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Multiplexed SSR marker analysis of *Diplocarpon coronariae* reveals clonality within samples from Middle Europe and genetic distance from Asian and North American isolates

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Abstract

Background: Apple blotch (AB) caused by *Diplocarpon coronariae* (Dc) has been established in Europe since 2010. AB is a serious apple disease, mostly in low input orchards and in cider production areas in Northern Italy, Switzerland, Austria and Germany. However, the epidemiology and population genetic structure of this pathogen is unknown.

Methods: We developed twelve Dc-specific microsatellite markers and screened DNA of both pure fungal isolates and infected apple leaves. The marker data of 313 European samples of Dc were compared to Dc isolates from Asia (n = 7) and the USA (n = 3).

Results: We found 31 distinct multilocus genotypes (MLGs) in European samples, and seven additional MLGs in the Asian and USA samples. The European samples had the typical genetic signature of a recently introduced species including high clonality, a low number of private alleles and one dominant MLG across all the sampling sites. All European MLGs were genetically distant from those MLGs of Asian and USA origin. Based on the lack of linkage disequilibrium observed, there is evidence that Dc undergoes regular cycles of sexual recombination in the European population, although the sexual stage (apothecia) has not been observed in Europe.

Conclusions: The twelve newly developed SSR markers reported here provide a useful tool to characterize the population genetic diversity and structure of Dc in Europe. Our study supports the hypothesis that Dc is a recently introduced pathogen in Europe, but of currently unknown origin. Dc has a large effective population size during field epidemics, so we believe that the pathogen has substantial evolutionary potential. Application of the SSR markers to large-scale and diverse Dc samples will help to better understand the epidemiology of AB, which has become a global apple disease, and will help guide effective mitigation strategies based on disease management and resistance breeding.

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Background

The fungal pathogen *Diplocarpon coronariae* (Dc), (Ellis & Davis) Wöhner & Rossman (Crous et al. 2020), formerly *Marssonina coronaria* (Ellis & Davis) Davis; teleomorph *Diplocarpon mali* (Harada & Sawamura) causes apple blotch (AB), with typical symptoms being a chlorotic leaf blotch accompanied by premature defoliation and black spotting on fruit (Wöhner and Emeriewen 2019). The pathogen was first described in 1903 in the USA (Davis 1903) and shortly after, in 1907 in Japan (Harada et al. 1974). A further report of AB was made in Korea (Kwang and Chong 1962), and Parmelee (1971) determined Dc in apple leaf samples collected in Canada and in herbarium specimens obtained from Romania. A report from Brazil (Leite Jr et al. 1986) and the literature cited therein indicated that AB had been a global threat to the health of apple trees for the previous 60 years, particularly in sub-tropical production regions. But it was not until the 1990s that Dc became a significant problem to apple production in China (Li et al. 2012), Korea (Lee et al. 1993), and India (Sharma 1999, Sharma et al. 2004). In Europe, Dc first emerged in 2001 in Italy in the Piedmont region (Tamietti and Matta 2003). Since 2013, reports of Dc outbreaks in Middle Europe have increased, affecting cider-production or low-input and organic orchards where scab-resistant cultivars are generally cultivated (Hinrichs-Berger and Muller 2013, Naef et al. 2013, Wöhner and Emeriewen 2019). More recently, Dc outbreaks have been reported in the Eastern USA (Aćimović and Donahue 2018, Villani 2018). These are the first reports of epidemics of AB in more than 100 years, since the disease was first described in the USA (Davis 1903).

China produces more than 45% of the global apple crop and there has been a tenfold increase in production during the last three decades (www.fao.org). However, annual yield loss due to Dc in China can be as high as 28% (Li et al 2012). The hypotheses as to why this pathogen has become so prevalent during the last two to three decades include (i) climate change, with warmer, moister weather conditions during summer favoring the spread of Dc into more temperate climate zones (Sharma et al. 2004), and (ii) the increased establishment of scab-resistant apple cultivars which led to a reduction in fungicide applications (Naef et al. 2013). Reduction in the use of fungicides may have allowed the resurgence of other minor apple pathogens including *Elsinoe pyri*, and *Alternaria alternata* that were previously controlled by the fungicides used against scab (Li et al 2012, Naef et al. 2013, Korsgaard et al. 2014). Possibly one of the two hypotheses, or a combination, allowed Dc to establish and spread rapidly in Europe. It is not clear, however, whether epidemics of AB are caused by the recent introduction of Dc from endemic regions (e.g. Asia), or the recent outbreaks are originating from an indigenous, so far latent European population of the pathogen.

Knowledge and understanding of the genetic variability of Dc has been limited, and thus far based solely on comparison of sequences of the Internal Transcribed Spacer (ITS) region of isolates from Korea and China (Lee et al. 2011). Production of apothecia and ascospores by Dc was reported in Japan and China (Gao et al. 2011), and it is thought that early infections are facilitated by ascospores from apothecia that are produced on overwintering leaf litter and are released the following spring. However, much of the life cycle, including overwintering and frequency of sexual reproduction, are still poorly defined (Wöhner and Emeriewen 2019). Sustainable control strategies cannot be developed without a basic understanding of the life cycle, epidemiological requirements and population genetic characteristics of the pathogen. For example, a deeper knowledge of the population genetics of *Venturia inaequalis* has provided helpful insights into the durability of apple resistance genes for apple scab management strategies (Guérin et al. 2007, Patocchi et al. 2020).

Simple sequence repeats (SSR) markers have been shown to be a powerful tool for population genetic studies of plant pathogens, including providing information on population divergence and gene flow (Jänsch et al. 2012, Gau et al. 2013, Grünwald et al. 2017). Knowledge of the population genetics can guide the development of new management strategies for Dc including breeding of AB resistant or tolerant apple cultivars. The aim of this study was (i) to develop Dc-specific (SSR) markers, and (ii) apply the SSR markers to determine the population genetic diversity and structure of Dc in Europe.

Materials and methods

Sampling and fungal isolation

To determine the genetic diversity of Dc in Europe, in 2016, 2017 and 2018 we collected representative Dc-infected apple leaves from a total of 34 sites across the apple production areas in Slovenia, Italy, Switzerland and Germany (Additional file 1: Table S1) by sampling 1–10 leaves from different trees at each site (Fig. 1,

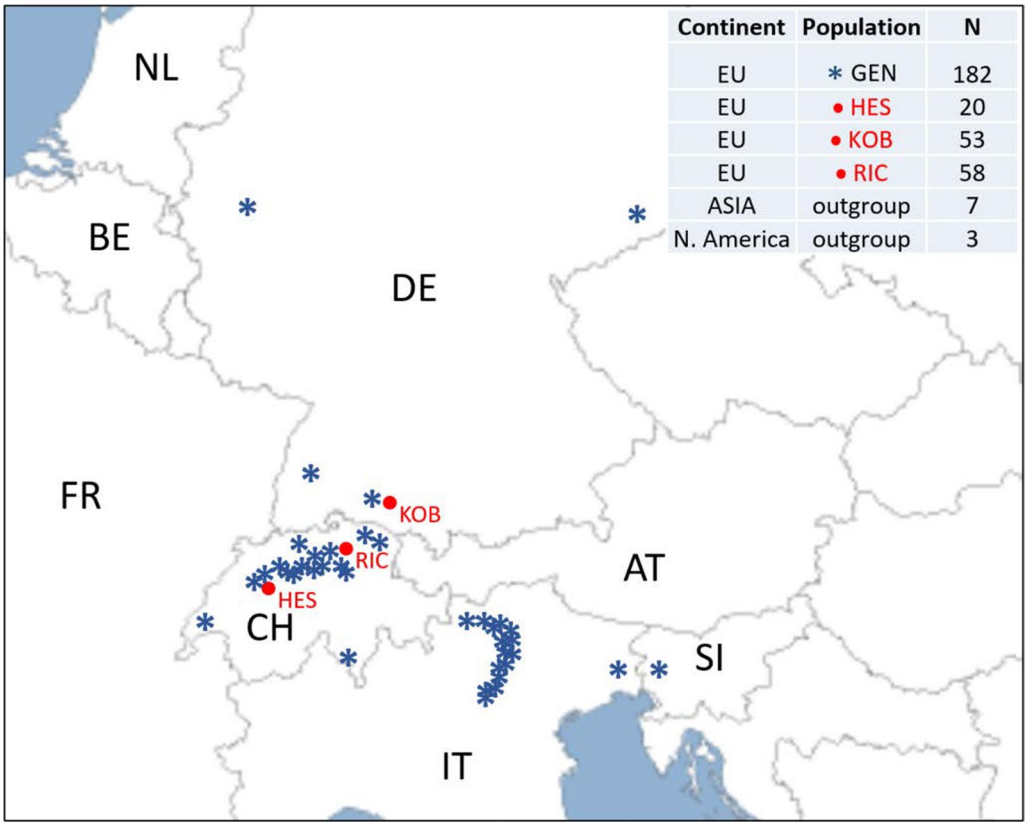

Fig. 1 Sample locations of populations of *Diplocarpon coronariae* (Dc) from 34 apple orchards in Europe (EU) indicated with a blue asterisk (n = 1–10 per site, total n = 182); the three more intensively sampled apple orchards are indicated with a red bullet (n ≥ 20 per orchard, EU_HES, EU_KOB and EU_RIC). A total of 313 European isolates of Dc were collected from the populations in Switzerland (CH), Germany (DE), Italy (IT) and Slovenia (SI) with an additional 10 isolates from Asia (China, Korea, Japan) and from North America (New York, USA) as outgroups as indicated in the legend

"EU_GEN"). The samples from two locations in Switzerland [CH-Au, (n = 13) and CH-Wädenswil (n = 27)] were collected in four and five orchards, respectively, with a distance of 300 to 3000 m between orchards. When more than one sample per orchard was collected, the samples were taken from trees at least 15 to 20 m distant from each other.

In order to determine the genetic variability within specific sites, we sampled three orchards more intensively. The intensively sampled orchards included an apple breeding block in Hessigkofen, Switzerland ("EU_HES"; https://pomaculta.org), a commercial cider production block in Rickenbach, Switzerland ("EU_RIC"; https://www.bioagrikultur.bio), and a pome fruit research station block in the Lake of Constance Region of Germany ("EU_KOB"; http://www.kob-bavendorf.de), collecting 20–58 leaves per orchard sampled from different trees across the blocks (Fig. 1).

In addition, we included 24 single spore cultures obtained from Dc lesions on freshly collected leaves and fruit (Additional file 1: Table S1). For isolation, acervuli on leaves were soaked in 20 µl of sterile water for 1 min. The surface of the acervuli was gently scraped with the tip of a pipette in order to release the spores, and the spore suspension taken up using a pipette, and spread on 1.8% (w/v) water agar. Agar blocks containing single spores that germinated after incubation for 18 h in the dark at room temperature (21–25 °C) were cut out using a needle under a stereo microscope at a magnification of 40–70× and transferred onto peptone potato dextrose agar (PPDA) (Lee et al. 2011) amended with 25 µg ml^{-1} each of chloramphenicol and tetracycline hydrochloride. AB-diseased apple fruit were disinfected with 70% (v/v) ethanol and rinsed with distilled water. Small pieces at the border of healthy and diseased parenchyma tissue were cut out and transferred onto PPDA and isolations made from single germinated propagules following the procedures described for leaf isolations.

To compare the genetic structure of European Dc isolates with isolates from regions with a longer history of

AB, we obtained DNA samples of four, two and three Dc isolates from Korea (Kyungpook National University and GBARES, Uiseong), China (Northwest A&F University, Yangling, Shaanxi), and the USA (Cornell University, NY) (Additional file 1: Table S1), respectively, as well as one Japanese isolate of Dc (NBRC 30405) obtained from the National Biology Resource Center (Shibuya-ku, Tokyo, Japan, http://www.nite.go.jp). Finally, for testing the specificity of the SSR markers, species closely related to Dc, notably *D. mespili* (CBS166.25), *D. rosae* (CBS163.1) and *D. earlianum* (CBS 162.32), were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands).

DNA extraction

Approximately 20 mg of leaf tissue (Additional file 1: Table S1) containing a single lesion were placed in a 2 ml tube with 15 to 20 glass beads (2 mm diameter). The samples were lyophilized in a freeze-dryer (Alpha 1–2, Martin Christ, Germany) and ground to a powder for 60 s in a bead beater (MP FastPrep-24 at 4.0 ms^{-1} MP Biomedicals, Santa Ana, CA, USA). The DNA was extracted using a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). Genomic DNA of 2-to-4-week-old fungal cultures grown on PPDA was extracted from approximately 50 mg of fresh mycelium and sclerotia-like structures using a Quick-DNA™ Fungal/Bacterial MiniPrep Kit (Zymo Research Corp, USA) (Additional file 1: Table S1).

Development of microsatellite markers

Genomic DNA of a 6-week-old culture of the Swiss isolate Dc CH01_Fibl_refDc was extracted as described above. The mycelium was ground in liquid N_2 using a mortar and pestle to avoid shearing of the genomic DNA. DNA yield was quantified using a Nanodrop (ThermoFisher, Waltham, MA, USA) and the integrity of DNA was verified on a 0.8% agarose gel. Five µg of genomic DNA was sent to Ecogenics GmbH (Balgach, Switzerland) and size-selected fragments of 500 to 2000 bp were enriched for SSR content by using magnetic streptavidin beads and biotin-labeled AGG-, GGT-, GATA- and GTAT-repeat oligonucleotides. The SSR-enriched library was sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the Nano 2 × 250 v2 format. Raw data were processed by Ecogenics GmbH, assembled in contigs and unique sequences selected. Primary selection criteria for the sequences were a microsatellite insert of at least six repeat units and flanking regions suitable for primer design.

Evaluation of the SSR markers and genotyping of field samples

Primers flanking the microsatellite motifs of 70 sequences were designed with a Tm of 60 °C using the tool Primer3 (https://sourceforge.net/projects/primer3). All primer pairs were screened for Dc specificity, i.e. reaction with Dc but absence of reaction with closely related fungal species, for length polymorphism between Swiss and Chinese Dc isolates, and for absence of reaction with DNA from healthy apple leaf tissue. To achieve this, each primer pair was assessed in a SYBR qPCR in a total volume of 10 µl containing 5 µl of KAPA SYBR Fast 2× (KK4618, Sigma-Aldrich, St. Louis, MO, USA), 3 µl of water, 1 µl of primer mix (containing forward and reverse primer, both pre-diluted in 1 mM Tris (pH 8.5) containing 0.01 mM Na_2EDTA at 2 µM each) and 1 µl of DNA extract. The PCR was performed in a 72-well rotor of a Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany). PCR conditions were 3 min at 95 °C for initial denaturation and enzyme activation, followed by 40 cycles with 5 s at 95 °C and 20 s at 60 °C, recording the fluorescence after each elongation. After cycling, dissociation of the amplification products was assessed by increasing the temperature from 55 to 90 °C. PCR products were also run on a 2% agarose gel in TAE containing ROTI®GelStain (3 µl/100 ml, Art. no. 3865.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for 1 h at 100 V. Size of amplicons was estimated with a 50 bp DNA ladder (Art. no. 2810.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

Twelve SSR markers were selected, and the forward primers labelled at the 5′ end either with FAM or HEX dyes (Table 1). SSR markers amplifying alleles of different size ranges were combined in four different multiplex PCR assays (three markers per multiplex assay). For the SSR fragment analysis, 1 µl of DNA extract was amplified in a total volume of 10 µl using 5 µl of Multiplex PCR Master Mix, 1 µl of Q-solution (Qiagen Multiplex PCR Kit) and 3 µl of a primer mix containing three primer pairs to give a final concentration of each primer of 0.1 to 0.3 µM. PCR cycling conditions were 15 min at 95 °C for initial denaturation and enzyme activation, followed by 35 cycles with 30 s at 95 °C, 60 s at 60 °C and 30 s at 72 °C, and a final elongation step for 10 min at 72 °C. Fragment analysis was performed on a 48-capillary ABI 3730xl DNA Analyzer, or on a 16-capillary ABI Prism 3130xl Genetic Analyzer (both ThermoFisher) at the Genetic Diversity Centre, ETH, Zurich, and Agroscope, Wädenswil (both in Switzerland). Each sample was made up with 10 µl of Hi-Di Formamide containing 0.1 µl of GeneScan 500 LIZ dye Size Standard (both Applied Biosystems) and 1 µl of a 75-fold diluted PCR product. The samples were denatured for 5 min at 95 °C and rapidly cooled before

Table 1 The SSR markers and primer sequences used to determine multilocus genotypes in *Diplocarpon coronariae* (Dc)

Marker and multiplex group	Accession no.[a]	Repeat motive	Amplicon size range[b]	No. of alleles[b]	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Combination 1						
Dc06	MT036090	AGT	168–216	5	FAM[c]-ACGGCTGGGAATAGATCGTC	GCGTGGCTCTGACAAGATTAC
Dc36	MT036095	TATG	227–387	4	HEX[d]-ACTCGCAGACGGAGAATACG	GGTTTCAAAGACCGGGTTGG
Dc59	MT036097	AGT	239–365	13	FAM[c]-ACGCCGTCCAATTGCTATCT	TCACAGGGAGGTTCCAGGAA
Combination 2						
Dc15	MT036092	AG	147–171	3	FAM-TAGACCTGTTCCGCCATCTG	AAACAGCTTGTCCGCGAAAC
Dc60	MT036098	CATA	238–318	8	HEX-GCAGTTGGAGGTCAGCTTGT	GCAGTTGGAGGTCAGCTTGT
Dc68	MT036100	ACT	327–472	8	FAM-GCCGGCCATGGGTCTATATC	AAGATCTGGCGGGTGATTGG
Combination 3						
Dc19	MT036093	AAG	117–159	5	FAM-GATTCGCAGCCCACTTTGAG	CGGTCAGATCCCACTCTTCC
Dc32	MT036094	TC/CA	209–385	8	HEX-AGCACGTCGTATCCTCTGAC	CAAGCGTTTTCATCTTGGGC
Dc62	MT036099	ATAC	204–372	9	FAM-GGAGTCGCGGTATGTGTGTA	TCCTGGCGAGTTGAAGTTGT
Combination 4						
Dc48	MT036096	GA	116–152	3	FAM-AACAATCGCCCGTACAAGTC	TGGGAAACGTACAGGATGGG
Dc09	MT036091	TCT	168–242	7	HEX-GCAATACGAGATGCTCGCTC	CGGGGTTTGCGATATCTGTG
Dc70	MT036101	GTAT	253–301	6	FAM-TTCCAGACCCGATAAGCTGC	CGAAGTATCCCGGAGAGCAC

The twelve markers were combined in four groups of three markers each for multiplexed PCR, each labelled with two different fluorescent dyes at the 5′end of the forward primers

[a] Accession no. refers to the original sequence the markers were designed from

[b] Number of alleles and amplicon size range is indicated for all samples tested (n = 323)

[c] FAM (6-carboxyfluorescein) was used as fluorescent dye for the short and long amplicons in each marker combination

[d] HEX (6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein) was employed as fluorescent dye for the fragment of intermediate size

loading on the sequencer. Amplicon lengths were scored from electropherograms with *Geneious* version 9.18 (Biomatters Ltd., Auckland, New Zealand) or *GeneMapper*™ version 5 (ThermoFisher, Waltham, MA, USA). Each PCR run and fragment length analysis contained at least two samples of the same Swiss Dc DNA extract. These samples were used for the normalization of slightly differing lengths of SSR alleles between the runs and the two laboratories where the genotyping was performed. Further, a panel of 14 DNA extracts from the one and 13 DNA extracts from the other laboratory representing different MLGs were tested at both sites to align possible shifts of SSR alleles among the laboratories.

Marker statistics and population genetic analysis

Samples with complete marker datasets for all the twelve loci were compiled in Excel (Additional file 1: Table S1) and an output file was generated with *GenAlEx* version 6.503 (Peakall and Smouse 2006). All statistical analyses were performed in *R* version 3.6.1 (R Core Team 2019). Marker statistics and population genetic diversity analysis were calculated using *poppr* version 2.8.6 (Kamvar et al. 2014), unless otherwise specified.

For all loci we estimated the overall number of alleles, expected heterozygosity (H_{exp}) (Nei 1978), the number of effective alleles ($A_E = 1/(1 - H_{exp})$), and genotypic evenness (Grünwald et al. 2003). We also calculated the number of private alleles in both, the European (n = 313) and worldwide samples (n = 323). Genotypic accumulation curves were calculated based on resampling (1000 permutations) to assess the suitability of the loci to discriminate between the different Dc isolates. The polymorphic information content (PIC) was calculated for each locus using the R package *polysat* (Clark and Jasieniuk 2011).

For population analyses, the observed number of multilocus genotypes (MLGs) and their frequency per population was calculated. We calculated the observed and expected numbers of MLGs, the latter at the smallest sample size of n > 10 based on rarefaction, as well as the clonal fraction, for the assessment of the genetic richness.

The genetic diversity within populations was estimated using the Shannon–Wiener Index (H) (Shannon 1948), the Stoddart and Taylor's Index (G) of MLG diversity (Stoddart and Taylor 1988), the Simpson's Index (λ) (Simpson 1949), and Nei's unbiased genotypic diversity (H_{exp}) (Nei 1978). The genotypic evenness was assessed using the E_5 index (Grünwald et al. 2003). To determine

linkage disequilibrium, the index of association within populations (I_A) and the standardized index of association ($\bar{r}_d$) and their probabilities based on resampling (1000 permutations) were calculated for both all and clone corrected data. The fixation index (F_{ST}) was calculated to determine possible population differentiation (Pembleton et al. 2013).

To further determine genetic structure, genetic relatedness among populations was explored based on Bruvo's genetic distance (Bruvo et al. 2004) using the *bruvo.boot* function in *poppr* on clone corrected data, taking into account the allelic constitution at each SSR locus. Using these data, a phylogenetic dendrogram based on the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was prepared with 1000 bootstrap replicates and a node label cut-off value of 70. In addition, a minimum spanning network was calculated for all data to visualize relationships among individuals using Bruvo's genetic distance for all MLGs. A Discriminant Analysis of Principal Components (DAPC) was also calculated to define the genetic clusters among the 323 Dc isolates using the R package *adegenet* (Jombart 2008). Subsequently, DAPC was performed for the European isolates alone. A stratified cross-validation as implemented in *adegenet* was used to determine the number of principal components to retain in the analysis. Genetic clusters were visually assessed by plotting the isolates according to the first two linear discriminant functions. Finally, DAPC-based population membership probabilities were calculated for each isolate.

Results

SSR marker development and genotyping

The Illumina MiSeq sequences of the Dc isolate from Switzerland were assembled from 1132 contigs or singletons containing a microsatellite insert with a tetra- or a trinucleotide of at least six repeat units or a dinucleotide of at least ten repeat units. Primer design flanking the SSR insert was possible in 949 of the microsatellites and was performed for 70 sequences with different repeat motifs of different length. Finally, twelve SSR markers were selected (Table 1). The SSR markers were screened against DNA of Dc from isolates from Switzerland and China and produced amplicons of a range of sizes in agarose gels, with no cross-reaction in SYBR qPCR with the healthy apple leaf tissue DNA, or with related species including *D. rosae*, *D. mespili* and *D. earlianum* (data not shown). The alignment of the twelve SSR markers with the contigs of the draft whole genome sequence of the Chinese Dc isolate NL1 (Cheng 2017) revealed that all but two (Dc60 and Dc70) were on different contigs (Additional file 2: Fig. S1). The two markers on the same contig were 3168 bases from each other (Additional file 2: Fig. S1).

A total of 330 DNA samples were obtained directly from Dc-infected apple leaf samples from Europe, and a further 33 DNA samples were extracted from single spore cultures—all were screened against the twelve SSR markers. A single allele was amplified per SSR marker with 288 (87.2%) of the directly extracted European AB DNA leaf samples. The remaining 42 samples either failed to amplify with all twelve markers or produced more than one allele with an SSR marker, indicating a mixed infection, and were thus excluded from further analysis. The DNA from the 25 European, the two Chinese and the reference Japanese single spore isolates produced only a single allele per locus, and amplified with all twelve SSRs (Additional file 1: Table S1). In contrast, the SSR markers Dc09, Dc15, Dc36 and Dc62 did not produce amplicons with DNA of the Korean and USA fungal isolates. Thus, a "null" allele was assigned to these sample-SSR marker combinations (Table 2, Additional file 1: Table S1).

Finally, a total of 313 European samples originating from Middle Europe and the Northern Mediterranean Region were included in the population genetic analysis and compared with seven samples originating from Asia and three samples from the USA as outgroups, making a grand total of 323 samples (Fig. 1). All isolates and their corresponding marker allele data is provided in Additional file 1: Table S1.

Locus-based statistics

All twelve SSR markers were polymorphic with allele numbers ranging from three (Dc15 and Dc48) to 13 (Dc59) (Table 2). Overall, 79 alleles were identified with the twelve SSR markers based on the analysis of all 323 samples (Table 2). The values of the expected heterozygosity (H_{exp}) were close to zero, reflected in the fact there were only a small number of effective alleles that dominated at each locus (Evenness values of approximately 0.3), with the exception of marker Dc70, which had an Evenness value of 0.5 indicating more equally abundant alleles at this locus (Table 2).

There were some notable differences in alleles between the European samples, and the Asian and USA samples: four markers (Dc06, Dc15, Dc19, Dc48) were represented by only one allele among the European samples (Table 2). The Asian and USA samples differed from European samples in having a greater number of private alleles (Table 2), with some markers having unique ranges of amplicon size in the population (e.g. Dc59, Table 2). In accordance with the number of alleles, the PIC values for the four markers (Dc06, Dc15, Dc19, Dc48) were zero for all European populations

Table 2 The number of alleles and private alleles, and the corresponding fragment lengths of the 313 European, the ten Asian and USA isolates obtained using the twelve SSR markers developed for *Diplocarpon coronariae* (Dc)

Locus	Europe (n = 313)		Asia + USA (n = 10)		All samples (n = 323)			
	No alleles (fragment lengths)	No private alleles	No alleles (fragment lengths)	No private alleles	No alleles	H_{exp} [a]	A_E [b]	Evenness [c]
Dc06	1 (168)	1	4 (186/204/213/216)	4	5	0.061	1.06	0.336
Dc36	2 (227/*231*)	1	3 (0*, *231*/239/387)	2	4	0.043	1.04	0.328
Dc59	7 (314/341/344/347/350/362/365)	7	6 (239/269/290/293/299/308)	6	13	0.176	1.21	0.326
Dc15	1 (*147*)	0	3 (0*/*147*/149/171)	2	3	0.031	1.03	0.334
Dc60	3 (282/286/290)	3	5 (238/242/258/278/318)	5	8	0.125	1.14	0.336
Dc68	4 (334/339/352/355)	4	4 (328/361/367/472)	4	8	0.130	1.15	0.350
Dc19	1 (*123*)	0	5 (117/*123*/129/150/159)	4	5	0.055	1.06	0.322
Dc32	4 (*209*/217/219/225)	3	5 (*209*/221/377/381/385)	4	8	0.079	1.09	0.310
Dc62	6 (312/336/340/344/348/372)	5	3 (0*/204/240/340)	3	9	0.091	1.10	0.311
Dc48	1 (*120*)	0	3 (116/*120*/156)	2	3	0.012	1.01	0.291
Dc09	4 (224/230/233/242)	4	3 (0*/168/208/212)	3	7	0.127	1.15	0.355
Dc70	4 (265/293/297/301)	4	2 (253/269)	2	6	0.310	1.45	0.507
Mean	3.2	2.7	3.8	3.4	6.6	0.104	1.12	0.342
Overall	38	32	46	41	79			

Locus genetic diversity characteristics are indicated for all samples combined. Shared alleles between the two groups are marked in italic. Absence of amplified marker products of Korean and USA isolates with some markers are indicated with 0*

[a] H_{exp}: expected Heterozygosity [Nei's gene diversity, (Nei 1978)]

[b] A_E: number of effective alleles ($A_E = 1/(1 - H_{exp})$)

[c] Evenness: evenness is a measure of the distribution of allele abundances, wherein a locus with equally abundant alleles yields a value equal to 1 and a locus dominated by a single allele is closer to zero

Table 3 The polymorphic information content (PIC) of 12 SSR markers developed for the population genetic study on *Diplocarpon coronariae* (Dc) isolates infecting apple in Europe, Asia and the USA (EU_GEN, EU_HES, EU_RIC, EU_KOB, ASIA_AS and USA_US, respectively)

Region/population	SSR marker											
	Dc06	Dc36	Dc59	Dc15	Dc60	Dc68	Dc19	Dc32	Dc62	Dc48	Dc09	Dc70
EU_GEN	0.00	*0.01*	*0.09*	0.00	*0.05*	*0.09*	0.00	*0.02*	*0.08*	0.00	*0.15*	*0.10*
EU_HES	0.00	0.00	*0.27*	0.00	*0.09*	0.00	0.00	0.00	0.00	0.00	0.00	0.00
EU_RIC	0.00	0.00	*0.03*	0.00	0.00	*0.03*	0.00	0.00	0.00	0.00	*0.03*	*0.41*
EU_KOB	0.00	0.00	*0.19*	0.00	*0.17*	*0.07*	0.00	*0.04*	*0.07*	0.00	*0.04*	*0.04*
ASIA_AS	*0.68*	*0.35*	*0.74*	*0.36*	*0.68*	*0.64*	*0.68*	*0.64*	*0.67*	*0.41*	*0.55*	*0.21*
USA_US	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Overall	*0.05*	*0.05*	*0.17*	*0.02*	*0.12*	*0.12*	*0.04*	*0.07*	*0.10*	*0.01*	*0.15*	*0.27*

PIC (polymorphic information content) measures the ability of a marker to detect polymorphisms among individuals of a population, and the higher that capacity the greater its value

(Table 3), but the remaining eight markers had a PIC value > 0 for EU_GEN. Markers Dc59 and Dc70 were > 0 for three and four of the European populations, respectively. For the Asian outgroup, the allelic informativeness and PIC values for all markers were higher than those of European samples, except for marker Dc70 (Table 3). Within the USA samples no allelic polymorphism was recorded (all USA isolates were clones of the same MLG).

Genetic diversity among populations

A total of 38 MLGs were identified, whereby 31 MLGs were assigned to the 313 European samples, six MLGs to the seven Asian samples, and one MLG to the three US

Table 4 Population genetic characteristics for populations of *Diplocarpon coronariae* on apple from Europe (EU), Asia and the USA

Region/population	N[a]	MLG[b]	CF[c]	eMLG ± SE[d]	H[e]	G[f]	λ[g]	H_{exp}[h]	E_5[i]	I_A[j]	$\bar{r}_d$[k]	Clone corrected[l]	
												I_A[j]	$\bar{r}_d$[k]
EU_GEN	182	23	0.87	3.51 ± 1.25	1.333	1.85	0.460	0.052	0.305	0.110$^{\cdot}$	0.018$^{\cdot}$	−0.520^{ns}	−0.078^{ns}
EU_HES	20	3	0.85	2.46 ± 0.53	0.687	1.65	0.395	0.036	0.660	−0.081^{ns}	−0.089^{ns}	−0.500^{ns}	−0.500^{ns}
EU_RIC	58	7	0.88	2.85 ± 0.82	1.038	2.25	0.556	0.051	0.686	−0.026^{ns}	−0.012^{ns}	−0.462^{ns}	−0.155^{ns}
EU_KOB	53	10	0.81	3.45 ± 1.15	1.134	1.82	0.449	0.054	0.387	0.169^{ns}	0.032^{ns}	−0.641^{ns}	−0.108^{ns}
EU total/overall	313	31	0.90	5.95 ± 1.60	1.537	2.20	0.546	0.060	0.329	−0.004^{ns}	−0.001^{ns}	−0.479^{ns}	−0.072^{ns}
ASIA_AS	7	6	0.14	n.a.[m]	1.748	5.44	0.816	0.739	0.937	2.828***	0.265***	2.332***	0.221***
USA_US	3	1	0.67	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]	n.a.[m]
Total/overall	323	38	0.88	4.06 ± 1.29	1.684	2.34	0.573	0.104	0.306	3.516***	0.359***	3.616***	0.339***

[a] N: number of isolates/samples

[b] MLG: number of multilocus genotypes

[c] CF: clonal fraction (1-(MLG/N))

[d] eMLG: expected number of MLG at the smallest sampling size > 10 based on rarefaction (± standard error on eMLG)

[e] H: Shannon–Wiener Index of MLG diversity (diversity in function of their relative abundance) (Shannon 1948)

[f] G: Stoddart and Taylor's Index of MLG diversity (Stoddart and Taylor 1988)

[g] λ: Simpson's Index (Simpson 1949)

[h] Hexp: Nei's gene diversity (expected heterozygosity) (Nei 1978)

[i] E5: Evenness (Grünwald et al. 2003)

[j] I_A: Index of association (Brown et al. 1980) with associated significance level ($^{ns}P \geq 0.1$; $^{\cdot}P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

[k] $\bar{r}d$: standardized index of association (Agapow and Burt 2001), with associated significance level (see I_A)

[l] Clone corrected: indices of association calculated for clone corrected data where the dataset is censored such that only one individual per MLG is represented per population

[m] n.a.: not applicable

samples (Table 4). The clonal fraction was visibly higher for European than for Asian samples. Genotype accumulation curves indicated that eleven and seven loci are sufficient for differentiating the MLGs at a global or European scale, respectively (Additional file 2: Fig. S2). The values of the genotypic and genetic diversity indices (H, G, λ, and H_{exp}) were higher for Asian samples suggesting higher genetic diversity compared to European samples. Evenness (E_5) showed higher values for EU_HES and EU_RIC than for EU-GEN and EU_KOB, but were highest for the Asian samples (Table 4). The frequency of MLGs per population (Fig. 2) showed two dominant European genotypes, MLG 38 (n = 207) and MLG 36 (n = 37), which together represented 78% of the total European sample. MLG 38 was predominant at all European sampling sites, representing 66% of all European samples. In contrast, MLG 36 that differed from MLG 38 at only locus Dc70 by one repeat of four bases (Additional file 1: Table S1) was predominant in the EU_RIC population.

Population structure and linkage disequilibrium

The dendrogram based on Bruvo's distance showed the vast majority of European genotypes clustered together, with the exception of MLG 27, MLG 28, and MLG 32 (found only in South Tyrol orchards) which grouped apart with a bootstrap node value of 96 (Fig. 3). In contrast, all outgroup genotypes from Asia and the USA clustered distant from the European genotypes, except for the genotype of the Japanese reference type strain of Dc, which grouped closer to the European genotypes compared to the Chinese, USA, and Korean genotypes (Fig. 3). Furthermore, the minimum spanning network supported the inter-continental grouping of the MLGs (Additional file 2: Fig. S3). Calculation of the fixation index F_{ST} indicated a clear and significant structuring of the sampled isolates among Europe, Asia and the USA, suggesting that the populations are genetically isolated from one another (Table 5). In contrast, there was little evidence of differentiation among the European populations with F_{ST} values of ≤ 0.100. Population EU_RIC was more distinct from the other populations in Europe, but had only slightly higher F_{ST} values (0.350–0.400, $P = 0.01$–0.02).

In a clonal population, genetic markers will exhibit linkage disequilibrium due to an absence of recombination. We calculated the index of association (I_A) and standardized index of association $\bar{r}_d$, which provide estimates of linkage disequilibrium. The observed values for the European population and all European subpopulation were not significantly different from zero (Table 3) nor significantly different ($P = 0.47$) to the expected value based on unlinked loci in a randomly

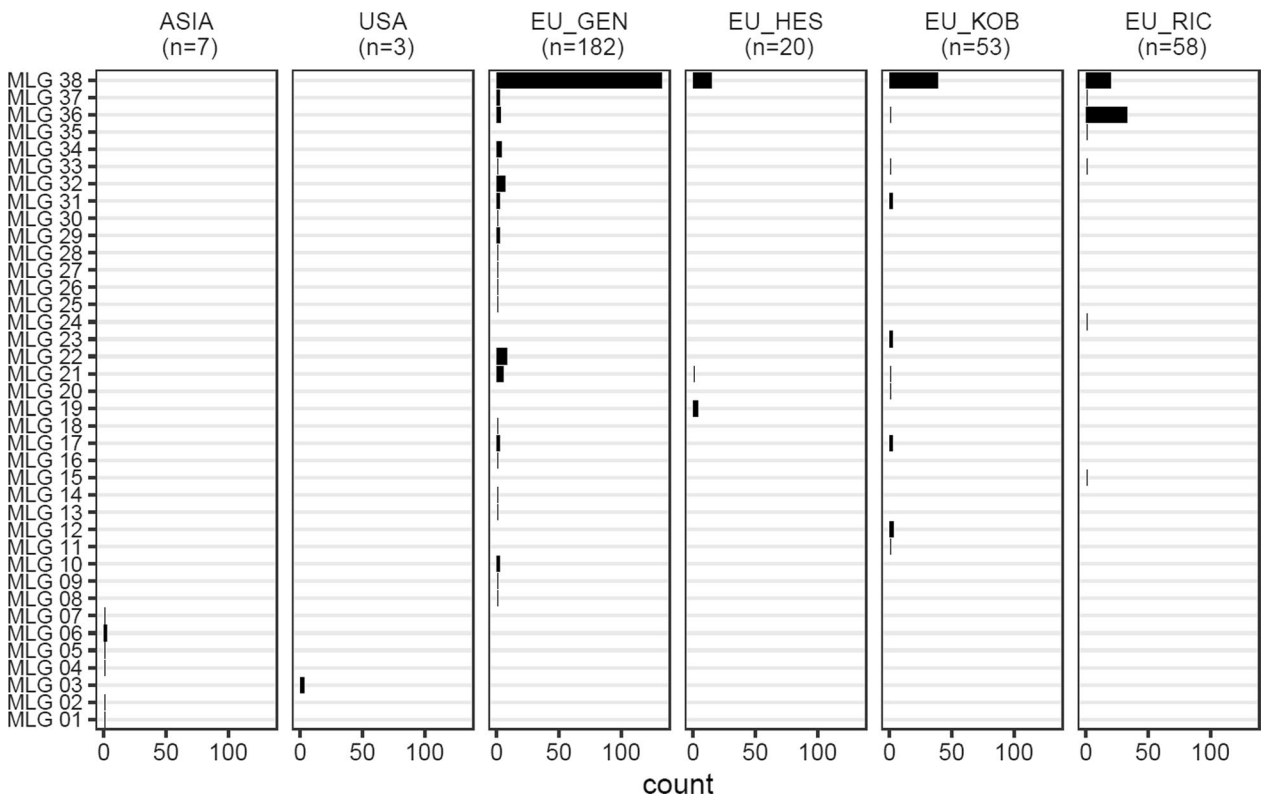

Fig. 2 Frequency of MLGs of *Diplocarpon coronariae* (Dc) grouped from 34 different apple orchards across Europe (EU_GEN), and from three additional, intensively sampled apple orchards in Europe (EU_HES, RIC and KOB) and from additional samples of *Dc* from Asia and the USA (n = 323, MLG = 38)

mating population (Additional file 2: Fig. S4) when calculated with all MLG data. The calculation of both association indices for clone corrected data gave non-significant differences ($P = 1.00$) as well but with negative values, which is an artifact of the calculation occurring in clonal populations of haploid organisms due to small sample size (Niklaus Grünwald and Zhian Kamvar, personal communication in July 2020). Thus, we fail to reject the null hypothesis of no linkage among the markers; in Europe Dc likely undergoes sufficiently frequent sexual reproduction to prevent linkage disequilibrium, while the population shows signs of a clonal, asexual component too.

The DAPC of all data resulted in retaining six principal components to maximize success of genetic cluster assignment (0.60), as estimated by cross-validation. The DAPC allowed discrimination into three distinct genetic clusters formed by isolates from Asia, the USA and Europe, respectively (Fig. 4). In the DAPC analysis of the European samples only, 14 principal components were retained to maximize success of genetic cluster assignment (0.47). Although most European isolates formed a single cluster in the analysis, isolates from EU_RIC clustered separately (Fig. 4).

Discussion

The twelve SSR markers were specific for Dc and could be combined in four multiplex assays to save cost and time. All twelve markers were polymorphic using all 323 isolates, but only eight were polymorphic for the European population, but proved sufficient to genotype the outgroup and European samples. Four of the twelve markers (Dc09, Dc15, Dc36, and Dc62) failed to produce amplicons with the Dc isolates from the USA and Korean (null alleles). The lack of amplification might have been due to mismatches of the primer sequences with the homologous genome sequences. Non-coding DNA regions containing SSR markers are less conserved than coding gene regions used for phylogeny, for example the EF-1 alpha promoter (Stielow et al. 2015), and this may result in higher sequence divergence in non-coding regions of genetically distant populations. In fact, the

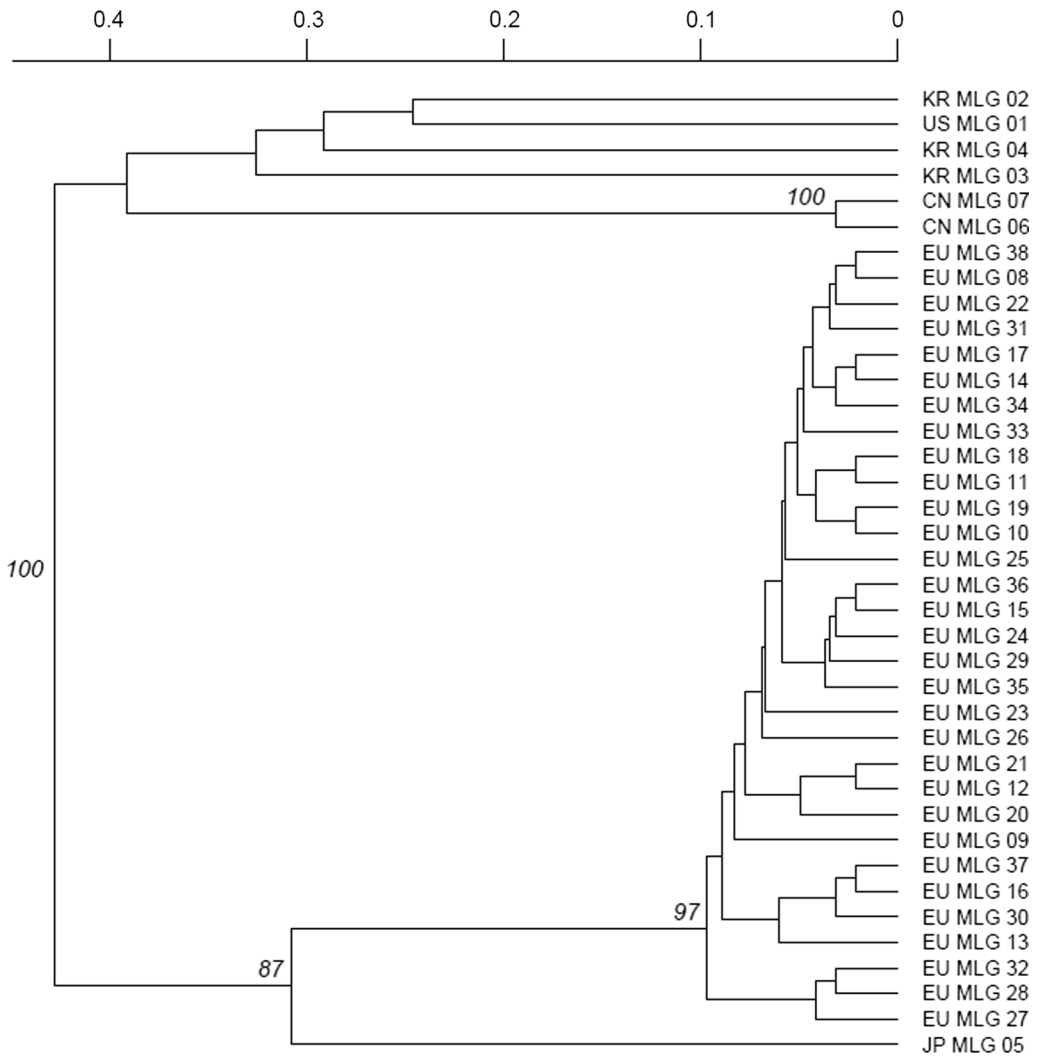

Fig. 3 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram of 323 isolates of *Diplocarpon coronariae* represented by 38 multilocus genotypes based on Bruvo's genetic distance. The UPGMA was calculated based on the length and number of the nucleotide repeats for each SSR locus with 1000 bootstrap replicates, and a node label cut-off value of 70. Outgroup MLGs are assigned to individual countries: Korea (KR), USA (US), China (CN), Japan (JP), whereas European MLGs are unitarily assigned (EU)

Table 5 Pairwise fixation indices (F_{ST}) indicating differentiation of populations of *Diplocarpon coronariae* from apple for all populations sampled in Europe, Asia and the USA (n = 323)

	EU_GEN	EU_HES	EU_RIC	EU_KOB	ASIA_AS	USA_US
EU_GEN (n = 182)	–	0.015	0.345	0.001	0.879	0.958
EU_HES (n = 20)	0.37	–	0.397	− 0.012	0.714	0.952
EU_RIC (n = 53)	0.11	0.01	–	0.357	0.822	0.936
EU_KOB (n = 58)	0.43	0.68	0.02	–	0.811	0.941
ASIA_AS (n = 7)	< 0.01	< 0.01	< 0.01	< 0.01	–	0.138
USA_US (n = 3)	< 0.01	< 0.01	< 0.01	< 0.01	0.20	–

F_{ST} values are presented above diagonal and the *P*-values (based on 1000 permutations) below the diagonal

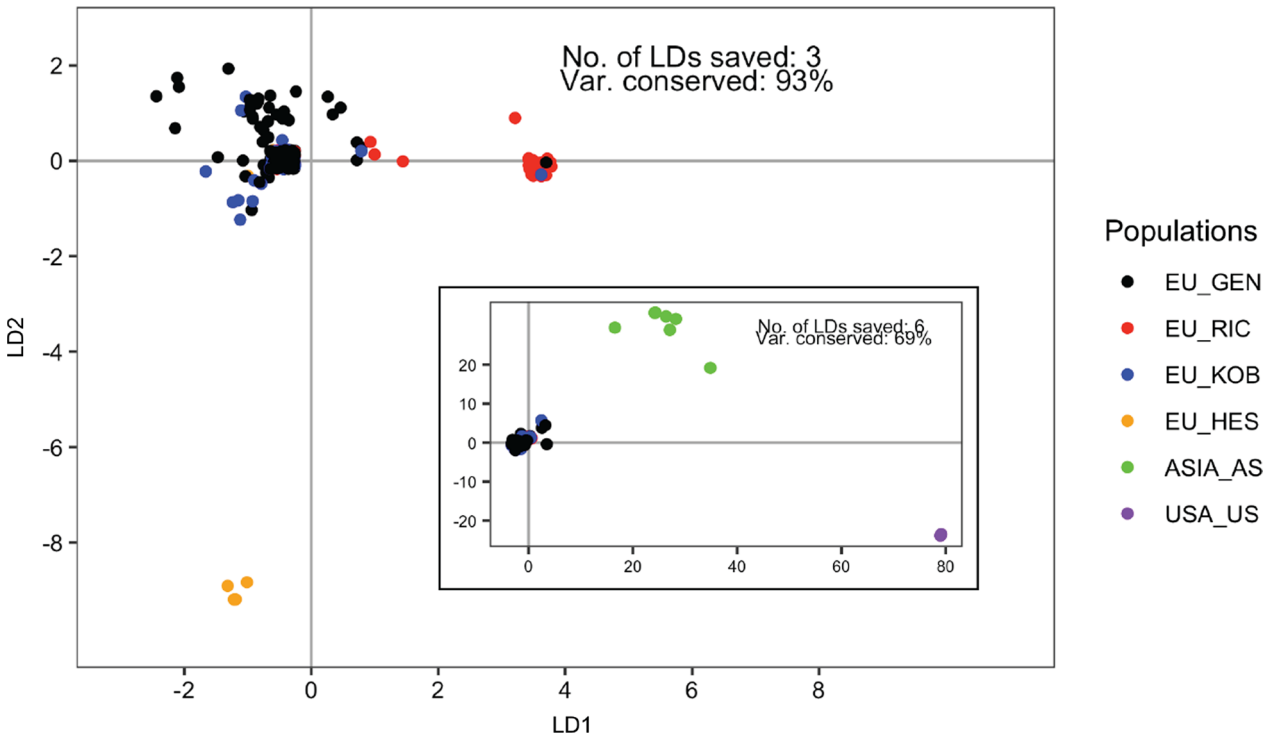

Fig. 4 Discriminant analysis of principal components of six populations (total 323 isolates) of *Diplocarpon coronariae* based on the allelic information of twelve SSR loci. The first two linear discriminant functions were used for plotting. The analysis was performed on the whole data set (inset panel) and on samples from the European populations only (main plot). Vertical and horizontal jitter (0.2 units each) was applied to draw the data points in order to reduce overplotting

alignments of the markers with homologous sequences of a Chinese Dc isolate NL1 (Cheng 2017), revealed several mismatches in the primer regions, besides the expected differences in number of SSR repeats. For future global studies of Dc populations, the primers need to be refined. Improvements might be achieved by either using degenerate bases at the mismatch positions of the primers or selecting primers targeting a more conserved region of the genome flanking the SSR regions.

Ideally, SSR markers should be spread across the chromosomes. Due to lack of an available, annotated and fully assembled whole genome sequence of Dc, the alignment with the draft genome of NL1 revealed that with the exception of two (Dc60 and Dc70) all of our 12 marker sequences were localized on different contigs. This implies that the genome regions of at least 10 markers might be sufficiently distant, and thus during the process of recombination, be independent from each other. Even specific alleles based on the two markers Dc60 and Dc70, such as represented in MLG 36 and MLG 21 were not found to be linked in the different samples and populations (Additional file 1: Table S1), suggesting free recombination of loci and independent mutation events.

The low number of alleles and MLGs, the high clonal fraction, as well as low values of gene diversity indicators (eMLG, H, G, λ, H_{exp}), and of Evenness (E_5) in the European populations are typical of clonal populations and support the hypothesis that Dc is a recently introduced pathogen in Europe. This is also supported by the observation that 66% of the Dc isolates from Europe belonged to one genotype (MLG 38), which occurred in all European regions sampled, and which likely indicates high gene flow of Dc within Europe associated with a low recombination rate. In contrast, the other dominant MLG, MLG 36 was mainly limited to one orchard (EU_RIC). However, the dominance of MLG 36, which was found elsewhere in Europe, might be a sampling bias due to the large sample size at the EU_RIC orchard (n = 58). Indeed, there is relatively low differentiation between European populations compared to the outgroup samples as shown by the UPGMA dendrogram and the three distinct clusters for European, Asian and USA samples in the DAPC analysis. This is also supported by the generally low F_{ST} values for European populations, again with the exception of EU_RIC, which can be explained by the high incidence of MLG 36 at this site.

Recently established populations of invasive species usually contain only a fraction of the genetic diversity that is observed at the center of origin for the species. This "founder-effect" can account for the presence of one or a few alleles or MLGs at an unusually high frequency. Similar to Dc, this has been shown for other recently introduced plant pathogens, for example, some pathogens introduced to Australia, including *Ascochyta rabiei* on chickpea (Leo et al. 2015) and *Alternaria brassicola* on *Cakile maritima* (Linde et al. 2010). In contrast, the frequency and distribution of MLGs in long established populations is expected to be more even. For example, SSR analysis of genetic variation supported the hypothesis that the origin of the fungal wheat pathogen *Phaeosphaeria nodorum* is located in the Fertile Crescent region that is also the center of origin of wheat (McDonald et al. 2012). In contrast, SSR analysis of populations of the barley scald pathogen *Rhynchosporium secalis* showed that lowest genetic diversity was present in the Fertile Crescent region, which is the origin of *Hordeum spontaneum*, the progenitor of cultivated barley (Linde et al. 2009). Their data support the hypothesis that *R. secalis* first evolved in Scandinavia, perhaps due to a host jump indicating a first bottleneck with a founder effect on *Hordeum secale*, and that regional populations of *R. secalis* in the Middle East, California, South Africa or New Zealand have undergone further recent population bottlenecks when they were introduced via infected seed through anthropogenic activities.

Similarly, SSR loci have been successfully used to reconstruct the global spread of the cercozoan potato pathogen *Spongospora subterranea* that likely originated from South America (Gau et al. 2013). Currently, there is no clear indication where the European Dc populations originated. The hypothesis that European populations of Dc are due to a recent introduction from Asia is not supported by our data both by reason of the few Asian genotypes available in our study, and the fact that those that were included, clustered separately to those from the USA and to the isolates from Europe. Future work on the population genetics of Dc from different regions of the world should be based not only on more isolates of Dc from different Asian regions, including India (a hot spot of AB) but also on a more uniform and comparable sampling approach to the populations and/or subpopulations.

In Europe, AB is a major problem in organic and/or low input meadow tree (cider) orchards. However, the increasing yield losses in conventional apple production during the last few decades in China (Li et al 2012) suggests that Dc is an emerging pathogen of global importance. McDonald and Linde (2002) identified factors that contribute to pathogen evolution and argued that the evolutionary potential is reflected in a pathogen's population genetic structure and reproductive characteristics. According to this framework, pathogens that pose the greatest risk are characterized by a high potential for gene flow, a mixed reproduction system, large effective population sizes and high mutation rates. The high potential of gene flow in Dc is well documented by its rapid dispersal across Europe, where it established populations within ten years after the first report of AB in Italy (Tamietti and Matta 2003). We speculate that this invasion pattern reflects a combined gene flow due to anthropogenic activities and natural dispersal. Agricultural practices, including exchange of propagation material between nurseries, and trade and commerce of production material in conjunction with intra- and intercontinental travel are notorious for spreading plant pathogens far beyond their native range limits. Some examples of fungal pathogens that have become exotic, endemic diseases in new regions include *Ceratocystis fimbriata* f. sp. *platani* in plane (Anselmi et al. 1994), *Cryphonectria parasitica* the cause of Chestnut blight (Prospero and Rigling 2012), and *Ophiostoma ulmi* the cause of Dutch elm disease (Brasier and Buck 2001), that were likely spread globally by human activity.

There is a limited knowledge of the life cycle of Dc. However, formation of apothecia and ascospore production was reported in Japan and China (Gao et al 2011), suggesting that sexual reproduction might play a significant role in the disease epidemiology. In other Ascomycota, natural dissemination is mainly caused by airborne ascospores that can be carried by the wind for considerable distances, from 45 m up to several kilometers (Aylor 1998; Linde et al. 2002; Holb et al. 2004). For the closely related *D. rosae* which causes black spot of rose, conidia are reported to be the main stage for overwintering. Sexually produced ascospores formed in apothecia were also observed but have rarely been reported (Knight and Wheeler 1977). The rarity of ascospore encounters with *D. rosae* has been attributed to the lack of conducive weather conditions for ascospore development. If the weather conditions are favorable for development of ascocarps, apothecia containing asci are observed in spring, but if the conditions are unfavorable, the fruiting bodies give rise to apothecial conidia (Aronescu 1934), thereby promoting the asexual life cycle of the fungus. Gao et al. (2011) observed that Dc primarily produces pseudoconidia in spring and only later, in May and June, did it form apothecia and ascospores under more favorable conditions. As reported by Wöhner and Emeriewen (2019), but also based on our observations, sexual reproduction of Dc (apothecia) has not been observed in Europe. Nevertheless, the fact that linkage disequilibrium is not detectable in the European pathogen population suggests that Dc is undergoing regular recombination,

and indicates that the life cycle may be partially sexual in Europe.

Considering these observations, we conclude that Dc populations in Europe most likely have a mixed reproduction system of sexual and asexual, clonal propagation. Mixed reproductive systems are common among fungi (Taylor et al. 1999) and it has been hypothesized that fungal pathogens with mixed reproduction system, a large effective populations size, a high potential for gene flow and high mutation rates, poses the highest risk for breaking host resistance (McDonald and Linde 2002). Adaptation through mutation could be especially problematic in the development of resistance to fungicides: thiophanate-methyl (which has some curative properties) resistant strains of Dc have already been reported in Japan (Tanaka et al. 2000).

AB epidemics result in large populations of Dc in late summer and autumn, so we suspect that there is a high potential for evolutionary change in Dc. However, we do not know exactly when and how the epidemics begin. Dc conidia from overwintered leaf litter were viable (Sharma et al. 2009) but buds and twigs are also suspected to be potential overwintering sites (Wöhner and Emeriewen 2019), all of which might contribute to primary infections. This is information needed in order to establish disease management practices aimed at reducing inoculum for new epidemics. In particular, infectious inoculum in buds or on wood would circumvent leaf litter removal measures and facilitate anthropogenic spread with planting material.

Based on the currently available, yet limited knowledge of the population biology of Dc, we assert that an integrated approach is needed to reduce the damage due to AB. Similar to the approach outlined for Septoria tritici blotch of wheat (McDonald and Mundt, 2016), resistance breeding, targeted use of fungicides according to prediction models, informed management of infected leaf litter including biological control, and trade with certified (pathogen free) propagation material across and within continents will be important pillars to successful and sustainable management of Dc.

Conclusions

AB is a burgeoning threat to apple production in Europe and in the USA—especially to ecologically sustainable production systems that are on the increase. Twelve new SSR markers were developed that allow differentiation of genotypes of Dc isolates, the causal agent of apple blotch. A subset of the markers is especially suited for genetic analyses at the European continental scale. All markers combined provided data that allowed conclusions to be drawn regarding the global relatedness of isolates. Our results show that Dc populations in Europe are genetically homogenous and clonal, and that the European genotypes are distinct from the Asian and USA genotypes. At present, the origin of the European populations of Dc remains unknown. However, a recent single or multiple introduction event is a likely scenario. An analysis of Dc isolates at a global scale, including India and the Near East will provide additional insights into likely origins of European Dc populations. Our data further suggest a mixed sexual and asexual reproduction of Dc in Europe. Combined with the large population size in field epidemics due to clonal mass multiplication there is a high evolutionary potential of Dc and the resulting adaptability of the population to changing environments may be expected.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43170-021-00039-6.

Additional file 1: Table S1. A list of all Dc samples and their geographic origin and amplicon sizes (bp) for each SSR marker.

Additional file 2: Figure S1. The location of the twelve SSR primer sequence regions of the Swiss Dc Isolate CH01 compared to the analogous sequence regions for the fully sequenced Chinese Dc Isolate NL1 (Genbank MZNU00000000.1). **Figure S2.** The genotype accumulation curves for twelve SSR markers used to screen all 323 isolates, and the eight SSR markers used to screen the European isolates only. **Figure S3.** The minimum spanning network of all samples grouped by MLGs based on Bruvo's genetic distance. **Figure S4.** The allelic diversity indices and linkage disequilibrium analysis for all the isolates and the clone corrected data. **Figure S5.** The DAPC-based population membership probabilities.

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Authors' contributions

LT, SB, YHR and HJS acquired the funding; TO, HJS, and AP conceived and designed the experiments; TO, AD, VL, AB, SGA, FK, YHR, HJS, and AP collected the Dc samples; TO, AD, and VL developed the methods and performed the analysis; LW and TO conducted the data analysis and designed the figures; TO wrote the manuscript with substantial input from AP, LW, and HJS; HJS, SGA, FK, BS, LW, AD, and AP reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Data of the sample collection, such as sampling date, origin, and the corresponding analytical results (fragment lengths) are listed and provided in Additional file 1: Table S1. The Illumina sequence information of the complete marker sequences, where the specific primer delimited marker regions are derived from, were deposited at Genbank (NCBI) with Accession No. MT036090-MT036101. Data files and the R Markdown file for all analyses are provided on https://github.com/dendrologicus/Dcoronariae_Popgen.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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