Genetic diversity of *Colletotrichum lupini* and its virulence on white and Andean lupin

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Lupin cultivation worldwide is threatened by anthracnose, a destructive disease caused by the seed- and air-borne fungal pathogen *Colletotrichum lupini*. In this study we explored the intraspecific diversity of 39 *C. lupini* isolates collected from different lupin cultivating regions around the world, and representative isolates were screened for their pathogenicity and virulence on white and Andean lupin. Multi-locus phylogeny and morphological characterizations showed intraspecific diversity to be greater than previously shown, distinguishing a total of six genetic groups and ten distinct morphotypes. Highest diversity was found across South America, indicating it as the center of origin of *C. lupini*. The isolates that correspond to the current pandemic belong to a genetic and morphological uniform group, were globally widespread, and showed high virulence on tested white and Andean lupin accessions. Isolates belonging to the other five genetic groups were mostly found locally and showed distinct virulence patterns. Two highly virulent strains were shown to overcome resistance of advanced white lupin breeding material. This stresses the need to be careful with international seed transports in order to prevent spread of currently confined but potentially highly virulent strains. This study improves our understanding of the diversity, phylogeography and pathogenicity of a member of one of the world's top 10 plant pathogen genera, providing valuable information for breeding programs and future disease management.

The fungal genus *Colletotrichum* contains many important plant pathogenic species that cause anthracnose and other pre- and post-harvest diseases in a wide variety of hosts. Among potential hosts are important fruit, cereal and legume crops such as strawberry, maize and soybean. Besides being of economic importance, *Colletotrichum* spp. have been widely used as model species to study plant-fungus interactions because of the diversity of lifestyles within this genus. *Colletotrichum* is listed in the top 10 of most important fungal plant pathogens worldwide. Within the genus, members of the *Colletotrichum acutatum* species complex are notorious and cause disease in many important crops. The most important morphological characteristic for members of this species complex are the acute ends of its conidia. Discrimination of *Colletotrichum* species solely based on morphological traits, however, is deemed unreliable due to the few and highly variable characteristics, the strong influence of environmental conditions and the high overlap between species. Therefore, a polyphasic approach, combining morphological and genetic data is recommended. Multi-locus phylogeny revealed a high diversity within the *C. acutatum* species complex, showing at least 32 different species divided among five clades. Although many species within the *C. acutatum* species complex have a broad host range, *Colletotrichum lupini*, belonging to clade 1, appears to be highly host specific on lupins (*Lupinus*).

Lupin anthracnose caused by *C. lupini* is the most important disease in lupin cultivation worldwide, affecting all economically important lupin species such as blue (*Lupinus angustifolius* L.), white (*L. albus* L.), Andean (*L. mutabilis* Sweet.), yellow (*L. luteus* L.) and ornamental lupin (*L. polyphyllus* Lindl.). The disease was first reported in 1912 in Brazil, but the fungal pathogen was identified much later. A first outbreak was reported in the 1940–1950s in North America and was followed by a more severe and globally widespread outbreak around the 1980s which is still persisting until this day. The disease is mainly dispersed via seeds, facilitating rapid spread through international seed transports, and within the crop by rain splash during the growing season. Even low amounts of initial inoculum can cause total yield losses making this disease highly destructive. Typical symptoms are stem twisting and necrotic lesions on stems and pods (Fig. 1). Current disease management is focused on planting certified disease-free seed and chemical protection. However, crop resistance could offer a more sustainable alternative. In blue lupin, anthracnose resistance is controlled by single resistance genes.
genes, whereas in white, Andean and yellow lupin no such single gene resistance is known and the observed quantitative resistance is considered to be polygenic. The increasing demand for plant-based protein is renewing the interest for lupins as a high quality protein crop, however, the current anthracnose pandemic, severely hampers cultivation.

The pathogen was first described as Gloesporium lupini, followed by C. gloeosporioides and C. acutatum until it was fully described as C. lupini. Currently two genetic groups (I and II) are distinguished within C. lupini based on vegetative compatibility groups (VCG), the ITS (internal transcribed spacer) region and multi-locus phylogeny of the ITS, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), CHS-1 (chitin synthase), HIS3 (histone), ACT (actin), TUB2 (β-tubulin 2), HMG (HMG box region) and APN/MAT1 (Apn2-Mat1-2-1 intergenic) loci. The TUB2 and GAPDH loci were shown to be the most informative within the C. acutatum species complex and APN/MAT1 the most informative within C. lupini, whereas classification based on the ITS region can be problematic due to low resolution within the complex. Although only two groups within C. lupini have been distinguished, with most of the reported strains belonging to group II, intraspecific diversity is thought to be greater as a high diversity was found in a Chilean C. lupini collection using random amplified polymorphic DNA (RAPD) markers and a distinct lupin infecting C. acutatum group was identified in Ecuador based on the ITS region. This suggests that highest intraspecific diversity is found in South America, which is believed to be the center of origin of members belonging to clade 1 of the C. acutatum species complex.

The overall aim of this study was to assess a worldwide collection of lupin-infecting Colletotrichum isolates through (i) multi-locus phylogeny, (ii) morphology and (iii) virulence on white and Andean lupin. Insights into
C. lupini diversity, phylogeography and plant-C. lupini interactions will improve our understanding of the current lupin anthracnose pandemic and support future disease management strategies and lupin breeding programs.

Results
Colletotrichum lupini comprises of six genetic groups supported by morphology. From the 50 sequenced isolates, 39 belonged to C. lupini (Table 1). A globally representative subset of 28 C. lupini isolates was characterized based on colony morphology (form, aerial mycelium, margin type and color of the reverse side) and 18 of those were further characterized for growth rate and conidial shape and size, revealing ten distinct morphotypes (A–J; Fig. 2, Table 2, Supplementary Figs. S1, S2). Despite certain variability, all observed conidia shared features typical for C. lupini (hyaline, smooth-walled, aseptate, straight and with one acute end) as described by Damm et al. Morphotype A was the most common and was observed for isolates from across the world (Europe, Australia, North- and South America), all belonging to genetic group II. Morphotypes B, C and G were observed for isolates from South Africa and morphotypes D, E, G, I and J were observed for isolates from South America.

Multi-locus phylogenetic analyses of 50 Colletotrichum isolates identified six distinct genetic groups within C. lupini (I–VI; Fig. 3, Supplementary Fig. S3). The combined sequence dataset contained 2251 characters (ITS: 1–496, GAPDH: 497–745, TUB2: 746–1200, APN/MAT1: 1201–2251) including alignment gaps. The APN/MAT1 locus showed the highest variability across the nucleotide data set, with 75.8% conserved sites for the whole data set (including out-groups) and 97.4% within C. lupini (Supplementary Table S1). The TUB2 and GAPDH loci showed 89.9% and 81.1% identical sites for the entire dataset and 97.8% and 98.4% identity within C. lupini, respectively. The ITS region showed the lowest variability with 97% identical sites across the whole dataset and 99.2% within C. lupini. As shown in Fig. 3, most C. lupini strains clustered with a high bootstrap support (BS) value of 79 and posterior probability (PP) of 1 with reference strains representing genetic group II (CBS 109221, IMI 375715 and RB221). Strains within group II showed a high identity among each other (> 99.9%) and showed morphotype A, except for Chilean strain JA15 showing morphotype D (Fig. 2). South African strain JA10 and Peruvian strain JA20, with morphotypes G and F, respectively, clustered together with a BS of 84 and PP of 1, forming a highly supported group (III). South African strains JA11 and JA12, with morphotypes C and B, respectively, clustered together with a BS of 98 and PP of 1, forming a highly supported group (IV). Ecuadorian strains JA18 and JA19 with distinct morphotypes I and J, respectively, showed 99.7% identity with reference strains of group II and clustered together with a BS of 60 (Fig. 3, Supplementary Fig. S3) and a PP of 1 in (Fig. 3), forming a distinct group (V). The reference strains for group I (CBS 109225 with morphotype H, CBS 109226 and CBS 509.97) are clustered together with a BS of 99 and PP of 1 and show 100% identity with each other and 99.6% identity with reference strains of group II. South American strains JA21, JA22 and CBS 109216, with morphotype E, cluster together with a BS of 98 and PP of 1 (Fig. 3) and a BS of 54 (Supplementary Fig. S3) forming a highly supported group (VI). JA21 and JA22 showed 99.8% and CBS 109216 showed 99.7% identity with reference strains of group I and 99.4% and 99.2% identity with references strains of group II, respectively.

Distinct virulence patterns on white and Andean lupin. Virulence assays performed on two white lupin (L. albus L.) accessions (Fedora and Blu-25) and two Andean lupin (L. mutabilis Sweet.) accessions (LUP 17 and LUP 100) revealed strong strain (p < 0.0001), lupin species (p < 0.0001) and strain × lupin species interaction effects (p < 0.0001). A strong accession effect was found within white lupin (p < 0.0001), whereas for Andean lupin there was no significant accession effect (p = 0.43). Strain (p < 0.0001) and strain × accession (p < 0.0001) interaction effects were found for both species. Strains belonging to genetic group II with morphotype A, caused severe disease on white lupin accession fedora and both Andean lupin accessions (Supplementary Fig. S4), showing standardized area under the disease progress curve (AUDPC) means ranging from 3.95 to 5 (Fig. 4). On the other tested tolerant white lupin accession Blu-25, AUDPC means for strains of group II with morphology A were more variable, with JA01 and IMI 375715 showing moderate (2.7–2.9) and Chilean strains JA16 and 17 showing high (3.8–4.1) virulence. Chilean strain JA15, also belonging to genetic group II but with a different morphology (D), caused low disease on LUP 100 and Blu-25 (1.9), showing a different virulence spectrum compared to the other tested strains of genetic group II. South African strains JA11 and JA12, belonging to genetic group IV with morphotypes C and B, respectively, showed a similar virulence spectrum on white lupin as strains of group II. JA10 and JA20, representing group III and morphotype G and F, respectively, were overall avirulent (< 2), with the exception of JA10 on Fedora, showing moderate virulence (2.95). Peruvian strain JA21, representing genetic group VI and morphotype E, caused low disease on white lupin (1.4–1.8), but severe disease on Andean lupin (4.25–5). A similar observation was found for the two Ecuadorian strains JA18 and JA19 of genetic group V and morphotypes I and J, respectively. These two strains caused low disease on white lupin and high disease on Andean lupin LUP 100. On Andean lupin LUP 17, however, a severe disease phenotype was only found for JA18 (3.6), whereas JA19 barely caused any disease symptoms (1.25). Similar to the observations for JA19, the Ukrainian strain CBS 109225 (genetic group I, morphotype H) caused severe disease on Andean lupin LUP 100 (3.36) and low disease on Andean lupin LUP 17 and white lupin (1.2–2). The C. tamarii and C. acutatum strains were avirulent across the lupin accessions (< 1.26).

Discussion
This study compared 39 C. lupini and 11 Colletotrichum spp. isolates collected from across the world to explore intraspecific diversity of C. lupini and to better understand the dynamics of the current lupin anthracnose pandemic and potential implications of further migrations of distinct pathogenic strains. Based on multi-locus phylogeny supported by isolate morphology, we identified four distinct genetic groups additional to previously
| Strain | Alternative code(s) | Species               | Host            | Origin                          | Year      | GenBank no. |
|--------|---------------------|-----------------------|-----------------|---------------------------------|-----------|-------------|
| JA01   |                    | Colletotrichum lupini | Lupinus albus   | Switzerland, Melikon            | 2018      | MW342515    |
| JA02   | C. lupini           | L. albus              | Germany         | Hattenhofen                     | 2019      | MW342516    |
| JA03   | C. lupini           | L. albus              | Germany         | Wittenhausen                    | 2018      | MW342517    |
| JA04   | C. lupini           | L. albus              | Germany         | Westerbork                      | 2018      | MW342518    |
| JA05   | C. lupini           | L. albus              | Russia          | Saint Petersburg                | 2018      | MW342519    |
| JA06   | C. lupini           | L. albus              | Australia       | WA, Dongara                     | 2004      | MW342520    |
| JA07   |                    | C. angustofolius      |                | South Africa                    | 1994      | MW342521    |
| JA08   | C. lupini           | L. luteus             | Australia       | WA, Mingenew                    | 2004      | MW342522    |
| JA09   |                    | C. lupini             | Australia       | WA, Yandanoooka                 | 2004      | MW342523    |
| JA10   | CMW 9930, SHK 788   | L. albus              | South Africa    | Bethlehem                       | 1995      | MW342524    |
| JA11   | CMW 9931, SHK 1033  | L. albus              | South Africa    | Stellenbosch                    | 1995      | MW342525    |
| JA12   | CMW 9933, SHK 2148  | L. albus              | South Africa    | Malmesbury                      | 1999      | MW342526    |
| JA13   | C. lupini           | L. mutabilis          | USA             | Florida, Martin County          | 2013      | MW342527    |
| JA14   | C. lupini           | L. hartwegii          | USA             | Florida, Martin County          | 2013      | MW342528    |
| JA15   | A-02                | L. albus              | Chile           | Cajon                           | 2009      | MW342529    |
| JA16   | A-10                | L. angustofolius      | Chile           | Cajon                           | 2009      | MW342530    |
| JA17   | A-24                | L. albus              | Chile           | Temuco                          | 2015      | MW342531    |
| JA18   | Lap1                | L. mutabilis          | Ecuador         | Montalvo                        | 2007      | MW342532    |
| JA19   | Lap18               | L. mutabilis          | Ecuador         | Pujili                          | 2007      | MW342533    |
| JA20   | C. mutabilis        | Peru, Carhuaz         | Peru            | Carhuaz                         | 2019      | MW342534    |
| JA21   | C. mutabilis        | Peru, Carhuaz         | Peru            | Carhuaz                         | 2019      | MW342535    |
| JA22   | C. mutabilis        | Peru, Carhuaz         | Peru            | Carhuaz                         | 2019      | MW342536    |
| CBS 109216 | BBA 63879 | C. lupini | Brazil          |                  | 2013      | JQ948156    |
| CBS 109221 | BBA 70352, RB172   | C. lupini | Germany         |                  | 2013      | JQ948169    |
| CBS 109225 | BBA 70884       | C. lupini | Ukraine        |                  | 2013      | JQ948155    |
| CBS 109226 | RB121, IMI 504884, H109, BBA 71249 | C. lupini | Canada, Nova Scotia |                  | 2013      | JQ948158    |
| CBS 509.97 | RB235, LARS 178    | C. lupini | France         |                  | 1996      | JQ948159    |
| IMI 375715 | 964A        | C. lupini | Australia, WA, Perth |                  | 1997      | JQ948161    |
| RB020  | PT30               | L. albus              | Portugal         | Azores                          | 1999      | MW342517    |
| RB042  | CBS 129944, CMG12   | C. lupini             | Portugal         | Lisbon                          | 1996      | JQ948159    |
| RB116  | CSL 1294           | L. polyphyllus        | UK               | York                            | 1997      | JQ948161    |
| RB122  | BBA 71310, C3      | C. lupini             | Poland           |                                | 1999      | MW342517    |
| RB123  | IMI 504885, SHK788  | L. albus              | South Africa    | Bethlehem                       | 1994      | MW342517    |
| RB124  | BBA 70555          | C. lupini             | Chile            |                                | 1994      | MW342517    |
| RB125  | C. lupini           | L. albus              | Austria          |                                | 1994      | MW342517    |
| RB127  | PT102              | Olea europea          | Spain            |                                | 1994      | MW342517    |
| RB147  | IMI 350308         | Lupinus sp.           | UK               | Kent                            | 1991      | MW342519    |
| RB221  | IMI 504893         | Lupinus sp.           | France           | Brittany                        | 2016      | MW342537    |
| RB226  | C. lupini           | Lupinus sp.           | France           | Brittany                        | 2016      | MW342537    |
| CBS 129814 | T.A6        | C. tamarillii         | Colombia         | Gundisnamarca                   | 2012      | JQ948181    |
| CBS 129955 | RB018, Tom-12     | C. tamarillii         | Colombia         | Antioquia, Santa Rosa           | 1998      | JQ948189    |
| CBS 211.78 | IMI 309622, RB184  | C. costaricensis      | Costa Rica       | Turrialba                       | 1998      | JQ948181    |
| CBS 134730 | RB237        | C. melonis            | Brazil           | Rio Grande do Brazil            | 2016      | KC204997    |

Continued
Table 1. Isolation details and GenBank accessions of Colletotrichum strains used in this study. YA strains from the FiBL culture collection characterized in this study, RB personal collection of Riccardo Baroncelli described in Dubrulle et al.39, CBS collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, IMI Culture collection of CABI Europe UK Centre, Egham, UK, ITS internal transcribed spacers 1 and 2 together with 5.8S rDNA, GAPDH glyceraldehyde-3-phosphate dehydrogenase, TUB2 β-tubulin 2, APN/ MAT1 Apn2-Mat1-2-1 intergenic. Codes in bold were used for morphology analysis in this study. Accession numbers in bold are newly determined in this study.

| Strain* | Alternative code(s) | Species | Host | Origin | Year | GenBank no.* |
|---------|---------------------|---------|------|--------|------|--------------|
| IMI 304,802 | RB216 | C. cuscutae | Cuscuta sp. | Dominica | 2011 | JQ948195 JQ948255 JQ948946 MK478340 |
| IMI 384,185 | CPC 189,37, RB218 | C. paraenseae | Caryocar brasiliense | Brazil | 1993 | JQ948191 JQ948521 JQ948942 MK478342 |
| CBS 130,239 | Frag NL-1 | C. nymphaeae | Fragaria x ananassa | The Netherlands | 1968 | JQ948250 JQ948580 JQ949901 MW342583 |
| IMI 360,928 | CPC 189,26, RB163 | C. nymphaeae | Fragaria x ananassa | Switzerland, Zurich | 1993 | JQ948243 JQ948573 JQ948984 MK478326 |
| CBS 12,2122 | BRIP 285,19, RB179 | C. simmondsii | Carica papaya | Australia | 1987 | JQ948276 JQ948606 JQ949927 MK478332 |
| CBS 369,73 | NRCC 10,084 | C. acutatum | L. angustifolius | New Zealand, Kanese | 1968 | JQ948350 JQ948681 JQ950081 MW342582 |
| CBS 370,73 | NRCC 10,088, RB187 | C. acutatum | Pinus Aridata | New Zealand, Tokorona | 1965 | JQ948351 JQ948682 JQ950002 MK478335 |

This study provides first solid evidence that, based on multi-locus phylogeny and morphology, genetic diversity within C. lupini is higher than previously shown. High-resolution genome-wide sequencing and an increased sampling density from especially the South American Andes region are now necessary to increase genetic resolution and to better understand C. lupini phylogeny and phylogeography. This could provide the basis for in-depth comparative genomic studies to identify effector gene clusters within the C. lupini genome. This study confirms that the current lupin anthracnose pandemic is caused by a genetically uniform group of highly virulent strains. The identification of strains with an increased virulence on tolerant white lupin breeding material and the observation of strain-specific virulence patterns should be taken into account in lupin resistance breeding programs.
Figure 2. *Colletotrichum lupini* morphology. Capital letters (A–J) indicate the different morphology types based on conidia shape and size and colony growth rate and morphology (see Table 2). Strain codes are followed by country of origin and roman numbers (I–VI) indicate genetic groups. Plates show the front and reverse of 14 day old colonies on PDA. Scale bars indicate 20 µm. Colors indicate strain origin: blue = Europe, green = South America, red = North America, orange = Southern Africa, dark blue = Australia.
Due to its seed-borne nature, caution should be taken when importing seeds, especially from South America, to prevent further introductions of potentially virulent strains across the world.

Methods

Fungal and plant material. A diverse collection of 39 *Colletotrichum lupini* and 11 closely related *Colletotrichum* spp. isolates, originating from Europe, Australia, Southern Africa and South and North America, was analyzed (Table 1). Nine isolates were collected from symptomatic lupin plants in this study, whereas the rest of the isolates was already identified as *C. lupini* or as other members of the *C. acutatum* species complex representing clades 1, 2 and 4. The *C. lupini* strains CBS 109225 (Ukraine), CBS 509.97 (France) and CBS 109226 (Canada) were chosen as reference for genetic group I, strains CBS 109221 (Germany), IMI 375715 (Australia) and RB221 (France) served as reference for genetic group II and the *C. acutatum* strains CBS 369.73 and CBS 370.73 were used as outgroup in the phylogenetic analysis. Inoculations were performed on two white lupin (*Lupinus albus* L.) accessions: Feodora (susceptible; breeder: Jouffrai Drillaud, France) and Blu-25 (tolerant; breeder: Semillas Baer, Chile), and two Andean lupin (*L. mutabilis*) accessions: LUP 17 and LUP 100 (genebank: IPK, Germany). Plant material can be requested at mentioned breeders and genebanks, who performed formal identification and gave permission to use the material for research purposes. The experimental research of the plant material used in this study complies with relevant institutional, national, and international guidelines and legislation.

Fungal isolation and culture conditions. Symptomatic (dried) lupin stem or pod tissue (Fig. 1) of 1–3 cm was surface sterilized (after rehydration in sterile ddH₂O for dried samples) for 5 s with 0.25% sodium hypochlorite solution and rinsed thrice for 5 s in sterile ddH₂O. Thin slices of 1 mm were cut and placed on PDA (potato dextrose agar, Carl Roth, Karlsruhe, Germany) amended with Tetracycline (0.02 g/l, Carl Roth) for 3 to 4 days at 22 °C in the dark. Single cultures were selected and grown on fresh PDA plates amended with Tetracycline for 4 to 6 days at 22 °C in the dark and suspected *Colletotrichum* species were sub-cultured. Single isolates were re-isolated on PDA plates amended with Tetracycline for 3 to 4 days at 22 °C in the dark and suspected *Colletotrichum* species were sub-cultured. Single

| Strain          | Morphotype | Genetic group | Growth rate (mm/day)a | Conidia L × W (µm)a | Conidia shapebc | Colony morphologyc |
|-----------------|------------|---------------|-----------------------|---------------------|-----------------|---------------------|
| IMI 375715, JA01, -06, -07, -13, -16, -17 | A          | II            | 6.2 ± 0.1             | 12 ± 2.1 × 4 ± 0.7  | Cylindrical to elliptical, occasionally clavate | Flat, circular, with entire margins, white-greyish cottony aerial mycelium, pale to orange on reverse, dark in center |
| JA12            | B          | IV            | 5.6 ± 0.1             | 13.3 ± 1.4 × 3.4 ± 0.5 | Cylindrical to elliptical, occasionally clavate | Flat, circular, with entire margins, white-brownish cottony aerial mycelium, pale on reverse |
| JA11            | C          | IV            | 5.5 ± 0.1             | 12 ± 1.7 × 4.5 ± 0.7 | Cylindrical to elliptical, occasionally clavate | Flat, circular, slightly filiform margins, white-greyish cottony aerial mycelium, pale on reverse, orange in center |
| JA15            | D          | II            | 5 ± 0                  | 9.7 ± 2.4 × 4.2 ± 1.0 | Cylindrical, occasionally roundish | Flat, circular, with entire margins, white-greyish cottony aerial mycelium, dark on reverse, pale at margins |
| CBS 109216, JA21, -22 | E          | VI            | 5.4 ± 0.3             | 8.5 ± 2.1 × 3.5 ± 0.7 | Cylindrical to clavate | Flat, circular, with entire margins, white-greyish cottony aerial mycelium, pale on reverse with black dots |
| JA20            | F          | III           | 4.2 ± 0.3             | 8.7 ± 1.1 × 3.2 ± 0.6 | Cylindrical, occasionally clavate | Flat, circular, with entire margins, white-greyish aerial mycelium, pale on reverse |
| JA10            | G          | III           | 4.9 ± 0.2             | 9.2 ± 1.7 × 3.6 ± 0.7 | Cylindrical to elliptical, occasionally clavate | Irregular and radially sulcate with aerial mycelia growth in the center, pale on reverse |
| CBS 109225      | H          | I             | 5.2 ± 0.1             | 8.5 ± 1.7 × 3.8 ± 0.8 | Cylindrical to clavate | Slightly irregular and thickly covered with wooly white-greyish aerial mycelia, pale on reverse |
| JA18            | I          | V             | 4.1 ± 0                | 10 ± 1.8 × 2.9 ± 0.7 | Cylindrical | Irregular, wooly white aerial mycelia on the margins, pale on reverse |
| JA19            | J          | V             | 6 ± 0.2                | 12.1 ± 1.8 × 2.4 ± 0.7 | Cylindrical | Irregular, white-greyish wooly aerial mycelium, pale on reverse with occasional black/orange dots |

Table 2. Growth rate, conidial size and shape, and colony morphology for the different morphotypes observed within *Colletotrichum lupini*. L length, W width. *Mean ± SD, see also Supplementary Fig. S2.* Observed conidia were rather variable in shape and size, but all conidia were hyaline, smooth-walled, aseptate, straight, with one end round and one end acute as described for *Colletotrichum lupini* in Damm et al.14. See also Fig. 2.
spore cultures were obtained and transferred to PDA and maintained at 22 °C in the dark as working cultures and stored at −80 °C in 25% glycerol for long-term storage.

Morphology. A globally representative subset of 28 C. lupini isolates was characterized based on colony morphology (form, aerial mycelium, margin type and color of the reverse side). From those, a subset of 18 isolates was further characterized for growth rate (mm/day), and conidial shape and size. Isolates were subcultured by placing a droplet of 5 μl spore suspension in the middle of three PDA plates and grown for 14 days at 22 °C in the dark. Culture diameter was recorded every 3 days. Photographs were taken from the front and reverse sides of the PDA plates after 14 days of incubation. Conidia were collected with a sterile spreader after flooding the Petri plate with 2 ml sterile ddH2O, the spore suspension was filtered with sterile cheese cloth and microscopic slides were prepared with sterile ddH2O. Conidia morphology was observed using light microscopy (DM2000-LED, Leica Microsystems, Wetzlar, Germany) equipped with a high definition camera (Gryphax Subra, Jenoptik AG, Jena, Germany). A minimum of at least 50 measurements were performed to determine conidia length and width. A principal component analysis (PCA) was performed on a subset of 17 representative C. lupini isolates, based on average conidia length and width, length width ratio, colony growth rate, form (circular = 1, most irregular = 4), aerial mycelia (no aerial mycelia = 1, most aerial mycelia = 4), color (palest = 1, darkest = 4) and filiform margin (yes = 1, no = 0), using R 4.0.361 and the FactoMineR package.

DNA extraction, PCR amplification and sequencing. Mycelium from single-spore cultures was collected after 7–10 days on PDA at 22 °C with a sterile spreader after flooding the Petri plate with 2 ml sterile ddH1O. Genomic DNA was isolated with a CTAB extraction protocol. Partial gene sequences were determined for the internal transcribed spacer (ITS) region using primers ITS5 and ITS4, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using primers GDF1 and GDR1, the β-tubulin 2 (TUB2) gene using primers

Figure 3. Multi-locus phylogeny of Colletotrichum lupini. Bayesian analysis tree inferred from the combined ITS, TUB2, GAPDH and APN/MAT1 sequence datasets of 50 Colletotrichum strains used in this study. Bootstrap support values (> 50) and Bayesian posterior probabilities (> 0.95) are given at each node. The tree is rooted to C. acutatum (CBS 369.73 and CBS 370.73). Strain codes are followed by host, country of origin and morphology (A–J). Grouping (I–VI) is based on phylogeny and morphology. Strains used for virulence assays are highlighted in bold. Clades indicate the different clades within the C. acutatum species complex.
Btub2Fd and Btub4Rd66 and the Apn2-Mat1-2-1 intergenic (APN/MAT1) spacer and partial mating type gene using Apnmat1F and Apnmat1R39. PCR was performed in a S1000 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to conditions described in Dubrulle et al.39 PCR products were verified by gel electrophoresis, purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and sent to Eurofins Genomics (Ebersberg, Germany) for sequencing. The obtained DNA sequences were analyzed and consensus sequences were generated using BioEdit v. 7.2.567.

**Phylogenetic analyses.** Alignments for each of the four loci, including sequences obtained in this study and downloaded from GenBank (Table 1), were performed with ClustalW using MEGA X48. Obtained multiple alignments where manually corrected and trimmed to obtain comparable sequences. Best-fit substitution models were determined for each locus separately and for the concatenated multi-locus alignment (ITS, TUB2, GAPDH and APN/MAT1). Phylogenetic analyses of the multi-locus alignment were based on Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was performed using RAxML v. 849 through the CIPRES science gateway portal70 using default parameters and 1000 bootstrap iterations. The BI analysis was performed with MrBayes v. 3.2.71 using a Markov Chain Monte Carlo (MCMC) algorithm using four chains and starting from a random tree topology. Substitution models for each locus were included for each partition. The analysis ran for 500,000 generations with trees sampled every 1000 generations to reach average standard deviations of split frequencies below 0.01. The first 25% of saved trees were discarded at the ‘burn-in’ phase and the 50% consensus trees and posterior probabilities (PP) were determined from the remaining trees. Bootstrap

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**Figure 4.** Virulence of *Colletotrichum lupini* strains on white (*Lupinus albus*) and Andean lupin (*L. mutabilis*). Anthracnose severity is expressed in standardized area under the disease progress curve (sAUDPC) and estimated means are shown. Strain codes are followed by abbreviated country of origin and morphotype (A–J). Different capital letters above bars indicate significant differences between strains (Tuckey-HSD, p < 0.05). Error bars indicate the standard error of the estimated mean.
support values (BS) from the ML analysis were plotted on the Bayesian phylogeny. Further phylogenetic analyses were performed with the unweighted pair group method with arithmetic mean (UPGMA) with 10,000 replicates in Mega X. All generated sequences were deposited in GenBank (Table 1) and alignments and trees in TreeBASE.

**Virulence.** Virulence tests were performed on white and Andean lupin with representative *C. lupini* strains (see Fig. 3). *C. tamarilloti* strain CBS 129814 and *C. acutatum* strain CBS 369.73 through stem-wound inoculation as described by Alkemade et al. 26, which was shown to highly correspond to field performance in Switzerland (r = 0.95). Disease scores ranging from 1 (non-pathogenic), 2 (low virulence) to 9 (highly virulent) were taken 4, 7 and 10 days post inoculation (dpi) and the standardized area under the disease progress curve was calculated (sAUDPC) 26. All inoculations were performed in a growth chamber (25 ± 2 °C, 16 h light and ~70% relative humidity) in a completely randomized block design with a minimum of six replicates per experiment.

**Statistical analysis.** Statistical analyses were performed with R 4.0.3 using the packages **lme4**27, **lmerTest**28 and **emmeans**24, following a mixed model with factors of interest (i.e. strain, lupin species, lupin accession) as fixed and replicated block nested in experiment as random factor. Datasets that did not follow assumptions of normality of residuals and homogeneity of variance were log10 transformed. Data are presented as estimated least-squares means using the aforementioned mixed model. A Tukey-HSD test (p ≤ 0.05) was applied for pairwise mean comparison of the different *Colletotrichum* strains within each lupin accession.

**Data availability**

The data that support the findings of this study are shown in this manuscript or, in the case of new sequences data, are openly available in Genbank at https://www.ncbi.nlm.nih.gov/genbank/ (for reference numbers see Table 1) and in Treebase at http://purl.org/phylo/treebase/phylows/study/TB2:527356?access-code=260136f8e616a0614b93528dd6fe6f&format=html.

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Author contributions
J.A.A., P.H. and M.M.M. conceived the original idea for this study. J.A.A. conducted the experiments and took the lead in manuscript writing. J.A.A. analyzed the data with contributions from P.H. and M.M.M. All authors significantly contributed to data interpretation and provided critical feedback that shaped the final version. J.A.A. designed the figures and tables with input from P.H., M.M.M., R.T.V. and M.R.F.

Competing interests
The authors declare no competing interests.

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