RESEARCH ARTICLE

Molecular Diversity of Anthracnose Pathogen Populations Associated with UK Strawberry Production Suggests Multiple Introductions of Three Different Colletotrichum Species

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Abstract

Fragaria × ananassa (common name: strawberry) is a globally cultivated hybrid species belonging to Rosaceae family. Colletotrichum acutatum sensu lato (s.l.) is considered to be the second most economically important pathogen worldwide affecting strawberries. A collection of 148 Colletotrichum spp. isolates including 67 C. acutatum s.l. isolates associated with the phytosanitary history of UK strawberry production were used to characterize multi-locus genetic variation of this pathogen in the UK, relative to additional reference isolates that represent a worldwide sampling of the diversity of the fungus. The evidence indicates that three different species C. nymphaeae, C. godetiae and C. fioriniae are associated with strawberry production in the UK, which correspond to previously designated genetic groups A2, A4 and A3, respectively. Among these species, 12 distinct haplotypes were identified suggesting multiple introductions into the country. A subset of isolates was also used to compare aggressiveness in causing disease on strawberry plants and fruits. Isolates belonging to C. nymphaeae, C. godetiae and C. fioriniae representative of the UK anthracnose pathogen populations showed variation in their aggressiveness. Among the three species, C. nymphaeae and C. fioriniae appeared to be more aggressive compared to C. godetiae. This study highlights the genetic and pathogenic heterogeneity of the C. acutatum s.l. populations introduced into the UK linked to strawberry production.
Introduction

*Fragaria × ananassa* (common name: strawberry) is a hybrid species cultivated worldwide belonging to the *Rosaceae* family. Since the 1980s, the UK strawberry industry has expanded rapidly representing a significant component of fruit production in the country [1]. Anthracnose is a major disease of cultivated strawberry, caused by two species complexes of the fungus referred to as *C. acutatum* and *C. gloeosporioides*. *C. acutatum* is considered to be the dominant cause of strawberry anthracnose, and the second most important pathogen of strawberry after *Botrytis cinerea* [2–7]. The *C. gloeosporioides* complex includes *C. fragariae*, which is now considered synonymous with a new species *C. theobromicola* [8]. However, researchers have often continued to use the name *C. fragariae* when referring to a pathogen that was associated with strawberry anthracnose [9–12]. *C. gloeosporioides* is found only occasionally on strawberry in Europe [3,7].

*C. acutatum s.l.* was described for the first time as a strawberry pathogen in California in 1983 [13], and has since appeared to have spread worldwide, including the UK, through runners and propagating material [2,6,14–16]. A first extensive genetic characterization of *C. acutatum s.l.* representing the global diversity of the pathogen led to its sub-division into genetic groups named from A1 to A9 [6, 17]. More recently, the *C. acutatum s.l.* has been sub-divided into more than 30 species based on multi-locus phylogeny [18].

The first record of *C. acutatum s.l.* in the UK was in 1978, on *Anemone* sp. grown in Jersey [19]. In 1982, the first incidence of anthracnose disease in strawberries caused by *C. acutatum s.l.* was recorded in the UK, and was attributed to the importation of infected strawberry runners from the USA [20]. DNA sequences in public databases suggest two UK isolates (CBS198.35 and CBS199.35) that were collected in 1935 from the host *Phormium* spp. (common name “New Zealand flax”) belong to *C. acutatum s.l.* [18, 21]. CABI database records during 1978 to 1983 shows the incidence of the pathogens various hosts and in different locations in the UK (http://www.herbimi.info). However, it seems highly improbable that the first outbreak on strawberry led to the wide dispersal of the pathogen. In 1993, Lovelidge proposed that the continued introduction of infected strawberry material from abroad was so common that the disease was destined to become endemic in the UK [14]. In subsequent years, further outbreaks have been reported on strawberry linked to the importation of infected propagation material mainly from mainland Europe and on other important crop hosts [20,22,23].

Strawberry anthracnose symptoms produced by the two *Colletotrichum* species complexes are similar and can be found on all parts of the plant [12]. Flower blight and fruit rot are common symptoms in the field [24], whereas lesions on stolons, petioles and leaves are mainly found in plant nurseries [15]. Crown symptomatology is characterized by reddish-brown necrotic areas [25] and in some cases stunting and chlorosis have been associated with root necrosis [15].

Research has been carried out to characterize *C. acutatum s.l.* populations related to strawberry in specific geographic areas including Israel, France, Bulgaria, Spain, Belgium and other European countries [2–5,7,26] and from specific regions of the USA [25]. Other research has attempted to characterize *C. acutatum s.l.* related to strawberry using isolates collected worldwide [3], both by genomic fingerprinting (such as RFLP, apPCR, etc.) and sequence analysis based on the ITS region. Results have highlighted the presence of at least one representative “clonal” population suggesting a single source of origin and, consequently, that the disease is spread through infected propagation material. However, ITS sequences alone or genomic fingerprinting are not suitable to discriminate among the newly assigned species designations.

In a recent study based on the analysis of more than two decades of anthracnose incidence data sets gathered by authorities responsible for plant health, trade was identified as the main
route of entry and establishment of *C. acutatum* in the UK strawberry production. Over this period, various nurseries were importing planting material into the UK, and at least 55 cases of infested material that was planted in the field through imports that were not intercepted by the border inspection posts, were identified [20].

The focus of the present study was to assess the extent of the genetic and pathogenic diversity of these introduced pathogen populations mainly utilising a unique collection of *C. acutatum s.l.* isolates established through the plant health inspection surveys from the early 1980s onwards. We focused on *C. acutatum s.l.* because previous reports from France, Israel, UK, Bulgaria and Spain had described this taxa as a major widely distributed pathogen, compared with other species such as *C. gloeosporioides s.l.* that occur less frequently in Europe [2–5,12]. A range of historic and contemporary *C. acutatum s.l.* isolates including those from worldwide strawberry crops, other plant hosts in the UK, as well as worldwide representatives from different hosts building on our previous work were accessed as reference sources for determining the genetic and species identities of isolates associated with UK strawberry anthracnose phytosanitary control work. Based on multi-locus phylogenetic analysis, we have identified 12 different haplotypes that belong to three different species *C. nymphaeae*, *C. godetiae* and *C. fioriniae* suggesting multiple introductions of the strawberry anthracnose pathogen. Pathogenic and growth characteristics of these haplotype representatives further highlight the heterogeneity of the introduced pathogen populations.

**Materials and Methods**

**Fungal isolates and culture conditions**

A diverse collection of *C. acutatum s.l.* was assembled for this study including: 67 isolates associated with strawberry production in the UK (obtained from the UK Food and Environment Research Agency, or FERA responsible for plant health within the Department for Environment, Food and Rural Affairs), 27 *C. acutatum s.l.* isolates collected from strawberry in other countries, and 13 isolates collected from other host species in the UK. For further comparison, 33 isolates were added to represent other genetic groups, and novel species from previous studies [6,17,18]. This included two isolates of *C. fruticola*, two isolates of *C. aenigma* (belonging to *C. gloeosporioides* species complex [8]) associated with strawberry, two UK isolates of *C. spinaciae* and one isolate each of *C. graminicola*, *C. higginsianum* [27] and *C. fioriniae* [28].

Sequence data of the markers was retrieved from the reference genome sequences available from Genbank for *C. graminicola* and *C. higginsianum* (accession numbers: ACOD01000000 and CACQ02000000, respectively) used among out-groups in the phylogenetic analysis (Fig 1). Details of the isolate collection used in the present study are provided in Table 1.

Cultures were maintained at 25°C on potato dextrose agar medium (PDA, Difco Laboratories, USA) for up to ten days under a 12 h light/12 h dark cycle. Long-term storage at 4°C involved cutting mycelial plugs from the edge of actively growing cultures on PDA and suspending them in sterile water.

**Characterization of genetic variation**

Genomic DNA was extracted according to the Chelex 100 protocol [29], with some modifications [30]. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, DE, USA).

Various target regions were used to characterise genetic diversity amongst the fungal isolates including: ITS region, partial sequence of the beta-tubulin 2 gene (TUB) (exons 3 through 6, including introns 2 through 4), partial sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, and partial sequence of the mating type gene (MAT1-2) (the intron
PCR amplification of the target regions for sequencing was carried out as described below using previously published primers under conditions standardised for routine work. For ITS, primers ITS1Ext and ITS4Ext [31] were used. The amplification program consisted of 2 min of initial denaturation (95°C), 30 cycles of amplification (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) and a final extension at 72°C for 5 min. For TUB, primers TB5 and TB6 [31] were used. The amplification program consisted of 2 min initial denaturation (95°C), 30 cycles of amplification (1 min at 94°C, 1 min at 65°C and 1 min at 72°C) and a final extension at 72°C for 5 min. For GAPDH, primers GDF1 and GDR1 [32] were used. The amplification program consisted of 2 min initial denaturation at 95°C, 35 cycles of amplification (1 min at 94°C, 1 min at 60°C and 30 sec at 72°C) and a final extension at 72°C for 3 min. For MAT1-2, primers HMGacuF2 and HMGacuR [21] for *C. acutatum s.l.* and primers HMGgloeF1 and HMGgloeR1 for *C. gloeosporioides s.l.* [33] were used. The amplification program consisted of 5 min initial denaturation at 95°C, 40 cycles of amplification (1 min at 95°C, 1 min between 48°C and 55°C and 30s at 72°C) and a final extension of 20 min at 72°C.

PCR products were separated using gel electrophoresis and purified using the QIAquick PCR purification kit (Qiagen, USA).

Sequencing of PCR products was carried out at the University of Warwick Genomics Centre, using an ABI Prism 7900HT or ABI3100 sequence detection system (Applied Biosystems, UK). PCR products were cleaned up and then quantified with reference to a ladder (Bioline EasyLadder I) containing DNA fragments of known concentration. One to five microliters of each sample (depending on DNA concentration) were used in sequencing reactions with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, UK). ABI trace files were analyzed and consensus sequences were generated using Geneious 7.1.6 [34]. All the sequences were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and were manually edited to optimise the alignment, as required. Multiple alignments were end trimmed in order to have comparable nucleotides.

Multiple sequence alignments were exported to MEGA5 [35] where best-fit substitution models were calculated for each separate sequence dataset. In order to evaluate whether the four sequenced loci were congruent and suitable for concatenation, tree topologies of 50% Neighbour-Joining bootstrap and maximum parsimony analysis (100,000 replicates) were separately performed for each gene and visually compared [36]. The multilocus concatenated alignment (ITS, TUB2, MAT1-2 and GAPDH) was performed with Geneious 7.1.6 [34]. A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes 3.2.1 [37] for combined sequence datasets. Models of nucleotide substitution for each gene determined by MEGA5 were included for each locus. The analysis in MrBayes ran for 5000000 of generations to reach a P value lower than 0.01 with two parallel searches using three heated and one cold Markov chain sampled every 100 generations; 25% of generations were discarded as burn-in. Further phylogenetic analysis was performed by
Table 1. Colletotrichum sp. strains used in this study with isolation details and GenBank accessions.

| Strain Code | Genus        | Species            | Genetic group [6] | Country       | Host                     | Accession numbers | ITs   | TUB    | MAT1-2  | GAPDH   |
|-------------|--------------|--------------------|-------------------|---------------|--------------------------|-------------------|-------|--------|---------|---------|
| B88         | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246514 KM251867 KM251969 KM252115 |
| NI90        | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | AF411766 AJ409294 KM251970 KM252116 |
| CSL 1079    | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246515 KM251868 KM251981 KM252118 |
| CSL 2546    | Colletotrichum | floriniae          | A3                | United Kingdom| Fragaria x ananassa      | KM246516 KM251870 KM251983 KM252120* |
| CSL 899     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246518 KM251872 KM251985 KM252122* |
| CSL 310     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246519 KM251873 KM251986 KM252123 |
| CSL 915     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246520 KM251874 KM251987 KM252124* |
| CSL 886     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | KM246521 KM251875 KM251988 KM252125 |
| CSL 919     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | KM246522 KM251876 KM251989 KM252126* |
| CSL 916     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | KM246523 KM251877 KM251990 KM252127* |
| CSL 918     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | KM246524 KM251878 KM251991 KM252128* |
| CSL 917     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria x ananassa      | KM246525 KM251879 KM251992 KM252129 |
| CSL 223     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246526 KM251880 KM251993 KM252130 |
| CSL 224     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246527 KM251881 KM251994 KM252131 |
| CSL 225     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246528 KM251882 KM251995 KM252132 |
| CSL 255     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246529 KM251883 KM251996 KM252133 |
| CSL 256     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246530 KM251884 KM251997 KM252134* |
| CSL 258     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246531 KM251885 KM251998 KM252135 |
| CSL 456     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria vesca           | KM246532 KM251886 KM251999 KM252136 |
| CSL 493     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria vesca           | KM246533 KM251887 KM252000 KM252137 |
| CSL 494     | Colletotrichum | godetiae           | A4                | United Kingdom| Fragaria vesca           | KM246534 KM251888 KM252001 KM252138 |
| CSL 604     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246535 KM251890 KM252003 KM252140 |
| CSL 607     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246538 KM251893 KM252006 KM252143 |
| CSL 608     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246539 KM251894 KM252007 KM252144 |
| CSL 872     | Colletotrichum | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa      | KM246541 KM251896 KM252009 KM252146 |

(Continued)
Table 1. (Continued)

| Strain Code | Genus              | Species            | Genetic group [6] | Country       | Host                  | Accession numbers |   |   |   |
|-------------|--------------------|--------------------|-------------------|---------------|-----------------------|-------------------|---|---|---|
| CSL 903     | Colletotrichum     | godetiae           | A4                | United Kingdom| Fragaria x ananassa  | KM246542 KM251897 KM252010 KM252147 |   |   |   |
| CSL 1001    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246543 KM251898 KM252011 KM252148 |   |   |   |
| CSL 1258    | Colletotrichum     | floriniae          | A3                | United Kingdom| Fragaria x ananassa  | KM246544 KM251899 KM252012 KM252149 |   |   |   |
| CSL 1259    | Colletotrichum     | floriniae          | A3                | United Kingdom| Fragaria x ananassa  | KM246545 KM251900 KM252013 KM252150* |   |   |   |
| CSL 1260    | Colletotrichum     | floriniae          | A3                | United Kingdom| Fragaria x ananassa  | KM246546 KM251901 KM252014 KM252151 |   |   |   |
| CSL 1261    | Colletotrichum     | floriniae          | A3                | United Kingdom| Fragaria x ananassa  | KM246547 KM251902 KM252015 KM252152 |   |   |   |
| CSL 1262    | Colletotrichum     | floriniae          | A3                | United Kingdom| Fragaria x ananassa  | KM246548 KM251903 KM252016 KM252153* |   |   |   |
| CSL 1305    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246549 KM251904 KM252017 KM252154 |   |   |   |
| CSL 1376    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246550 KM251905 KM252018 KM252155 |   |   |   |
| CSL 1377    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246551 KM251906 KM252019 KM252156 |   |   |   |
| CSL 1378    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246552 KM251907 KM252020 KM252157 |   |   |   |
| CSL 1379    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246553 KM251908 KM252021 KM252158 |   |   |   |
| CSL 1380    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246554 KM251909 KM252022 KM252159 |   |   |   |
| CSL 1381    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246555 KM251910 KM252023 KM252160 |   |   |   |
| CSL 1382    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246556 KM251911 KM252024 KM252161 |   |   |   |
| CSL 1383    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246557 KM251912 KM252025 KM252162 |   |   |   |
| CSL 1384    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246558 KM251913 KM252026 KM252163 |   |   |   |
| CSL 1385    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246559 KM251914 KM252027 KM252164 |   |   |   |
| CSL 1386    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246560 KM251915 KM252028 KM252165 |   |   |   |
| CSL 1387    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246561 KM251916 KM252029 KM252166 |   |   |   |
| CSL 1388    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246562 KM251917 KM252030 KM252167 |   |   |   |
| CSL 1389    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246563 KM251918 KM252031 KM252168 |   |   |   |
| CSL 1390    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246564 KM251919 KM252032 KM252169 |   |   |   |
| CSL 1391    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246565 KM251920 KM252033 KM252170 |   |   |   |
| CSL 1392    | Colletotrichum     | nymphaeae          | A2                | United Kingdom| Fragaria x ananassa  | KM246566 KM251921 KM252034 KM252171 |   |   |   |

(C Assuming the table continues)
Table 1. (Continued)

| Strain Code | Genus           | Species          | Genetic group [6] | Country       | Host                      | Accession numbers         |
|-------------|-----------------|------------------|-------------------|---------------|---------------------------|---------------------------|
| CSL 1393    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246567 KM251922 KM252035 KM252172 |
| CSL 1394    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246568 KM251923 KM252036 KM252173 |
| CSL 1395    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246569 KM251924 KM252037 KM252174 |
| CSL 1396    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246570 KM251925 KM252038 KM252175 |
| CSL 1397    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246571 KM251926 KM252039 KM252176 |
| CSL 1398    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246572 KM251927 KM252040 KM252177 |
| CSL 1429    | Colletotrichum  | godetiae         | A4                | United Kingdom| Fragaria x ananassa       | KM246573 KM251928 KM252041 KM252178 |
| CSL 1441    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246574 KM251929 KM252042 KM252179 |
| CSL 1442    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246575 KM251930 KM252043 KM252180 |
| CSL 1443    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246576 KM251931 KM252044 KM252181 |
| CSL 1444    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246577 KM251932 KM252045 KM252182 |
| CSL 1449    | Colletotrichum  | godetiae         | A4                | United Kingdom| Fragaria x ananassa       | KM246578 KM251933 KM252046 KM252183 |
| CSL 2064    | Colletotrichum  | godetiae         | A4                | United Kingdom| Fragaria x ananassa       | KM246579 KM251934 KM252047 KM252184 |
| CSL 1002    | Colletotrichum  | godetiae         | A4                | United Kingdom| Fragaria x ananassa       | KM246580 KM251935 KM252048 KM252185 |
| CSL 892     | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | KM246584 KM251938 KM252053 KM252188 |
| IMI 299103  | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria vesca            | JQ948231 JQ949882 KM252069 JQ948561 |
| PD88-857    | Colletotrichum  | nymphaeae        | A2                | United Kingdom| Fragaria x ananassa       | JQ948232 JQ949883 KM252100 JQ948562 |

C. acutatum sensu lato from strawberry worldwide

C2897 Colletotrichum nymphaeae A2 Australia Fragaria x ananassa AJ300558 AJ314718 KM251967 KM252113
CSL 397 Colletotrichum nymphaeae A2 USA Fragaria x ananassa AF411765 AJ409296 KM251968 KM252114
CSL 1053 Colletotrichum godetiae A4 Netherlands Fragaria x ananassa AJ536210 KM251869 KM251982 KM252119
CSL 891 Colletotrichum nymphaeae A2 Portugal Fragaria sp. EF622184 KM251889 KM252002 KM252139
CSL 511 Colletotrichum nymphaeae A2 France Fragaria x ananassa KM246536 KM251891 KM252004 KM252141
CSL 729 Colletotrichum nymphaeae A2 Switzerland Fragaria x ananassa KM246537 KM251892 KM252005 KM252142
CSL 1430 Colletotrichum godetiae A4 Norway Fragaria vesca KM246585 KM251939 KM252054 KM252189
CSL 1432 Colletotrichum godetiae A4 Norway Fragaria x ananassa KM246586 KM251940 KM252055 KM252190

(Continued)
Table 1. (Continued)

| Strain Code | Genus                  | Species   | Genetic group [6] | Country     | Host                     | Accession numbers                     | ITS | TUB   | MAT1-2 | GAPDH     |
|-------------|------------------------|-----------|-------------------|-------------|--------------------------|---------------------------------------|-----|-------|--------|-----------|
| PJ7 [28]    | Colletotrichum         | fioriniae | A3                | New Zealand | Fragaria x ananassa      | genome: JARH00000000                   |     |       |        |           |
| CSL 1020, IMI 301119 [18] | Colletotrichum | nymphaeae  | A2                | Kenya       | Fragaria vesca           | JQ948266 JQ949917 KM252070 JQ948596 |     |       |        |           |
| IMI 311743 [18] | Colletotrichum       | nymphaeae  | A2                | USA         | Fragaria x ananassa      | JQ948258 JQ949909 KM252071 JQ948588 |     |       |        |           |
| IMI 335544 | Colletotrichum         | nymphaeae  | A2                | Italy       | Fragaria x ananassa      | KJ018636 KJ018648 KM252072 KJ018660 |     |       |        |           |
| IMI 345026 [18] | Colletotrichum       | godetiae  | A4                | Spain       | Fragaria x ananassa      | JQ948424 JQ950075 KM252073 JQ948755 |     |       |        |           |
| CSL 1005, IMI 345027 | Colletotrichum     | nymphaeae  | A2                | France      | Fragaria x ananassa      | AJ536199 KM251946 KM252074 KM252198 |     |       |        |           |
| IMI 345028 | Colletotrichum         | nymphaeae  | A2                | Colombia    | Fragaria x ananassa      | AF090853 KM251947 KM252075 KM252199 |     |       |        |           |
| IMI 345029 | Colletotrichum         | nymphaeae  | A2                | Costa Rica  | Fragaria x ananassa      | KM246591 KM251948 KM252076 KM252200 |     |       |        |           |
| CSL 1034, IMI 345030 | Colletotrichum     | nymphaeae  | A2                | Costa Rica  | Fragaria x ananassa      | AJ536203 KM251949 KM252077 KM252201 |     |       |        |           |
| IMI 345031 | Colletotrichum         | nymphaeae  | A2                | Italy       | Fragaria x ananassa      | KM246592 KM251950 KM252078 KM252202 |     |       |        |           |
| IMI 345578 | Colletotrichum         | fioriniae  | A3                | New Zealand | Fragaria ananassa        | JQ948334 JQ949985 KM252080 JQ948664 |     |       |        |           |
| CSL 1046, IMI 346326 | Colletotrichum     | simmondsii | A2                | Australia   | Fragaria x ananassa      | AJ536208 KM251952 KM252081 KM252204 |     |       |        |           |
| IMI 345585 [18] | Colletotrichum       | salicis   | A7                | New Zealand | Fragaria x ananassa      | JQ948476 JQ950127 KM252084 JQ948807 |     |       |        |           |
| CSL 1090, IMI 348160 | Colletotrichum     | nymphaeae  | A2                | USA         | Fragaria x ananassa      | AJ536200 KM251953 KM252086 KM252205 |     |       |        |           |
| IMI 348177 [18] | Colletotrichum       | nymphaeae  | A2                | USA         | Fragaria x ananassa      | KM246593 KM251954 KM252087 KM252206 |     |       |        |           |
| IMI 348490 | Colletotrichum         | nymphaeae  | A2                | France      | Fragaria x ananassa      | KM246594 KM251955 KM252088 KM252207 |     |       |        |           |
| CSL 1086, IMI 348498 | Colletotrichum     | nymphaeae  | A2                | France      | Fragaria x ananassa      | KM246595 KM251956 KM252089 KM252208 |     |       |        |           |
| CSL 1049, IMI 348499 | Colletotrichum       | fioriniae  | A3                | France      | Fragaria x ananassa      | AJ536220 KM251957 KM252090 KM252209 |     |       |        |           |
| IMI 360928 [18] | Colletotrichum       | nymphaeae  | A2                | Switzerland | Fragaria x ananassa      | JQ948243 JQ949894 KM252091 JQ948573 |     |       |        |           |
| Strains isolated from different hosts in UK |
| RB-MAL-03 | Colletotrichum         | godetiae  | A4                | United Kingdom | Malus domestica     | KF834206 KF834207 KM252049 KF834208 |     |       |        |           |
| RB-MAL-04 | Colletotrichum         | godetiae  | A4                | United Kingdom | Malus domestica     | KM246582 KM251936 KM252050 KM252186 |     |       |        |           |
| CSL 1294 | Colletotrichum         | lupini    | A1                | United Kingdom | Lupinus polyphyllus | AJ300561 KM251944 KM252059 KM252194 |     |       |        |           |
| CSL 287 [18] | Colletotrichum       | acutatum  | A5                | United Kingdom | Statice sp.         | JQ948389 JQ950040 KM252060 JQ948720 |     |       |        |           |
| RB-VIT-01, CBS 129951 [22] | Colletotrichum | godetiae  | A4                | United Kingdom | Vitis vinifera     | KF834203 KF834204 KM252061 KF834205 |     |       |        |           |

(Continued)
Table 1. (Continued)

| Strain Code | Genus          | Species          | Genetic group [6] | Country       | Host                   | Accession numbers                  | ITS     | TUB     | MAT1-2  | GAPDH   |
|-------------|----------------|------------------|-------------------|---------------|------------------------|------------------------------------|---------|---------|---------|---------|
| CSL 455     | Colletotrichum | nymphaeae        | A2                | United Kingdom| Photinia sp.           | JQ948217 JQ949868 KM252063 JQ948547 |
| JCS1, CBS   | Colletotrichum | floriniae        | A3                | United Kingdom| Tulipa sp.             | AJ749680 KM251945 KM252064 KM252195 |
| 129948 [18] |                |                  |                   |               |                        |                                    |         |         |         |         |
| CSL 302a    | Colletotrichum | floriniae        | A3                | United Kingdom| Nandina domestica     | AJ749670 AJ748626 KM252065 KM252196 |
| CSL 473 [19]| Colletotrichum | floriniae        | A3                | United Kingdom| Liriodendron tulipifera| JQ948345 JQ949996 KM252066 JQ948675 |
| CSL 318 [18]| Colletotrichum | floriniae        | A3                | United Kingdom| Magnolia sp.           | JQ948346 JQ949997 KM252067 JQ948676 |
| IMI 350308  | Colletotrichum | lupini           | A1                | United Kingdom| Lupinus sp.            | AJ300561 KM251951 KM252079 KM252203 |
| CBS 198.35  | Colletotrichum | kinghornii       | A7                | United Kingdom| Phormium sp.           | JQ948454 JQ950105 KM252083 JQ948785 |
| PD93-1748,  | Colletotrichum | godetiae         | A4                | United Kingdom| Prunus avium          | JQ948408 JQ950059 KM252101 JQ948739 |
| CBS 126527  |                |                  |                   |               |                        |                                    |         |         |         |         |

**Isolates from different host worldwide and used as references for genetics groups / species**

| Strain Code | Genus            | Species          | Genetic group [6] | Country       | Host                   | Accession numbers                  | ITS     | TUB     | MAT1-2  | GAPDH   |
|-------------|------------------|------------------|-------------------|---------------|------------------------|------------------------------------|---------|---------|---------|---------|
| PT250, CBS  | Colletotrichum   | rhombiforame     | A6                | Portugal      | Olea europaea          | JQ948457 JQ950108 KM251971 JQ948788* |
| 129953 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| PT135, CBS  | Colletotrichum   | nymphaeae        | A2                | Portugal      | Olea europaea          | JQ948201 JQ949852 KM251972 JQ948531 |
| 129945 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| PD85-694, CBS | Colletotrichum | chrysanthemi     | A2                | Netherlands   | Chrysanthemum sp.      | JQ948272 JQ949923 KM251973 JQ948602 |
| 126519 [18] |                |                  |                   |               |                        |                                    |         |         |         |         |
| PD89-582, CBS | Colletotrichum | simmondsii       | A2                | Netherlands   | Cyclamen sp.           | JQ948281 JQ949932 KM251974 JQ948611* |
| 126524 [18] |                |                  |                   |               |                        |                                    |         |         |         |         |
| PT227, CBS  | Colletotrichum   | acutatum         | A5                | Portugal      | Olea europaea          | JQ948364 JQ950015 KM251975 JQ948695* |
| 129952 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| Tom-21, CBS | Colletotrichum   | tamarilloi       | A8                | Colombia      | Cyphomandra betacea    | JQ948188 JQ949839 KM251976 JQ948518 |
| 129954 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| Tom-12, CBS | Colletotrichum   | tamarilloi       | A8                | Colombia      | Cyphomandra betacea    | JQ948189 JQ949840 KM251977 JQ948519 |
| 129955 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| CBS 193.32  | Colletotrichum   | godetiae         | A4                | Greece        | Olea europaea          | JQ948415 JQ950066 KM251978 JQ948746* |
| [18]        |                  |                  |                   |               |                        |                                    |         |         |         |         |
| PT30        | Colletotrichum   | lupini           | A1                | Portugal      | Lupinus albus          | AJ300561 AJ292250 KM251979 KM252117* |
| CR46, CBS   | Colletotrichum   | floriniae        | A3                | Portugal      | Vitis vinifera         | JQ948343 JQ949994 KM251980 JQ948673* |
| 129947 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| 9178        | Colletotrichum   | salicis          | A7                | Norway        | Vaccinium corymbosum   | KM246583 KM251937 KM252051 KM252187* |
| MP1, CBS    | Colletotrichum   | salicis          | A7                | USA           | Acer platanoides       | JQ948466 JQ950117 KM252052 JQ948797* |
| 129972 [18] |                  |                  |                   |               |                        |                                    |         |         |         |         |
| PJ8         | Colletotrichum   | acutatum         | A5                | New Zealand   | Pyrus pyrifolia        | KM246587 KM251941 KM252056 KM252191* |
| ATCC MYA-663| Colletotrichum   | floriniae        | A3                | USA           | Malus domestica        | KM246589 KM251943 KM252058 KM252193* |
| HY09        | Colletotrichum   | lupini           | A1                | Canada        | Lupinus albus          | KJ018635 KJ018647 KM252062 KJ018659* |
| JL198       | Colletotrichum   | godetiae         | A4                | Serbia        | Olea europaea          | AJ749689 AJ748613 KM252068 KM252197* |
| AR3787, CBS | Colletotrichum   | phormii          | A7                | South Africa  | Phormium sp.           | JQ948453 JQ950104 KM252082 JQ948784* |

(Continued)
| Strain Code | Genus            | Species | Genetic group | Country      | Host              | Accession numbers         | Abbreviation |
|------------|------------------|---------|---------------|--------------|-------------------|---------------------------|--------------|
| CBS 607.94 | Colletotrichum   | salicis | A7            | Netherlands  | Salix sp.         | JQ948460 JQ950111 KM252085 JQ948791* |              |
|            |                  |         |               |              |                   |                           |              |
| ALM-NRB-30K| Colletotrichum   | godetiae| A4            | Israel       | Prunus dulcis     | DQ003129 KM251960 KM252094 KM252212* |              |
|            |                  |         |               |              |                   |                           |              |
| CBS 101611 | Colletotrichum   | sp. 1   |              | Costa Rica   | Fern              | JQ948196 JQ949847 KM252095 JQ948526* |              |
|            |                  |         |               |              |                   |                           |              |
| BBA 70884 | Colletotrichum   | lupini  | A1            | Ukraine       | Lupinus albus     | JQ948155 JQ949806 KM252096 JQ948485* |              |
|            |                  |         |               |              |                   |                           |              |
| STE-U 164 | Colletotrichum   | acutatum| A5            | South Africa | Pinus radiata     | JQ948356 JQ950007 KM252097 JQ948687* |              |
|            |                  |         |               |              |                   |                           |              |
| STE-U 5303| Colletotrichum   | laticophilum | A2    | India         | Hevea brasilensis | JQ948289 JQ949940 KM252098 JQ948619 |              |
|            |                  |         |               |              |                   |                           |              |
| CBS 122122 | Colletotrichum   | simmondsii | A2   | Australia     | Carica papaya     | JQ948276 JQ949927 KM252099 JQ948606* |              |
|            |                  |         |               |              |                   |                           |              |
| CBS 211.78 | Colletotrichum   | costaricense |              | Costa Rica | Coffea sp.        | JQ948181 JQ949832 KM252102 JQ948511 |              |
|            |                  |         |               |              |                   |                           |              |
| DPI 11711 | Colletotrichum   | brisbanense | A2   | Australia     | Capsicum annuum   | JQ948291 JQ949942 KM252103 JQ948621 |              |
|            |                  |         |               |              |                   |                           |              |
| DPI 13483 | Colletotrichum   | simmondsii | A2   | Australia     | Carica papaya     | JQ948277 JQ949928 KM252104 JQ948607* |              |
|            |                  |         |               |              |                   |                           |              |
| ATCC 38896| Colletotrichum   | nymphaeae | A2   | Netherlands   | Nymphaeae alba    | JQ948199 JQ949850 KM252105 JQ948529 |              |
|            |                  |         |               |              |                   |                           |              |
| CBS 797.72 | Colletotrichum   | fioriniae | A3   | New Zealand   | Pinus radiata     | KM246598 KM251961 KM252106 KM252213* |              |
|            |                  |         |               |              |                   |                           |              |
| OCO-ARC-4 | Colletotrichum   | sp. 2   |              | USA          | Citrus x sinensis | EU647305 KM251962 KM252107 EU647318* |              |
|            |                  |         |               |              |                   |                           |              |
| STF-FTP-10 | Colletotrichum   | sp. 2   |              | USA          | Citrus x sinensis | EU647306 KM251963 KM252108 EU647319 |              |
|            |                  |         |               |              |                   |                           |              |
| Coll-25    | Colletotrichum   | scovillei | A2   | Taiwan        | Capsicum annuum   | KJ018637 KJ018649 KM252109 KJ018661 |              |
|            |                  |         |               |              |                   |                           |              |
| Coll-154   | Colletotrichum   | scovillei | A2   | Taiwan        | Capsicum annuum   | DQ410028 KM251964 KM252110 KM252214 |              |
|            |                  |         |               |              |                   |                           |              |
| Isolates as out-group | | | | | | | |
| CSL 311    | Colletotrichum   | fruticola | OG  | USA          | Fragaria x ananassa | KM246512 KM251865 KM251965 KM252111* |              |
| CSL 386    | Colletotrichum   | fruticola | OG  | USA          | Fragaria x ananassa | KM246513 KM251866 KM251966 KM252112* |              |
| CSL 780    | Colletotrichum   | aenigma  | OG  | UK           | Fragaria x ananassa | KM246517 KM251871 KM251984 KM252121* |              |
| CSL 869    | Colletotrichum   | aenigma  | OG  | UK           | Fragaria x ananassa | KM246540 KM251895 KM252008 KM252145* |              |
| CSL 593    | Colletotrichum   | spinaciae | OG  | UK           | Spinacia oleracea  | KM246596 KM251958 KM252092 KM252210 |              |
| CSL 739    | Colletotrichum   | spinaciae | OG  | UK           | Spinacia oleracea  | KM246597 KM251959 KM252093 KM252211 |              |
| M1.001     | Colletotrichum   | graminicola | OG  | USA          | Zea mais          | genome: ACOD0100000000 |              |
| IMI 349063 | Colletotrichum   | higginsianum | OG  | Trinidad and Tobago | Brassica chinensis | genome: CACQ0200000000 |              |

Abbreviation
CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands IMI: Culture collection of CABI Europe UK Centre, Egham, UK CSL: Culture collection of The Food and Environment Research Agency, DEFRA, York, UK OG: out-group* strains used for pathogenicity tests

doi:10.1371/journal.pone.0129140.001
Comparison of fungal growth in culture

The 67 fungal isolates collected from strawberry in the UK were compared with a subset of other isolates (chosen based on genetic, host and geographic diversity) including 49 isolates of *C. acutatum s.l.* and four isolates of *C. gloeosporioides s.l.* for *in vitro* growth studies on PDA (Potato Dextrose Agar, BD Difco). For experiments, a 7 mm diameter mycelial plug excised from the edge of an actively growing PDA culture was placed at the centre of a fresh PDA plate. In the growth experiment, two perpendicular colony diameters were measured daily and colony radius was calculated from cultures incubated at four different temperatures (15°C, 20°C, 25°C and 30°C) in darkness. Data corresponding to the linear growth phase were subjected to analysis of variance of regression in order to create growth curves for each isolate at each temperature. In both tests three plates were used as replicates. Statistical analysis was performed by SIGMAPLOT 10 program (Sigmaplott Software, USA). Colony characters were recorded after 15 days of incubation at 25°C under 12 h light/ 12 h dark cycle.

Pathogenicity tests

Representative isolates (highlighted with asterisks in Table 1) of each *C. acutatum s.l.* group isolated from strawberry in UK, together with reference isolates from other hosts, were used for pathogenicity tests on the generally susceptible strawberry cultivar Elsanta [38]. A conidial suspension was prepared for each isolates by flooding 10-day-old PDA culture plates with sterile deionised water. Spore concentration was adjusted to $10^5$ spores ml$^{-1}$ and $10^6$ spores ml$^{-1}$ for fruit and crown inoculation, respectively [7,38]. Unripe fruits (white fruit beginning to turn pink, as shown in Fig 3A) [39] were inoculated with a 5μl drop of conidial suspension. Before inoculation, fruit surfaces were disinfected for 5 min using NaClO (1% active chlorine) in 50% EtOH, washed three times in sterilized water, blotted dry and placed in a tray with moist sand on the bottom to prevent movement of the fruits during further procedures. After inoculation, fruits were incubated at 25°C under 12 h light/ 12 h in dark cycle.

Disease symptoms were evaluated 7 days after inoculation (d.a.i.) (Fig 3B) by recording the incidence of disease (% of infected fruits), and the aggressiveness of lesion development using...
the following severity scale: 0, no visible lesions; 1, lesions on less than 33% of fruit surface; 2, lesions covering 33–66% of fruit surface; and 3, lesions covering more than 66% of fruit surface. Three fruits inoculated with sterile distilled water (SDW) as well as fresh fruits served as non-inoculated controls. Four independent replicates were tested for each fungal isolate, consisting of three inoculated fruits for each replicate. At the end of the experiment, *Colletotrichum* isolates were re-isolated from infected fruits and cultured on PDA to confirm colony characteristics.

The capability of the isolates to produce crown rot symptoms was evaluated by injecting the crowns of three-months-old strawberry plants (Fig 3C) with 0.2 mL conidial suspension using a syringe [4,7]. Plants were placed in glasshouse at 23°C with 16h light / 8h darkness. After 24 days (d.a.i.), plants were evaluated for the presence of crown tissues with red-brownish discoloration, wilting and collapse of the plant, typical symptoms of *Colletotrichum* crown rot, according to the following severity scale: 0, no lesions; 1, crown tissues discoloration but no wilting or collapse; 2, wilting or collapse of part of the plant; and 3, plant death. Crowns of all plants were sectioned and examined for the presence of red-brownish lesions (Fig 3D). Crown infection was confirmed by re-isolation of the pathogen. Three plant crowns injected with SDW as well as untouched plants served as negative controls for each replicates. The experiment was independently replicated three times, with six plants for each replicate.

Values of disease severity were used to calculate a Disease Index (DI, average severity) according to the following formula: \( \Sigma v n / N \), where \( v \) represents the numeric value of the class, \( n \) is the number of plants or fruits assigned to the class, \( N \) is the total number of the plants or fruits assessed. Data for pathogenicity tests on both fruits and plants were subjected to analysis of variance ANOVA and means compared using Tukey’s multiple range test by Systat11 (Systat Software, USA).
Results

Characterization of genetic variation, and species identification

Phylogenetic trees were constructed using combined ITS, TUB2, GADPH and MAT1-2 sequence data set consisting of 148 *Colletotrichum* isolates (Table 1). As shown in Fig 1, most of the *C. acutatum* s.l. isolates (49/67) were identified as belonging to *C. nymphaeae* (= A2 genetic group), based on clustering with high bootstrap value with the reference isolates CBS 797.72, PT135, IMI345028 and other genetically similar isolates (identical sites = 1422/1438 or 98.9%; pairwise identity = 99.9%). A smaller proportion of isolates in the diversity collection (12/67) were identified as belonging to *C. godetiae* (= A4 genetic group) based on genetic clustering with reference isolates ALMNRB-30K, CBS 193.32 and JL198 (identical sites = 1411/1438 or 94.6%; pairwise identity = 99.4%). And finally, six isolates were identified as belonging to *C. fioriniae* (= A3 genetic group) based on clustering with the reference isolate ATCC 56813 (identical sites = 1436/1443 or 99.5%; pairwise identity = 99.9%).

Molecular characterisation of 67 *Colletotrichum* isolates collected from strawberry in the UK along with the reference isolates representing the host and geographic diversity (Figs 1 and 2) suggests that there have been multiple introductions of the anthracnose pathogen belonging to different *Colletotrichum* species into the country. Three different species *C. nymphaeae*, *C. godetiae* and *C. fioriniae* were identified based on sequence from four loci [6,17,18]. Incidence of these species is shown in Fig 2, where *C. nymphaeae* corresponds to 73%, followed by *C. godetiae* (18%) and *C. fioriniae* (9%). GAPDH is the locus that shows the highest variability across the nucleotide dataset, with 24.1% identical sites for the entire set of data (out-group included) and 59.3% within *C. acutatum* s.l. The MAT1-2 gene also shows a high variability with 34.4% identical sites of which 78.6% in *C. acutatum* s.l. TUB and ITS loci show lower percentage of variable sites. In detail, TUB has 58.1% of identical sites in the final alignment and 80.7% only considering *C. acutatum* s.l. While ITS has 77.8% and 92.4% of conserved nucleotides, respectively with and without out-groups. Based on the nucleotide variability referred to above, four haplotypes of *C. nymphaeae*, three haplotypes of *C. fioriniae*, and five haplotypes of *C. godetiae* were identified further highlighting the multiple introductions of the pathogens belonging to these species into the UK.

Fungal growth in plate culture

Radial growth data of *C. acutatum* s.l. and *C. gloeosporioides* s.l. isolates were subjected to analysis of variance of regression in order to obtain growth curves that were all statistically significant ($R^2 \geq 0.9447$ and $P<0.0001$), with the only exception of one isolate showing a $R^2 = 0.770$ (*C. nymphaeae* CSL224 at 30°C). The slope for each isolate (three replicates for each isolate) belonging to the same species were averaged, in order to detect the hypothetical optimal growth temperature, and results are shown in Table 2. Almost all species, particularly those containing isolates from strawberry in the UK namely *C. nymphaeae*, *C. fioriniae*, and *C. godetiae* had highest growth rates at 25°C that was considered as optimum temperature. It is pertinent to mention that higher levels of strawberry anthracnose incidence in the UK have been reported in the southwest and southeast regions, where relatively high temperatures are most often reached [20]. However, *C. phormii*, *C. kinghormii* and *C. rhombiforme* showed the highest growth rate at the temperature of 20°C and they were not able to grow at 30°C. Interestingly, these three species are evolutionarily closely related, suggesting a specific adaptation to different environmental conditions compared to other members of the same complex. With respect to *C. gloeosporioides* s.l. isolates (*C. aenigma* CSL780 and CSL 869; *C. fruticola* CSL 311 and *C. acutatum* Associated with Strawberry in the UK

PLOS ONE | DOI:10.1371/journal.pone.0129140 June 18, 2015 14 / 21
CSL386), used as out-groups, all the four isolates showed the highest growth rate at all the tested temperatures when compared with all the other isolates.

*C. nymphaeae* isolates developed white cottony aerial mycelium, light brownish conidial masses with peculiar colony colour from dark grey to dark brown. Twelve isolates belonging to *C. godetiae* were characterized by white aerial mycelium, and yellow pigmentation to white colour on the reverse side of the culture. *C. fioriniae* isolates were dark red on the reverse side of the cultures with orange conidial masses in large drops on the colony surface, and conidiomata formed directly on the hyphae. However, these characters are often difficult to describe reliably, and can change following sub-culturing or based on the length and type of storage. Thus, there is a need for further development of molecular methods for reliable and rapid diagnosis and monitoring of the pathogen populations belonging to different species associated with strawberry production in a specific geographic location.

### Characterisation of variation in pathogenicity

Thirty-four *C. acutatum* s.l. isolates were chosen for pathogenicity tests on fruits and plants, including six representative isolates from each of the three species described above related to strawberry production in the UK (highlighted with "*" in Table 1 and in bold in Fig 1), and one or more isolates representative of all the major species of the *C. acutatum* complex. Four *C. gloeosporioides* s.l. isolates that were isolated from strawberry infected tissues from UK (CSL 780 and CSL 869, *C. aenigma*) and USA (CSL 311 and CSL 386, *C. fruticola*) were included in the experiments as an out-group.

*C. acutatum* s.l. isolates varied in aggressiveness on both host tissues. In the fruit assays, among the three species identified from the strawberry production systems in the UK, *C. nymphaeae* and *C. fioriniae* were more aggressive compared to *C. godetiae*. This was particularly

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Table 2. Radial growth (mm h⁻¹) of each *Colletotrichum* species at different temperatures.

| Species                  | 15°C | 20°C | 25°C | 30°C |
|--------------------------|------|------|------|------|
| out-group                |      |      |      |      |
| *C. aenigma*             | 0.112 ± 0.001 | 0.199 ± 0.002 | 0.261 ± 0.008 | 0.124 ± 0.011 |
| *C. fruticola*           | 0.118 ± 0.004 | 0.209 ± 0.005 | 0.238 ± 0.019 | 0.150 ± 0.008 |
| *C. rhombiforme*         | 0.091 ± 0.001 | 0.135 ± 0.001 | 0.111 ± 0.002 | 0.000 ± 0.000 |
| *C. kühnhornii*          | 0.073 ± 0.001 | 0.108 ± 0.001 | 0.077 ± 0.002 | 0.000 ± 0.000 |
| *C. phormii*             | 0.106 ± 0.001 | 0.166 ± 0.001 | 0.139 ± 0.002 | 0.000 ± 0.000 |
| *C. salicis*             | 0.094 ± 0.002 | 0.142 ± 0.003 | 0.163 ± 0.005 | 0.004 ± 0.000 |
| *C. godetiae*            | 0.094 ± 0.002 | 0.147 ± 0.002 | 0.179 ± 0.004 | 0.035 ± 0.005 |
| *C. acutatum*            | 0.054 ± 0.004 | 0.087 ± 0.006 | 0.148 ± 0.004 | 0.058 ± 0.005 |
| *C. fioriniae*           | 0.081 ± 0.003 | 0.136 ± 0.005 | 0.185 ± 0.004 | 0.083 ± 0.006 |
| *Colletotrichum* sp. 2   | 0.085 ± 0.002 | 0.140 ± 0.001 | 0.178 ± 0.002 | 0.075 ± 0.002 |
| *C. lupini*              | 0.086 ± 0.001 | 0.130 ± 0.003 | 0.152 ± 0.009 | 0.058 ± 0.002 |
| *Colletotrichum* sp. 1   | 0.083 ± 0.001 | 0.132 ± 0.001 | 0.138 ± 0.001 | 0.043 ± 0.001 |
| *C. tamarilloi*          | 0.069 ± 0.001 | 0.123 ± 0.002 | 0.148 ± 0.003 | 0.007 ± 0.000 |
| *C. simmondsii*          | 0.040 ± 0.003 | 0.092 ± 0.008 | 0.112 ± 0.014 | 0.089 ± 0.011 |
| *C. laticiferum*         | 0.058 ± 0.002 | 0.113 ± 0.001 | 0.161 ± 0.001 | 0.121 ± 0.002 |
| *C. nymphaeae*           | 0.077 ± 0.001 | 0.135 ± 0.002 | 0.159 ± 0.004 | 0.063 ± 0.005 |
| *C. chrysanthemi*        | 0.050 ± 0.001 | 0.083 ± 0.001 | 0.111 ± 0.001 | 0.087 ± 0.002 |
| *C. scovillei*           | 0.036 ± 0.001 | 0.105 ± 0.001 | 0.115 ± 0.001 | 0.062 ± 0.002 |

* Values represent the average + SD of slopes (growth rates expressed as mm h⁻¹) of all isolates belonging to the same species, three replicates for each isolate. The optimal temperature for each species is indicated in bold.

doi:10.1371/journal.pone.0129140.t002
noticeable for isolates originating from strawberry as reflected by the fruit disease index range for *C. nymphaeae* (2.08–3.00), *C. fioriniae* (1.92–2.75) and *C. godetiae* (0.75–2.08). Interestingly, with isolates originating from other hosts, *C. nymphaeae* isolates were less aggressive (0.67–1.67), and one or more isolates belonging to *C. fioriniae* (2.00–2.17) as well as *C. godetiae* (2.17) showed fruit disease index in the range of the strawberry isolates. Among the other species tested within the *C. acutatum* complex, *C. acutatum* s.s., *C. simmondsii* and Colletotrichum sp.2 included one or more isolates originating from non-strawberry hosts that showed medium level of aggressiveness with fruit disease index ranging from 1.17 to 2.08. Whereas, *C. lupini* (0.08–0.75), *C. phormii* (0.58), *C. salcis* (0.17–0.67), and *C. rhombiforme* (0.67) along with *Colletotrichum* sp.1 (0.33) isolates originating from various hosts other than strawberry were much less aggressive as reflected by the fruit disease index. The *C. gloeosporioides* s.l. isolates tested showed a fruit disease index ranging from 1.50 to 2.50 (Table 3).

In the *in vitro* assays, anthracnose fruit rot symptoms were observed (e.g. Fig 3B) for various isolates tested with different levels of aggressiveness, as shown by the disease index ranging from 0.08 to 3.0 (Table 3). The variation in aggressiveness among different isolates was clearly reflected by the differences in incidence which ranged from 8.33 to 100% with only 4 out of 38 isolates showing 91.7 to 100% as well as the lesion type which ranged from 0.1 to 3.0 (S1 Table). When lesion morphology was evaluated, different kinds of lesions could be distinguished on fruits, ranging from brown ones containing orange drops of conidia to those entirely covered with aerial mycelium, with different lesion size. *C. nymphaeae* CSL899 was the most aggressive on strawberry fruits with the highest disease index (3.0, corresponding to symptoms covering more than 66% of fruit surface).

In the plant assays, varying degrees of crown rot symptoms were recorded 24 d.a.i, as reflected by the disease index range shown in Table 3. Symptom severity was generally low, with no isolate scoring higher than 2 (wilting and collapse of plant). Among the three species identified from UK strawberry production systems, *C. fioriniae* isolates originating from strawberry showed a higher range of disease index (0.72–1.00) compared to *C. nymphaeae* (0.5–0.83) and *C. godetiae* (0.39–0.67). The *C. gloeosporioides* s.l. isolate CSL 311 (*C. fruticola* from strawberry in USA) showed the highest disease index (1.6), this isolate was also amongst the most aggressive on fruit (Table 3). *Colletotrichum* isolates were recovered from all crowns showing symptoms.

**Discussion**

The UK strawberry industry has expanded rapidly in recent years, and this appears to correlate with increasing losses attributed to anthracnose caused by *Colletotrichum* spp. [6]. This study provides the first molecular characterization of *C. acutatum sensu lato* diversity related to strawberry production in the UK, combined with pathogenic characterization. A collection of 148 isolates representative of UK and global diversity of *C. acutatum* s.l. populations has been assembled. The isolates were chosen based on host association, geographic distribution, phylogenetic relationships and biological diversity.

On the basis of four sequence loci (ITS, TUB, GAPDH, and MAT1-2), the *C. acutatum sensu lato* isolates were assigned to three newly designated species *C. nymphaeae*, *C. godetiae* and *C. fioriniae* following a recent taxonomic re-assessment [18]. According to available literature, *C. nymphaeae* is the most common and *C. godetiae* is also often reported in European and American strawberry fields [6]. These two species were also the most representative in our dataset of isolates related to strawberry in the UK. *C. fioriniae* has a worldwide distribution and is common on strawberry but only a few isolates were identified in our collection, and this group was not commonly present in the fields in the UK. *C. simmondsii*, *C. acutatum sensu*...
Table 3. Variability in aggressiveness of *Colletotrichum* species isolates on strawberry fruits and plants.

| Isolate | Species | Isolation source | Origin | Fruit Disease Index* | Plant Disease Index* |
|---------|---------|------------------|--------|----------------------|----------------------|
| CSL 256 | C. nymphaeae | Fragaria | UK | 2.50 abcd | 0.50 bc |
| CSL 899 | C. nymphaeae | Fragaria | UK | 3.00 a | 0.83 abc |
| CSL 915 | C. nymphaeae | Fragaria | UK | 2.08 abcd | 0.61 bc |
| ATCC 38896 | C. nymphaeae | Nymphaeae | Netherlands | 0.67 defg | 0.28 bc |
| CSL 455 | C. nymphaeae | Photinia | UK | 1.08 bcdefg | 0.56 bc |
| PT135 | C. nymphaeae | Olea | Portugal | 1.67 abcd | 0.89 abc |
| CSL 916 | C. godetiae | Fragaria | UK | 1.92 abcd | 0.39 bc |
| CSL 918 | C. godetiae | Fragaria | UK | 0.75 cdefg | 0.39 bc |
| CSL 919 | C. godetiae | Fragaria | UK | 2.08 abcd | 0.67 bc |
| ALM-NRB-30K | C. godetiae | Prunus | Israel | 0.25 fg | 0.11 c |
| CBS 193.32 | C. godetiae | Olea | Greece | 0.75 cdefg | 0.28 bc |
| JL198 | C. godetiae | Olea | Serbia | 2.17 abcede | 0.39 bc |
| CSL 1259 | C. foriniae | Photinia | UK | 1.08 bcdefg | 0.39 bc |
| CSL 1262 | C. foriniae | Fragaria | UK | 1.92 abcddefg | 1.00 ab |
| CSL 2546 | C. foriniae | Fragaria | UK | 2.67 ab | 0.72 bc |
| CBS 797.72 | C. foriniae | Pinus | New Zealand | 1.08 bcd | 0.39 bc |
| ATCC MYA-663 | C. foriniae | Malus | USA | 2.00 abcd | 0.83 abc |
| CR46 | C. foriniae | Vitis | Portugal | 2.17 abcede | 0.33 bc |
| PJ8 | C. acutatum | Pyrus | New Zealand | 2.08 abcd | 0.72 bc |
| PT227 | C. acutatum | Olea | Portugal | 1.42 abcd | 0.78 abc |
| STE-U-164 | C. acutatum | Pinus | South Africa | 0.83 cdefg | 0.28 bc |
| CBS 12122 Colletotrichum sp. 1 | C. simmondsii | Carica | Australia | 0.25 ef | 0.22 bc |
| CBS 294.67 | C. simmondsii | Carica | Australia | 1.17 abcddefg | 0.61 bc |
| PD89-582 | C. simmondsii | Cyclamen | Netherlands | 1.83 abcd | 0.44 bc |
| BBA 70884 | C. lupini | Lupinus | Ukraine | 0.58 efg | 0.33 bc |
| HY09 | C. lupini | Lupinus | Canada | 0.08 g | 0.17 bc |
| PT30 | C. lupini | Lupinus | Portugal | 0.75 cdefg | 0.56 bc |
| 9178 | C. salis | Vaccinium | Norway | 0.50 efg | 0.28 bc |
| CBS 607.94 | C. salis | Salix | Netherlands | 0.67 defg | 0.17 bc |
| MP1 | C. salis | Acer | USA | 0.17 fg | 0.22 bc |
| CBS 101611 | Colletotrichum sp. 1 | Fern | Costa Rica | 0.33 efg | 0.06 c |
| OCO-ARC-4 | Colletotrichum sp. 2 | Citrus | USA | 1.42 abcd | 0.11 c |
| AR3787 | C. phormii | Phormium | South Africa | 0.58 efg | 0.22 bc |
| PT250 | C. thomobiforme | Olea | Portugal | 0.67 defg | 0.33 bc |
| CSL 780 | C. aenigma | Fragaria | UK | 2.50 abcd | 0.50 bc |
| CSL 869 | C. aenigma | Fragaria | UK | 1.92 abcd | 0.72 bc |
| CSL 311 | C. fruticola | Fragaria | USA | 2.50 abcd | 1.56 a |
| CSL 386 | C. fruticola | Fragaria | USA | 1.50 abcd | 0.22 bc |

Disease Index data related to aggressiveness on strawberry fruits and crowns of representative *Colletotrichum* isolates.

*: Different letters within the same column correspond to significantly different values (ANOVA; P < 0.05). The values are the averages ± SD of four independent replicates, three fruits for each replicate and of three independent replicates, six plants for each replicate. Disease Index was calculated according to the following formula: Σvn/N, where v represents the numeric value of the class, n is the number of fruits or plants assigned to the class, N is the total number of the plants assessed.

+: 0, no visible lesions; 1, lesions on less than 33% of fruit surface; 2, lesions covering 33–66% of fruit surface; and 3, lesions covering more than 66% of fruit surface.

#: 0, no lesions; 1, crown tissues discoloration but no wilting or collapse; 2, wilting or collapse of part of the plant; and 3, plant death.

doi:10.1371/journal.pone.0129140.t003
stricto, C. salicis and C. miyabeana are common on strawberry in Oceania and have only been found sporadically in Europe. Isolates belonging to these species have not been detected on strawberry in the UK. The variability observed within the UK C. acutatum sensu lato species fits in part with previous reports of C. acutatum on strawberry within specific geographic regions. For example, in France, Israel, Bulgaria and Spain, the majority of strawberry anthracnose pathogen isolates clustered in the same species C. nymphaeae, and almost no intra-specific diversity was observed within each country [2–5]. A different situation has been observed on Belgian isolates, where the population represented: 33% isolates belonging to C. nymphaeae, 5% C. fioriniae, 50% C. godetiae, 3% C. acutatum s.s. and 6% C. salicis. A possible explanation to C. acutatum s.l. status in the UK might be recent introduction (late 70s) from a limited number of sources. The reason for the differences in the occurrence of various Colletotrichum species associated with strawberry production in different geographic locations still remains unclear, but the source of importation of the planting material and local trade have been heavily implicated [4,7].

The pathogenicity assays used in this work are based on a study in Belgium [7] in view of the similar molecular diversity of the anthracnose pathogen populations associated with strawberry production. These assays with the isolates representing the molecular diversity not only revealed variability in aggressiveness in different species described within C. acutatum s.l., but also complex patterns both between and within the species. For example, based on isolates originating from strawberry, C. fioriniae and C. nymphaeae appear equally aggressive on fruits with C. nymphaeae isolates indicating a degree of host-preference. Both C. fioriniae and C. godetiae included isolates originating from other hosts that showed comparable levels of aggressiveness to isolates from strawberry. Similar situation was observed with at least some non-strawberry isolates belonging to species such as C. acutatum s.s. and C. simmondsii. Furthermore, at least one C. godetiae isolate from strawberry was much less aggressive compared to others. These patterns suggest that some Colletotrichum species such as C. fioriniae and C. godetiae include populations that are capable of infecting a wider range of hosts, also influenced by environmental conditions. Further studies using a wider set of isolates of these three species and appropriate pathological and biological assays are required to gain additional insights into the evolution of pathogenicity in relation to field symptoms as well as any differential responses to host varieties and fungicides locally used in the UK strawberry production systems.

The study has highlighted the genetic and pathogenic heterogeneity of the introduced anthracnose pathogen populations belonging to three different Colletotrichum species emphasising the need for effective phytosanitary procedures linked to pathogen monitoring and characterisation to generally limit the entry of non-native pathogens. This also underlines the requirement of reliable and rapid diagnostic tools for further research and application in strawberry anthracnose management. The recent release of a whole genome sequence of C. fioriniae isolated from strawberry [28] along with the newly characterised isolates, based on multi-locus sequence and aggressiveness information reported here, represents a useful platform for further research into the genetic basis of C. acutatum s.l.—strawberry interactions.

Supporting Information

S1 Table. Variability in aggressiveness of Colletotrichum species isolates on strawberry fruits and plants. a 0, no visible lesions; 1, lesions on less than 33% of fruit surface; 2, lesions covering 33–66% of fruit surface; and 3, lesions covering more than 66% of fruit surface. b no lesions; 1, crown tissues discoloration but no wilting or collapse; 2, wilting or collapse of part of the plant; and 3, plant death.

(XLSX)
Acknowledgments

The authors would like to dedicate this work to Maurizio Forti (University of Pisa), who passed away in December 2013 and to Dez Barbara (University of Warwick) who passed away in July 2012. The authors would like to thank Fera and the University of Warwick for funding this research and providing the strains set. They are especially thankful to: Ulrike Damm (CBS-KNAW Fungal Biodiversity Centre–The Netherlands), Paul Cannon and Alan Buddie (CABI—UK), Gunn Mari Stømeng (Norwegian University of Life Sciences—Norway), Katherine LoBuglio (Harvard University Herbaria, USA), Peter R. Johnston (Manaaki Whenua Landcare Research—New Zealand), James Cunnington (Institute for Horticultural Development—Australia), Amy Rossman (USDA-ARS–USA), Stanley Freeman (ARO Volcani Center—Israel), Daniel Buchvaldt Amby (University of Copenhagen–Denmark), Natalia Peres (University of Florida–USA) and Sheu Zong-ming (AVRDC–The World Vegetable Center–Taiwan) for kindly providing reference isolates.

Author Contributions

Conceived and designed the experiments: RB S. Sreenivasaprasad. Performed the experiments: RB AZ S. Sarrocco. Analyzed the data: RB S. Sarrocco GV SAS MRT EH. Contributed reagents/materials/analysis tools: GV CRL S. Sreenivasaprasad. Wrote the paper: RB AZ S. Sarrocco SAS MRT GV EH S. Sreenivasaprasad.

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