chasmothecia morphology or ITS sequences is not always available to clearly assign a given isolate or population to an existing or novel *Erysiphe* species. The precise characterization of the causal agent of powdery mildew in a given crop is key for its control, particularly for resistance breeding. Therefore, efforts should be continued to expand the understanding of legume powdery mildews. To this aim, we targeted the powdery mildew infecting *S. coronaria*, a semiperennial legume with high potential as a protein source in pasture and forage which has been largely neglected so far.

Phylogenetic analysis of the powdery mildew samples collected on sulla clustered them together with the sequence of a powdery mildew collected on *Coronilla varia* on an independent clade between the *E. guarinonii*/*E. trifoliorum* and *E. palczewskii* clades. Interestingly, a detailed analysis of *Erysiphe* spp. phylogeny placed the *C. varia* sequence in a similar location [40] confirming that the sequences obtained on *S. coronaria* and *C. varia* correspond to a separate clade. The high similarity between the ITS sequences of the powdery mildew collected on *C. varia* and on *S. coronaria* and the phylogenetic analysis strongly suggest that all these powdery mildews correspond to the same *Erysiphe* species. Measurements of conidia and conidial foot cells of the sulla-infecting powdery mildew partially overlap those of other *Erysiphe* species (Figure 3 and Table 3) although they clearly

differentiate from them, supporting the assumption that powdery mildew samples collected on *S. coronaria* belong to a distinct species as observed from the phylogenetic analysis. However, these data are not enough to ascribe these isolates to a specific *Erysiphe* species, confirming the limited taxonomic resolution of these morphological parameters [41]. The sole reported species on *Hedysarum* spp. have been described as *E. hedysari* based on their chasmothecia morphology [16,20]. As far as we know, our study is the first to report the molecular characterization of powdery mildew isolates on plants from the *Hedysarum* clade. However, the sexual stage of these isolates could not be detected on the samples examined despite examining older samples and after maintaining infected leaves at distinct temperatures. Therefore, it is impossible to ascertain whether the isolates collected on *S. coronaria* belong to *E. hedysari* as previously reported [16,20] or correspond to an as-yet undescribed *Erysiphe* species.

High specialization was observed for the powdery mildew of sulla. The sulla isolate only infected the *S. coronaria* accessions. None of the accessions from the other legume genera were infected by the isolate collected on sulla. In turn, all studied *S. coronaria* accessions were heavily infected by the sulla isolate but not by any other isolates. This level of specificity was not observed for the *E. pisi* and *E. trifoliorum* isolates collected on pea, grasspea, vetches or annual medic species, respectively, that could be infected by more than one isolate. None of the studied isolates infected faba bean (*V. faba*), alfalfa (*M. sativa*), or any of the *Cicer* or *Phaseolus* species studied, but we cannot comment on their eventual specificity against other powdery mildew species not included in this study. This is not surprising, as to the best of our knowledge, no significant powdery mildew damage occurs on alfalfa, faba bean or chickpea. Occasional powdery mildew infections on alfalfa have been reported in research nurseries and greenhouses but not in commercial fields [42]. Similarly, only sporadic infections were observed on faba bean. In both cases, the causal agent has been ascribed to *E. pisi* [42,43]. Only sporadic infections have also been observed on chickpea and ascribed to *Leveillula taurica*, *E. pisi* or other undetermined *Erysiphe* sp. [44]. By contrast, powdery mildew, caused by *E. diffusa* isolates, can be important on common bean under specific conditions [45]. Only the *E trifoliorum* isolates collected on *Lathyrus* and *Medicago* could infect some of the fenugreek accessions. In the literature, *Leveillula taurica*, *Erysiphe polygoni* and *E. trifoliorum* have been reported as the causal agents of powdery mildew on fenugreek [46,47]. All this confirms the little understanding on the causal agent and host range of the powdery mildew of many legume species, in spite of the implications in epidemiology and management. Knowledge of the host range is important to determine whether other crops could be affected by a given *Erysiphe* species. Furthermore, it is possible that alternative hosts could provide a means of overwintering of the pathogen, providing inocula to initiate epidemics in successive years.

A previous report on the host range of rust infecting *Vicia* species [48] agrees with the differential response to powdery mildew isolates seen here, where faba bean was not infected by any of the tested isolates, whereas several vetch species could be infected by several isolates. *Uromyces viciae-fabae* isolates from faba bean only infected faba bean, whereas isolates from vetch or lentil infected different *Vicia* spp. The host range expansion of powdery mildew fungi might be important to the understanding of their phylogenetic history and sometimes sheds light on the evolution of the hosts. The subgenus *Vicia* is smaller, less variable morphologically and comprises almost exclusively annuals including the most agriculturally important species *V. faba* and *V. sativa*. It is relevant that none of the powdery mildew isolates studied infected faba bean, and only a few slightly infected *V. sativa*, both of which belong to the most evolved subgenus *Vicia*. In contrast, species such as *V. articulata*, *V. monanthos*, *V. villosa*, and *V. cracca* of subgenus *Vicilla* that are considered more primitive and diverse [49] could be infected by several powdery mildew isolates, as found with the less specialized rusts [48]. This trend suggests a possible cospeciation of powdery mildew with host, as earlier suggested for rusts [50,51].

All sulla accessions studied were highly susceptible to the sulla powdery mildew. A small variation in levels of resistance to powdery mildew has been reported in field trials [9]

but no details were provided on the actual levels of resistance, apart from stating that the cv. Grimaldi and the ecotype Tarifa were the most resistant among the studied accessions. However, Grimaldi was reported as susceptible under greenhouse conditions [14]. All this reinforces the need to first clarify the causal agent of sulla powdery mildew, and then to standardize resistance screenings to discern the actual levels of resistance available for breeding. Accordingly, we performed, for the first time, the molecular characterization of sulla-infecting powdery mildews and studied their host ranges. Altogether, we demonstrate that the sulla-infecting powdery mildew belongs to a highly specific species, presumably *E. hedysari,* which showed high specificity to its host. Beside clarifying the status of the sulla-infecting powdery mildew as an independent species, these results will be helpful for future breeding for powdery mildew resistance in sulla.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12081852/s1, Table S1: Host range: response of legume species to isolates of powdery mildew.

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References

1. Ruisi, P.; Siragusa, M.; Di Giorgio, G.; Graziano, D.; Amato, G.; Carimi, F.; Giambalvo, D. Pheno-morphological, agronomic and genetic diversity among natural populations of sulla (*Hedysarum coronarium* L.) collected in Sicily, Italy. *Gen. Res. Crop Evol.* **2011**, *58*, 245–257. [CrossRef]
2. Bonanno, A.; Di Grigoli, A.; Mazza, F.; De Pasquale, C.; Giosuè, C.; Vitale, F.; Alabiso, M. Effects of ewes grazing sulla or ryegrass pasture for different daily durations on forage intake, milk production and fatty acid composition of cheese. *Animal* **2016**, *10*, 2074–2082. [CrossRef]
3. Akbarian, M.M.; Mojaradi, T.; Shirzadi, T. Effects of *Hedysarum coronarium* L. (sulla) as a green manure along with nitrogen fertilizer on maize Production. *Agritech* **2021**, *41*, 95–106. [CrossRef]
4. Erwin, M.; Foster, K.; Skinner, P.; Nichols, P.; Nutt, B.; Snowball, R.; Beatty, R. Sulla and Purple Clover as Fodders. Limits on Seed Production. RIRDC Publication No 06/049. 2006. Available online: https://www.agrifutures.com.au/wp-content/uploads/publications/06-049.pdf (accessed on 18 April 2022).
5. Crocker, G.; Hackney, B. Sulla. Primefact 745. State of New South Wales through NSW Department of Primary Industries, 2008. ISSN 1832-6668. Available online: www.dpi.nsw.gov.au/primefacts (accessed on 29 April 2022).
6. Leto, G.; Todaro, M.; di Noto, A.M.; Alicata, M.L. Comparison of sulla-hay and sulla-silage in the lactating ewes and their effects on milk and cheese characteristics. *Small Rum. Res.* **2002**, *45*, 301–306. [CrossRef]
7. Annicchiarico, P.; Ruisi, P.; Di Miceli, G.; Pecetti, L. Morpho-physiological and adaptive variation of Italian germplasm of sulla (*Hedysarum coronarium* L.). *Crop Pasture Sci.* **2014**, *65*, 206–213. [CrossRef]
8. Córdoba, E.M.; Nadal, S.; Román, B.; Gónzalez-Verdejo, C.I. Collection, characterization and evaluation of wild (*Hedysarum coronarium* L.) populations from Andalusia (southern Spain). *Aust. J. Crop Sci.* **2013**, *7*, 165–172.
9. Flores, F.; Gutiérrez, J.; López, J.; Moreno, M.T.; Cubero, M.T. Multivariate analysis approach to evaluate a germplasm collection of *Hedysarum coronarium* L. *Gen. Resour. Crop Evol.* **1997**, *44*, 545–555. [CrossRef]
10. Marghali, S.; Panaud, O.; Lamy, F.; Ghariani, S.; Sarr, A.; Marrakchi, M.; Trifi-Farah, N. Exploration of intra- and inter-population genetic diversity in *Hedysarum coronarium* L. by AFLP markers. *Gen. Resour. Crop Evol.* **2005**, *52*, 277–284. [CrossRef]
11. Fernández-Aparicio, M.; Prats, E.; Emeran, A.A.; Rubiales, D. Characterisation of resistance mechanisms to *Erysiphe betae* in *Beta vulgaris*. *Phytopathology* **2009**, *99*, 385–389. [CrossRef]
12. Fondevilla, S.; Rubiales, D. Powdery mildew control in pea. A review. *Agron. Sustain. Devel.* **2012**, *32*, 401–409. [CrossRef]
13. Trabanco, N.; Pérez-Vega, E.; Campa, A.; Rubiales, D.; Ferreira, J.J. Genetic resistance to powdery mildew in common bean. *Euphytica* **2012**, *186*, 875–882. [CrossRef]
14. Louati-Namouchi, I.; Louati, M.; Chriki, A. A quantitative study of some agronomic characters in Sulla (*Hedysarum coronarium* L.). *Agronomie* **2000**, *20*, 223–231. [CrossRef]

15. Rubiales, D.; Fondevilla, S.; Chen, W.; Gentzbittel, L.; Higgins, T.J.V.; Castillejo, M.A.; Singh, K.B.; Rispail, N. Achievements and challenges in legume breeding for pest and disease resistance. *Crit. Rev. Plant Sci.* **2015**, *34*, 195–236. [CrossRef]
16. Braun, U.; Takamatsu, S. Phylogeny of *Erysiphe*, *Microsphaera*, *Uncinula* (Erysipheae) and *Cystotheca*, *Podosphaera*, *Sphaerotheca* (Cystotheceae) inferred from rDNA ITS sequences—Some taxonomic consequences. *Schlechtendalia* **2000**, *4*, 1–33.
17. Braun, U.; Kruse, J.; Wolcan, S.M.; Murace, M. Three new species of the genus *Erysiphe* (Ascomycota, Erysiphales) on legumes and some new combinations. *Mycotaxon* **2010**, *112*, 173–187. [CrossRef]
18. Watson, M.J. *Hedysarum coronarium*—A legume with potential for soil conservation and forage. *N. Z. J. Agric. Res.* **1982**, *16*, 189–193.
19. Chen, M.M. *Forest Fungi Phytogeography: Forest Fungi Phytogeography of China, North America, and Siberia and International Quarantine of Tree Pathogens*; Pacific Mushroom Research & Education Center: Sacramento, CA, USA, 2003; ISBN 0-9723916-6-5.
20. Braun, U. Descriptions of new species and combinations in *Microsphaera* and *Erysiphe* (V). *Mycotaxon* **1984**, *19*, 374–383.
21. Rubiales, D.; Carver, T.W.L. Defence reactions of *Hordeum chilense* accessions to three *formae speciales* of cereal powdery mildew fungi. *Can. J. Bot.* **2000**, *78*, 1561–1570. [CrossRef]
22. Attanayake, R.N.; Glawe, D.A.; Dugan, F.M.; Chen, W. *Erysiphe trifolii* causing powdery mildew of lentil (*Lens culinaris*). *Plant Dis.* **2009**, *93*, 797–803. [CrossRef]
23. Lebeda, A.; Mieslerova, B.; Sedlarova, M. First report of *Erysiphe palczewskii* on *Caragana arborescens* in the Czech Republic. *Plant Pathol.* **2008**, *57*, 779. [CrossRef]
24. Meeboon, J.; Takamatsu, S. Notes on powdery mildews (Erysiphales) in Japan: I. *Erysiphe Sect. Erysiphe*. *Mycoscience* **2015**, *56*, 257–266. [CrossRef]
25. Meeboon, J.; Takamatsu, S. Notes on powdery mildews (Erysiphales) in Thailand III. *Erysiphe species on Fabaceae, Fagaceae, Hydrangeaceae and Lamiaceae. Tropical Plant Pathol.* **2017**, *42*, 239–249. [CrossRef]
26. Qiu, P.; Tang, S.; Guan, G.; Li, Y.; Takamatsu, S.; Liu, S. Revisiting a poorly known powdery mildew species: Morphology and molecular phylogeny of *Erysiphe longissima*. *Mycoscience* **2019**, *60*, 250–254. [CrossRef]
27. Raeder, U.; Broda, P. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1985**, *1*, 17–20. [CrossRef]
28. Madeira, F.; Pearce, M.; Tivey, A.R.N.; Basutkar, P.; Lee, J.; Edbali, O.; Madhusoodanan, N.; Kolesnikov, A.; Lopez, R. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic Acids Res.* **2022**, *50*, W276–W279. [CrossRef]
29. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780. [CrossRef]
30. Lemoine, F.; Correia, D.; Lefort, V.; Doppelt-Azeroual, O.; Mareuil, F.; Cohen-Boulakia, S.; Gascuel, O. NGPhylogeny.fr: New generation phylogenetic services for non-specialists. *Nucleic Acids Res.* **2019**, *47*, W260–W265. [CrossRef]
31. Criscuolo, A.; Gribaldo, S. BMGE (Block Mapping and Gathering with Entropy): A new software for selection of phylogenetic informative regions from multiple sequence alignments. *BMC Evol. Biol.* **2010**, *10*, 210. [CrossRef]
32. Castresana, J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **2000**, *17*, 540–552. [CrossRef]
33. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
34. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
35. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526. [PubMed]
36. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321. [CrossRef]
37. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef]
38. Prats, E.; Llamas, M.J.; Rubiales, D. Characterisation of resistance mechanisms to *Erysiphe pisi* in *Medicago truncatula*. *Phytopathology* **2007**, *97*, 1049–1053. [CrossRef]
39. Sánchez-Martín, J.; Rubiales, D.; Prats, E. Resistance to powdery mildew (*Blumeria graminis* f.sp. *avenae*) in oat seedlings and adult plants. *Plant Pathol.* **2011**, *60*, 846–856.
40. Takamatsu, S.; Ito, H.A.; Shiroya, Y.; Kiss, L.; Heluta, V. First comprehensive phylogenetic analysis of the genus *Erysiphe* (Erysiphales, *Erysiphaceae*) I. The *Microsphaera* lineage. *Mycologia* **2015**, *107*, 475–489. [CrossRef] [PubMed]
41. Takamatsu, S. Studies on the evolution and systematics of powdery mildew fungi. *J. Gen. Plant Pathol.* **2018**, *84*, 422–426. [CrossRef]
42. Mohan, S.K.; Bijman, V.P.; Miller, D.R. Powdery Mildew Caused by *Erysiphe pisi* on Alfalfa in Idaho and Oregon. *Plant Dis.* **2001**, *85*, 678. [CrossRef]
43. Cook, R.T.A.; Fox, R.T.V. *Erysiphe pisi* var. *pisi* on faba beans and other legumes in Britain. *Plant Pathol.* **1992**, *41*, 506–512. [CrossRef]
44. Dugan, F.M.; Attanayake, K.; Glawe, D.; Chen, W. *Powdery Mildew of Chickpea. Compendium of Chickpea and Lentil Diseases and Pests*; American Phytopathological Society: Saint Paul, MN, USA, 2011; pp. 51–52.

45. Leitão, S.T.; Almeida, N.F.; Moral, A.; Rubiales, D.; Vaz Patto, M.C. Identification of resistance to rust (*Uromyces appendiculatus*) and powdery mildew (*Erysiphe difussa*) in Portuguese common bean germplasm. *Plant Breed.* **2013**, *132*, 654–657. [CrossRef]
46. Worku, J. Effects of Powdery Mildew (*Leveillula Taurica* and *Erysiphe Polygoni*) on yield and yield components of fenugreek in the mid-altitudes of Bale, South Eastern Ethiopia. *J. Plant. Sci.* **2017**, *5*, 65–67. [CrossRef]
47. Bradshaw, M.; Braun, U.; Götz, M.; Jurick, W. Phylogeny and taxonomy of powdery mildew caused by *Erysiphe* species on *Lupinus* hosts. *Mycologia* **2022**, *114*, 76–88. [CrossRef]
48. Rubiales, D.; Sillero, J.C.; Emeran, A.A. Response of vetches (*Vicia* spp.) to specialized forms of Uromyces vicia-fabae and to *Uromyces pisi*. *Crop Prot.* **2013**, *46*, 38–43.
49. Maxted, N. An ecogeographical study of *Vicia* subgenus *Vicia*. In *Systematic and Ecogeographic Studies on Crop Genepools*; International Plant Genetic Resources Institute: Rome, Italy, 1995; Volume 8.
50. Leppik, E.E. Evolutionary specialization of rust fungi (Uredinales) on the Leguminosae. *Ann. Bot. Fenn.* **1972**, *9*, 135–148.
51. Van der Merwe, M.; Walker, J.; Ericson, L.; Burdon, J.J. Coevolution with higher taxonomic host groups within the *Puccinia/Uromyces* rust lineage obscured by host jumps. *Mycol. Res.* **2008**, *112*, 1387–1408. [CrossRef] [PubMed]