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Genetic diversity analysis of the cucurbit powdery mildew fungus *Podosphaera xanthii* suggests a clonal population structure

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ABSTRACT

The sexual stage of *Podosphaera xanthii* is rarely found worldwide. However, chasmothecia are frequently recorded in northern Italy, suggesting the presence of an actively mating population. With the aim of investigating the genetic structure of the Italian population with respect to populations from other countries, genetic diversity analysis was performed both on 92 isolates from European and American countries by multilocus sequence typing (MLST) and on 59 isolates by amplified fragment length polymorphism (AFLP) methods. Mating type frequencies were tested for random mating and two-locus linkage disequilibrium (LD) analysis was performed. Results showed very low levels of genetic diversity: MLST showed no variations in eight housekeeping gene fragments and, accordingly, UPGMA dendrogram from AFLP data showed a high similarity (0.91–1.00 simple matching similarity coefficient) between isolates. Moreover, the random mating test showed no deviations from mating-type 1:1 ratio in the Italian population but deviations were observed in populations from Europe and American countries while two-locus LD analysis showed the presence of significant LD. The results suggest that the populations of *P. xanthii* are likely to be predominantly clonal, and asexual reproduction, producing a huge amount of conidia, appears to be the predominant type of reproduction of the species.

Introduction

Among the over 200 diseases affecting cucurbits (Zitter *et al.* 1996), powdery mildew is considered the most important widespread disease limiting cucurbit production. Although there are some indications of records of *Leveillula taurica* as a causal species of cucurbit powdery mildew (El Ammari & Wajid Khan 1983; Branzanti & Brunelli 1992; Vakalounakis *et al.* 1994), the most common pathogens causing the disease are two obligate biotrophic ascomycetes fungi: *Podosphaera xanthii* (Castagne) U. Braun

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and N. Shishkoff (Braun & Takamatsu 2000) and *Golovinomyces orontii* (Castagne) Heluta (1988). In the North of Italy, as in many areas of the world, *P. xanthii* is the predominant species causing cucurbit powdery mildew (Branzanti & Brunelli 1992; Pirondi *et al.* 2015a). Furthermore, in this area, an abundant production of chasmothecia has been confirmed, since they can be easily collected from senescent leaves and soil samples (Pirondi *et al.* 2015b). This is a very interesting observation from the epidemiological point of view, especially since the epidemiological relevance of the sexual reproduction in *P. xanthii* remains unknown (Pérez-García *et al.* 2009).

In the literature, the sexual stage of *P. xanthii* has been found in Italy (Marras & Corda 1977; Branzanti & Brunelli 1987; Pirondi *et al.* 2015b), Germany (Ulbrich & Smolka 1994), Hungary (Nagy 1976), Bulgaria (Velkov & Masheva 2002), the United States (McGrath 1994), Greece (Vakalounakis & Klironomou 1995), France (Bardin *et al.* 1997), and Morocco (Endo *et al.* 2012), but has never been observed in Spain (Álvarez & Torés 1995) and in the South of Italy (Miazzi *et al.* 2011). Previous studies (Branzanti & Brunelli 1992; Pirondi *et al.* 2015b) reported the presence of *P. xanthii* chasmothecia exclusively, thus, this is considered to be the most important and widespread species causing powdery mildew on cucurbits (Pérez-García *et al.* 2009). Therefore, we wondered whether sexual reproduction in populations of North Italy would have a significant impact on the genetic diversity of this pathogen.

Since some geographical differences in chasmothecia production of *P. xanthii* are evident, it becomes extremely interesting to investigate the genetic structure of *P. xanthii* populations to determine whether there is any evidence of sexual recombination in the areas where chasmothecia were found and then understand the role that sexual reproduction, and thus recombination, could play in the life cycle of this species. Information regarding the genetic structure within *P. xanthii* populations is scarce. By performing Random Amplification of Polymorphic DNA (RAPD) analysis, Bardin *et al.* 1997 revealed a low degree of polymorphism in 28 isolates of *P. xanthii* from different countries, and cluster analysis did not separate any group. Similarly, RAPD analysis conducted on *P. xanthii* isolates from southern Spain revealed no variation among isolates (A.P., pers. comm.). In contrast, RAPD analysis performed by Miazzi and coworkers (2011) revealed high genetic variation among 82 isolates of *P. xanthii* from Apulia (southern Italy) but, in this case too, cluster analysis did not separate groups and no markers could be associated with host plants and geographical origin of the isolates. Furthermore, within a study on strobilurin resistance in *P. xanthii* populations from southern Spain, Fernández-Ortuño *et al.* (2008) sequenced ITS regions from 25 isolates; only one nucleotide substitution was found in two isolates and in different positions. In addition, an Amplified Fragment Length Polymorphism (AFLP) study conducted by Naruzawa *et al.* (2011) showed high variability within 22 isolates of *P. xanthii* from Brazil with a Jaccard similarity coefficient ranging between 0.23 and 0.69. However, the dendrogram obtained showed no separation of groups in relation to races, geography and host plants. Interestingly, in the same work, the same isolates did not show any sequence variation within the 5.8S and ITS region.

Pathogen populations must constantly adapt to changes in their environment to survive. In agricultural ecosystems, environmental changes may include introduction of resistant varieties, applications of fungicides and fertilizers, irrigation, and crop rotation. It is clear that agricultural systems impose strong directional selection on pathogen populations. *P. xanthii* is a difficult to control pathogen (McGrath 2001; Pérez-García *et al.* 2009) and, given the versatility shown by the species to adapt not only to environmental conditions but also to overcome different control strategies, understanding the genetics of pathogen populations is a crucial aspect in anticipating how populations will evolve in response to new control strategies. With the fragmentary information regarding the genetic diversity of *P. xanthii* populations, the aim of this study was: 1) to investigate the genetic structure of *P. xanthii* populations using both Multilocus Sequence Typing (MLST) and AFLP techniques; 2) to determine whether populations of *P. xanthii* in Italy could be randomly mating with respect to populations from other countries where chasmothecia were rarely/never found. To answer this last question, mating type (MAT) frequencies were tested for random mating and AFLP data were analyzed by two-locus linkage disequilibrium analysis.

Material and methods

Isolate collection and identification

Podosphaera xanthii isolates were obtained from infected cucurbit plants from orchards in the North of Italy (Bologna and Mantova provinces). Furthermore, isolates from the South of Italy, South-Central Spain, France, the Czech Republic, Bulgaria, the United States, Canada and Central–South America were also included in the study. The *P. xanthii* isolates used in this study are listed in Table 1. Fungal material was collected from crops in fields or plastic tunnels. From all the material collected, monoconidial isolates were obtained and maintained *in vitro* as described by Álvarez & Torés (1997). Finally, part of the fungal biomass was used for DNA extraction and part was preserved at −80 °C using silica gel as described by Pérez-García *et al.* (2006). To confirm that we were working with isolates of *P. xanthii*, conidia were inspected for lateral position of germ tubes and the presence of fibrosin bodies, as typical identifying features of *P. xanthii* conidia (Kable & Ballantyne 1963; Zaracovitis 1965; Lebeda 1983; Braun & Cook 2012), under light microscopy.

DNA isolation

Biomass of the powdery mildew monoconidial isolates was recovered 15–20 d after inoculation and DNA extraction was carried by using the cetyltrimethylammonium bromide (CTAB) method (Stewart & Via 1993) as modified by Robinson *et al.* (2002), already used for this pathogen by Vela-Corcía *et al.* (2014).

Primer design for MLST analysis

To address fungal diversity analysis by MLST, we planned to sequence the following gene regions: α-tubulin (*TUB1*), chitin synthase I (*CSI*), intergenic spacer (*IGS*), translation elongation factor α (*EF1a*) and mitochondrial small subunit of ribosomal DNA (*mt SSU rDNA*). As the genome of *Podosphaera*

Table 1 – Isolates of *P. xanthii* used in this study.

Isolate	Location	Year of collection	Host plant
GI18	Bologna (Italy)	2009	*Cucurbita pepo*
RA23	Ravenna (Italy)	2010	*Cucurbita pepo*
210	Bologna (Italy)	2010	*Cucurbita pepo*
410	Bologna (Italy)	2010	*Cucurbita pepo*
810	Sermide (Italy)	2010	*Cucumis melo*
2310	Moglia (Italy)	2010	*Cucurbita pepo*
2610	Cadriano (Italy)	2010	*Cucurbita pepo*
2710	Cadriano (Italy)	2010	*Cucumis sativus*
3210	Altedo (Italy)	2010	*Cucurbita pepo*
4010	Bologna (Italy)	2010	*Cucurbita maxima*
4311	Bologna (Italy)	2011	*Cucurbita pepo*
4811	Imola (Italy)	2011	*Cucurbita pepo*
4911	Sermide (Italy)	2011	*Cucumis melo*
5011	Sermide (Italy)	2011	*Cucumis melo*
5211	Novellara (Italy)	2011	*Cucumis melo*
5311	Sermide (Italy)	2011	*Cucumis melo*
5411	Sermide (Italy)	2011	*Cucurbita maxima*
5611	Sermide (Italy)	2011	*Cucumis melo*
5711	Sermide (Italy)	2011	*Cucumis melo*
5811	Sermide (Italy)	2011	*Citrullus lanatus*
6111	Ragusa (Italy)	2011	*Cucumis melo*
6211	Ragusa (Italy)	2011	*Cucumis melo*
6311	Moglia (Italy)	2011	*Cucumis melo*
6411	Moglia (Italy)	2011	*Cucumis sativus*
6511	Moglia (Italy)	2011	*Cucumis sativus*
6611	Imola (Italy)	2011	*Cucumis sativus*
6711	Altedo (Italy)	2011	*Cucurbita moschata*
7811	Cadriano (Italy)	2011	*Cucurbita pepo*
IO12	Castelnuovo Rangone (Italy)	2012	*Cucurbita pepo*
AT12	San Miniato (Italy)	2012	*Cucubita maxima*
EL12	Sestola (Italy)	2012	*Cucurbita pepo*
F.1–5	Monopoli (Italy)	2011	*Cucumis melo*
F.1–7	Monopoli (Italy)	2011	*Cucumis melo*
F.2–1	Monopoli (Italy)	2011	*Cucumis melo*
F.2–2	Monopoli (Italy)	2011	*Cucumis melo*
F.2–3	Monopoli (Italy)	2011	*Cucumis melo*
F.2–4	Monopoli (Italy)	2011	*Cucumis melo*
F.3–2	Monopoli (Italy)	2011	*Cucumis melo*
SSa12	Alghero (Italy)	2012	*Cucurbita pepo*
SSb12	Alghero (Italy)	2012	*Cucurbita pepo*
3_11	Olomouc-Holice (Czech Republic)	2011	*Cucurbita pepo*
42_11	Konecchlumi (Czech Republic)	2011	*Cucurbita pepo*
49_11	Olomouc-Holice (Czech Republic)	2011	*Cucurbita maxima*
60_11	Kvasice (Czech Republic)	2011	*Cucurbita pepo*
81_11	Novy Jicin-Kojetin (Czech Republic)	2011	*Cucumis melo*
BUa12	Krumovo (Bulgaria)	2012	*Cucumis melo*
BUb12	Krumovo (Bulgaria)	2012	*Cucumis melo*
BUc12	Saedinenie (Bulgaria)	2012	*Cucurbita maxima*
BUd12	Saedinenie (Bulgaria)	2012	*Cucurbita maxima*
SF60	Greece	1999	*Cucurbita pepo*
SF61	Greece	1999	*Cucumis sativus*
2086	Greece	1997	*Cucumis melo*
221088	Almeria (Spain)	2006	*Cucurbita pepo*
311127	Murcia (Spain)	2006	*Cucumis melo*
311119	Murcia (Spain)	2006	*Cucumis melo*
311120	Murcia (Spain)	2006	*Cucumis melo*
311287	Murcia (Spain)	2008	*Cucumis melo*
221231	Almeria (Spain)	2008	*Cucurbita pepo*
211242	Almeria (Spain)	2008	*Cucumis melo*
221301	Almeria (Spain)	2002	*Cucurbita pepo*
311251	Murcia (Spain)	2008	*Cucumis melo*
311271	Murcia (Spain)	2008	*Cucumis melo*
811301	Badajoz (Spain)	2008	*Cucumis melo*

(*continued on next page*)

Table 1 – (continued)

Isolate	Location	Year of collection	Host plant
711319	Ciudad Real (Spain)	2008	*Cucumis melo*
711349	Ciudad Real (Spain)	2008	*Cucumis melo*
23775	Almeria (Spain)	2004	*Cucumis sativus*
SF218	Malaga (Spain)	1999	*Cucurbita pepo*
31874	Murcia (Spain)	2004	*Cucumis melo*
81695	Badajoz (Spain)	2003	*Cucumis melo*
311128	Murcia (Spain)	2006	*Cucumis melo*
98SM65	Almeria (Spain)	1998	*Cucumis melo*
00SM39	Bouches-du-Rhône (France)	2000	*Cucumis melo*
04SM1	Bouches-du-Rhône (France)	2004	*Cucumis melo*
04SM2	Bouches-du-Rhône (France)	2004	*Cucumis melo*
SM1R2	Vaucluse (France)	1987	*Cucumis melo*
085M9	France	2008	*Cucumis melo*
MX12	Sinaloa (Mexico)	2012	*Cucumis sativus*
Scr48.2	Argentina	1993	*Cucurbita maxima*
Scc187.1	Martinique (French Caribbean)	1994	*Cucumis sativus*
Scc76.2	Quebec (Canada)	1994	*Cucumis sativus*
Sm41.1	USA	1993	*Cucumis melo*
Scc140.1	California (USA)	1994	*Cucumis sativus*
S-9	New York (USA)	2012	*Cucurbita maxima*
11.13-X	New York (USA)	2012	*Cucurbita maxima*
US1	New York (USA)	2012	*Cucurbita pepo*
US2	New York (USA)	2012	*Cucurbita pepo*
US3	New York (USA)	2012	*Cucurbita pepo*
US4	New York (USA)	2012	*Cucurbita pepo*
US5	New York (USA)	2012	*Cucurbita pepo*
US6	New York (USA)	2012	*Cucurbita pepo*
US7	New York (USA)	2012	*Cucurbita pepo*
US8	New York (USA)	2012	*Cucurbita pepo*

xanthii was not yet sequenced, isolation of fragments from these genes was carried out by designing primers on conserved gene regions (exons) of other powdery mildew fungi whose genome sequences were recently released (Spanu *et al.* 2010): *Blumeria graminis* (www.blugen.org), *Golovinomyces orontii*, and *Erysiphe pisi* (on-line databases of Max Planck Institute for Plant Breeding Research at Cologne, Germany, http://www.mpipz.mpg.de/14157/fungal_genomes). In addition, the Internal Transcribed Spacer (ITS) region, the sterol 14-α-demethylase (*CYP51*) and β-tubulin (*TUB2*) gene fragments were also amplified and sequenced. From the different isolates, each marker was sequenced three times from three independent PCR amplifications. Features of the primer set used for MLST analysis and GenBank accession numbers (A.N.) for each sequenced gene fragment are shown in Table S2.

AFLP analysis

AFLP analysis was performed by following the method described by Vos *et al.* (1995) with minor modifications. Genomic DNA was digested at 37 °C for 1 h using two restriction enzymes: *Tru1*I (*Mse*I) (frequent cut) and *Eco*RI (rare cut) supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). MSE and ECO adapters were then ligated to digested genomic DNA and ligation reaction was carried out by incubation at 37 °C for 3 h. Subsequently, 450 µl of double distilled water were then added to each ligation mixture and the pre-amplification reaction with primers M01 (Eco RI + A) and M02 (MseI + C) was then performed. Pre-amplification products were diluted 1:5 and six primer combinations were used to perform selective amplification reactions: M48 + E31, M48 + E32, M47 + E24, M48 + E24, M50 + E24, M50 + E20 (www.keygene.com). Standard AFLP amplification protocols, with 0.4 u of GoTaq Promega® DNA polymerase[1] for each sample, were used. Amplified fragments were separated by electrophoresis on 5 % polyacrylamide gels, subsequently stained with the silver method (Bassam *et al.* 1991).

In order to obtain information about the differences in AFLP profiles observed among isolates, some polymorphic bands were randomly selected, manually excised from gels and re-amplified with the corresponding selective primers. Each PCR product was then cloned in a pGEM plasmid using the pGEM-T Vector System kit (Promega, Madison, WI, USA) following the manufacturer's instructions. After extraction, plasmid inserts were sequenced with universal primers SP6 and T7 at Macrogen Europe (Amsterdam, The Netherlands) and obtained sequences were then submitted to GenBank. Blastn and Blastx algorithms were performed in order to find similarity to sequences deposited in the NCBI database.

Mating type identification

To identify the idiomorph at the locus *MAT* (*MAT 1-1-1* or *MAT 1-2-1*) of each *Podosphaera xanthii* isolate, a Multiplex-PCR was performed with the idiomorph-specific primers aboxF2/aboxR2 and hmgF2/hmgR2 described by Pirondi *et al.* (2015b.)

[1] Details about PCR reactions, including PCR thermal protocol are available from the authors.

Data analysis

After sequencing, consensus sequences were obtained for each isolate and marker using Contig Express software (Vector NTI Advance 10.3.0, Life Technologies, Carlsbad, CA, USA) and then aligned with Clustal Omega Multiple Alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) in order to find any genetic variation.

Each of the six AFLP gels was manually analyzed by giving a score of one or zero for the presence or the absence of common bands, respectively. Only reproducible bands were scored and both monomorphic and polymorphic bands were considered. Furthermore, for each primer combination, PCR amplifications were randomly repeated on eight isolates to estimate the error rate. Statistical analysis of data was carried out under the following assumptions: 1) co-migrating bands were homologous; 2) different band positions represent different loci. AFLP binary matrix obtained from all primer combinations was then converted in a distance matrix and cluster analysis was performed by the unweighted pair group with arithmetic mean (UPGMA) method based on simple matching similarity coefficient by using NTSYSpc 2.2 software and a dendrogram was generated.

Estimation of genetic diversity parameters was carried out by analyzing the data matrix with POPGENE 1.31 software (Yeh & Boyle 1997). Isolates were divided in three populations based on their geographical origins. In particular, two groups, one European (Italy and other European country populations) and one American (North and Center-South American isolates), were created with two and one populations, respectively. Estimation of genetic diversity parameters was performed both on the entire data-set and on each of the three populations by calculating: percentage of polymorphic loci (P), observed number of alleles (na), effective number of alleles (ne) (Hartl & Clark 1989), Nei's gene diversity (h) (Nei 1973) and phenotypic diversity using the Shannon's (1949) information index (I). Moreover, to examine the genetic variation among and within populations (F_{st}), the following indexes were calculated: total genetic diversity (H_t), diversity within populations (H_s), diversity within groups (H_c), diversity among populations (G_{st}), diversity among groups (G_{cs}) calculated as $(H_t-H_c)/Ht$. In addition, Nei's analysis of gene diversity in subdivided populations (Nei 1972) was used to estimate genetic identity and genetic distance between the three populations.

Tests for random mating

Once the *MAT* idiomorph for each isolate had been identified, *MAT* frequencies were analyzed by the chi-square (χ^2) test using GraphPad Prism 6.01 software (GraphPad Software Inc.®, La Jolla, CA, USA) to highlight significant deviations from the null hypothesis (H_o) of MAT 1:1 ratio in each population. When sample sizes are small, the exact binomial test is more accurate than the χ^2 test (Sokal & Rolhf 1995; Linde *et al.* 2003; Rau *et al.* 2005). For this reason, with the same software, the exact binomial test was performed in addition to χ^2 test. Statistical analysis was performed on frequencies of the 59 isolates used for AFLP and LD analysis. *MAT* frequencies were tested for random mating in both entire and clone-corrected samples (described below).

Because of the abundant and easy collection of chasmothecia in North of Italy (Branzanti & Brunelli 1992; Pirondi 2015b) we planned to detect some evidence of sexual recombination in Italian populations with respect to populations from other European countries (Bardin *et al.* 1997; Perez-Garcia *et al.* 2009; Křístková *et al.* 2009) and from North America (McGrath 1994; McGrath *et al.* 1996) where chasmothecia are considered to be rare. For this purpose, two-locus LD analysis (Weir 1979) was performed on each of the three populations. As suggested by Kohli & Kohn (1998), to reduce the statistical error that can be introduced by very frequent fragments in the populations, LD was calculated only by considering loci with allele frequency <0.95. Moreover, to eliminate the effects of asexual reproduction, data for LD analysis were clone-corrected by including only one representative of each unique genotype as the clone-corrected sample is considered to be more conservative with respect to rejecting the null hypothesis of random mating (Maynard Smith *et al.* 1993; Kohli & Kohn 1998).

Results

Genetic diversity analysis of Podosphaera xanthii *populations*

Several PCR approaches, based on the amplification of different housekeeping genes, were adopted to investigate the genetic structure of *Podosphaera xanthii*. However, after sequence alignment, no sequence polymorphisms were identified in eight housekeeping loci.

On the other hand, AFLP analysis revealed the presence and/or absence of polymorphisms and common main bands in each AFLP gel. These differences were scored for the binary matrix construction. In total, from the selected six primer pairs, 169 loci were produced, of which 66 were polymorphic (39.05 %) across the 59 isolates. Markers selected from each primer combination and the relative percentages of polymorphism are illustrated in Table 2.

Cluster analysis with UPGMA method using simple matching similarity coefficient was then performed and a dendrogram showing genetic similarity was obtained (Fig 2). As shown in the dendrogram, genetic similarity was very high as it ranged from 0.91 to 1.00, and isolates did not cluster in groups according to geography, host plant, year of collection or mating type. Minimum genetic similarity was observed between isolates 6711 and 311120 while maximum similarity of 1.00 was observed in four groups. The first group consisted of isolates 811301, 711349, and 211242 (all from Spain), the second group of isolates 311287 (Spain), 2086 (Greece), 5211, 6411, 2610, 4311, 4811, EL12, AT12, GI18 (all from the North of Italy), and US2 (U.S.A.), the third group of isolates 3210 (North of Italy) and US5 (U.S.A.) and the fourth group of isolates US1 and 11.13-X (all from the U.S.A.). The estimated error rate was about 1 %.

Although, in general, a low degree of genetic diversity was observed, AFLP analysis was able to reveal some polymorphisms among *P. xanthii* isolates. Sequences related to mobile genetic elements as transposons and retro-transposons, as expected for genotypes that are mainly clonally reproduced,

Table 2 – AFLP analysis in *P. xanthii*. Number of markers selected for AFLP analysis and percentage of markers that were polymorphic.

Primers	M48-E24	M47-E24	M50-E20	M50-E24	M48-E31	M48-E32	Total
Markers	30	36	25	23	28	27	169
Polymorphic markers	10	10	8	8	10	20	66
Polymorphism (%)	33.33	27.77	32.00	34.78	35.71	74.07	39.05

were found. Polymorphisms from isolates 31119 and 5311 showed similarity to a polyprotein associated with a Long Terminal Repeat (LTR) transposable element (GenBank A.N. KJ698672 and KJ698673); from 31874 to a transposon (A.N. KJ720653); from 41.1 with the subunit one of cytochrome c oxidase (A.N. KJ698677). The other two isolates showed similarities to different proteins: isolate 085M9 to an ABC transporter protein (A.N. KJ698675) and isolate 311119 to the guanylate binding protein I (A.N. KJ698679).

Genetic diversity parameters were calculated both on the entire AFLP data-set and separately on the three populations (Table 3). The highest number of observed alleles (na) was shown by the Italian population while the lowest values were observed in American isolates. Mean Nei's gene diversity (*h*) was 0.0304 and mean Shannon's information index (*I*) was 0.0614. According to total diversity, all three populations showed low levels of diversity. Mean gene diversity (Nei (1973) and Shannon's (1949) information index at each locus are presented in Table S2.

As illustrated in Table 4, genetic diversity within groups (H_c) and within populations (Hs) showed low values, according to total genetic diversity (H_t). Genetic diversity among populations was low and, moreover, the diversity among European and American populations (G_{cs}) was 12.08 %.

Genetic relationships among the three populations were assessed by calculating the Nei's genetic distance index between populations using the pairwise method (Nei 1972). As illustrated in Table 5, genetic identity between the three populations was very high as it ranged from 0.9953 to 0.9978.

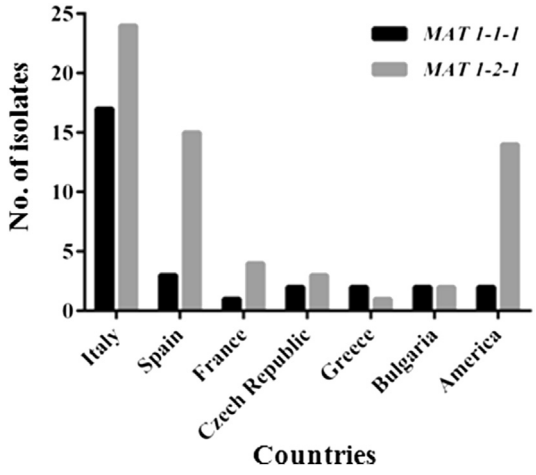

Fig 1 – Distribution of MAT idiomorphs in *P. xanthii* populations from European countries and the American continent. Mating type idiomorphs were molecularly identified by a Multiplex-PCR. Differences in MAT idiomorph distribution were observed, especially in the Italian population that showed a higher number of MAT 1-1-1 idiomorphs than Spanish and American populations.

Tests for random mating

Primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 were used to identify the MAT idiomorph (*MAT 1-1-1* or *MAT 1-2-1*, respectively) of the different *Podosphaera xanthii* isolates and thus to estimate the MAT frequencies in relation to the geographical origin of the isolates. MAT distribution of the 92 isolates in each country is illustrated in Fig 1. Analyzing the MAT frequencies of isolates used for AFLP analysis, differences were not statistically significant in Italian population as both chi-square and exact binomial tests revealed no deviations from the 1:1 ratio and the H_o was rejected both when the entire set and the clone-corrected sample was analyzed (Table 6). This is consistent with the records of abundant chasmothecia in this area (Pirondi 2015b). On the other hand, in the case of other European countries and the American continent, significant deviations in MAT frequencies were observed from 1:1 ratio as both chi-square and exact binomial tests rejected the H_o.

Two-locus LD analysis was performed on the three populations both on the entire and on the clone-corrected data set. Significant association among fragments was observed in each population but the null hypothesis of random association could not be rejected for many pairs of fragments in χ^2 tests (with 1 ° of freedom) in all three populations (Table 7). Of 595 tests for associations between pairs of fragments for 35 fragments in the entire sample of 22 isolates from Italy, significant association was observed since 94.78 % of tests of LD were significant ($P < 0.05$). However, when only the clone-corrected sample was analyzed (15 isolates), the number of significant pairwise comparisons was reduced to 67.22 %. Populations from other European countries and from the American continent showed lower percentages of significant associations among fragments than the Italian population in the entire samples but, as for the Italian population, the percentage was reduced when only the clone-corrected sample was analyzed. In particular, the null hypothesis of random association was rejected for 43.84 % and 31.37 % of pairwise comparisons between fragments of the clone-corrected samples of Europe and America respectively.

Discussion and conclusions

In this study, the genetic diversity of *Podosphaera xanthii* was investigated both by MLST and AFLP methods. Even if the two techniques are based on different methodologies, the

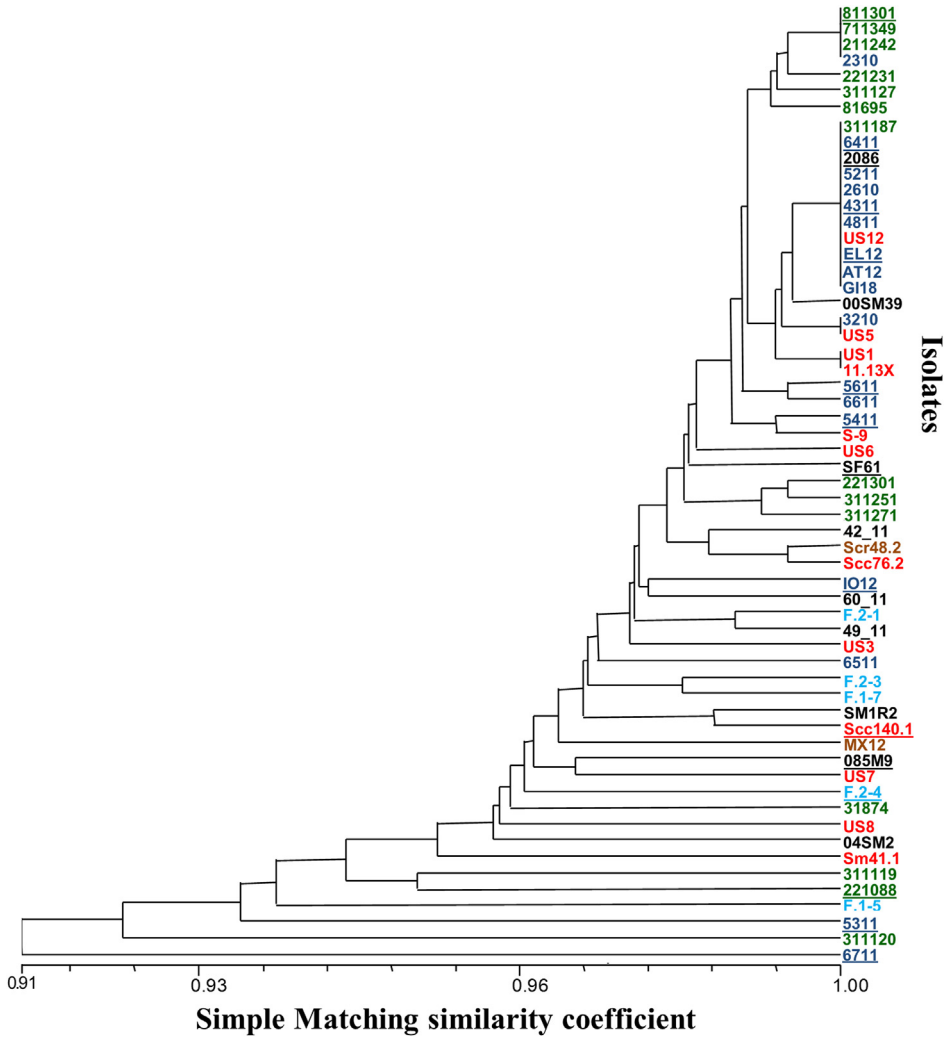

Fig 2 – Dendrogram obtained from cluster analysis showing genetic diversity of *P. xanthii* isolates. The binary matrix was converted to a distance matrix by NTSYS software and then cluster analysis was performed using UPGMA algorithm with simple matching similarity coefficient. Genetic similarity within isolates was very high as it was distributed between 0.91 and 1.00. The colours of the isolates denote their different geographical origin: North of Italy (dark blue), South of Italy (light blue), Spain (green), other European countries (black), North America (red), Central and South America (brown). Moreover, underlined isolates represent *MAT* 1-1-1 idiomorphs while *MAT* 1-2-1 idiomorphs are in plain text.

Table 3 – Genetic diversity of *P. xanthii* populations from Italy, other European countries and the American continent estimated by AFLP method. The parameters were calculated using Popgene Version 1.31 (Yeh & Boyle 1997; http://www.ualberta.ca/~fyeh/).

Population	Population size	na[a]	ne[b]	*h*[c]	*I*[d]	Polymorphic loci	*P* (%)
Italy	22	1.2071	1.0375	0.0298 (0.0693)	0.0550 (0.1193)	35	20.71
European countries	23	1.2189	1.0375	0.0296 (0.0689)	0.0553 (0.1176)	37	21.89
America	14	1.1065	1.0331	0.0237 (0.0764)	0.0397 (0.1228)	18	10.65
Total	59	1.3550	1.0365	0.0304 (0.0600)	0.0614 (0.1051)	60	35.5

Standard deviation is indicated in brackets.
a na: observed number of alleles.
b ne: expected number of alleles.
c h: Nei's gene diversity. Standard deviation is indicated in brackets.
d *I*: Shannon's information index.

Table 4 – Genetic diversity and differentiation between *P. xanthii* populations from European and American continents.

Group	H_t[a]	H_c[b]	H_s[c]	G_{st}[d]	G_{sc}[e]
Europe	0.0312	–	0.0278	0.1079	–
America	0.0209	–	0.0177	0.1497	–
Total	0.0285	0.0238	0.0271	0.0516	0.1208

a H_t total genetic diversity.
b H_s diversity within populations.
c H_c diversity within groups.
d G_{st} diversity among populations calculated as (Ht-Hs)/Ht.
e G_{cs} diversity among groups calculated as (Ht-Hc)/Ht.

obtained results were congruous. The high genetic similarity within isolates demonstrated by AFLP analysis is in agreement with the absence of genetic diversity in housekeeping gene fragments determined by MLST. Results from both methodologies suggest that *P. xanthii* populations have a very low genetic diversity as no genetic differentiation was observed between the three populations analysed. These results are in clear contrast to those obtained using MLST in other powdery mildew fungi such as *Blumeria graminis* (Inuma *et al.* 2007) and *Erysiphe necator* (Brewer & Milgroom 2010).

Given the absence of genetic variation observed in the populations of *P. xanthii* by MLST, genetic diversity was addressed by the AFLP technique. Using 59 of the 92 isolates analysed by MLST, genetic similarity proved to be again very high as it ranged between 0.91 and 1.00. Unlike the AFLP study by Naruzawa *et al.* (2011) performed only on Brazilian strains, the isolates analyzed in this study showed a higher similarity. The dendrogram obtained from cluster analysis did not group isolates according to geographical origin, host plants, climate areas, cultivation systems or mating types. Considering the epidemiology of this pathogen and the role that winds may play in the spread of this species, the absence of separate geographical groups could make sense. Accordingly, differences among isolates are very small as they are all distributed in a very narrow range of similarity coefficients. The low diversity shown by cluster analysis was also confirmed by the estimation of the genetic diversity parameters as genetic diversity within and among populations was also very low. These data suggest the presence of a predominantly clonal population structure. However, even if the sexual stage of *P. xanthii* is considered to be rare worldwide, chasmothecia were easily collected in the North of Italy and no deviations from MAT ratio 1:1 were observed in the area by Pirondi (2015b), suggesting the presence of actively mating populations. Accordingly, in tests for random mating, no deviations from MAT 1:1 ratio were observed in populations from Italy. On the contrary, MAT frequencies from populations of other European countries and from the American continent deviated significantly from the 1:1 ratio and rejected the H_o, in accordance with the rare or sporadic recording of the sexual stage in these areas (McGrath *et al.* 1996; Krístková *et al.* 2009; Pérez-García *et al.* 2009; Miazzi *et al.* 2011).

Table 5 – Nei's original measures (Nei 1972) of genetic identity (above diagonal) and genetic distance (below diagonal).

Population	Italy	Europe	America
Italy	–	0.9978	0.9953
Europe	0.0022	–	0.9970
America	0.0047	0.0030	–

Table 6 – Mating type distributions of *P. xanthii* populations analyzed by AFLP and LD. The numbers in brackets indicates the frequency of each idiomorph.

Populations analyzed by AFLP and LD	Mating type distributions[a]					
	N	MAT 1-1-1	MAT 1-2-1	χ^{2b}	p[c]	p[d]
Italy						
Uncorrected	22	9 (0.41)	13 (0.59)	0.73	0.39	0.262
Clone-corrected	15	7 (0.47)	8 (0.53)	0.07	0.80	0.598
European countries						
Uncorrected	23	5 (0.22)	18 (0.78)	7.35*	0.007	0.005*
Clone-corrected	18	3 (0.17)	15 (0.83)	8.00*	0.005	0.004*
America						
Uncorrected	14	1 (0.07)	13 (0.93)	10.29*	0.001	0.001*
Clone-corrected	11	1 (0.09)	10 (0.91)	7.36*	0.007	0.006*

a Asterisk (*) indicates deviation from mating type 1:1 ratio ($P < 0.05$).
b χ^2 value based on a 1:1 ratio with 1 ° of freedom.
c Probability of a greater χ^2 value under the null hypothesis of 1:1 ratio (1 ° of freedom).
d Probability value as obtained with an exact binomial analysis to test whether mating type frequencies deviate significantly from a 1:1 ratio.

LD analysis among pairs of loci was performed on isolates from the three populations both on the entire set and, to better underline the genetic structure caused by sexual reproduction, on the clone-corrected sample. Results showed the presence of significant LD in all three populations. However, when clone-correction was applied, the percentage of significant LD decreased and the 32.78 %, 56.16 %, and 68.63 % of the pairwise associations were not significant in populations from Italy, Europe and America respectively. This is probably caused by the loss of statistical power, as with a very small sample size, the clone-correction reduces the power to reject the null hypothesis and this affected the robustness of LD analysis. For this reason, it could be interesting to analyze more populations coming from European countries where chasmothecia were sporadically recorded such as Bulgaria (Velkov & Masheva 2002), the Czech Republic (Krístková *et al.* 2009) and Greece (Vakalounakis & Klironomou 1995). This could contribute to better understand the contribution of sexual recombination to the observed random associations. Moreover, it is important to observe that when clone-correction was applied, the percentage of significant LD in the Italian population decreased considerably, demonstrating the importance of asexual reproduction even in the presence of chasmothecia.

The lack of genetic diversity revealed by MLST could be caused by genetic drift. This evolutionary force may have occurred in *P. xanthii*, for example when the pathogen became pathogenic to cucurbits as a consequence of a founder effect.

Table 7 – Percentage of association among pairs of fingerprint fragments in both entire and clone-corrected samples.

Population	No. of isolates	No. of fragments	No. of pairwise comparisons	% of significant comparisons
Italy				
Entire	22	35	595	94.78
Clone-corrected	15	35	595	67.22
Europe				
Entire	23	37	666	55.25
Clone-corrected	18	37	666	43.84
America				
Entire	14	18	153	44.44
Clone-corrected	11	18	153	31.37

This process, which causes genetic loss, may have occurred relatively recently, thus being responsible for the lack of genetic diversity observed in housekeeping genes. Fungi of the *Erysiphaceae* family probably originated during the Cretaceous period (Mori *et al.* 2000a 2000b; Takamatsu & Matsuda 2004; Takamatsu *et al.* 2010). Within the five tribes forming the family, tree-parasitic fungi take basal position and herb-parasitic fungi have derived positions (Takamatsu *et al.* 2010), suggesting that the early host plants of the *Erysiphaceae* were trees (Mori *et al.* 2000a, 2000b) and multiple host shifts from trees to herbs may have then occurred during the Tertiary period (Takamatsu & Matsuda 2004). Also, in the last decade, the species infecting the Cucurbitaceae family was considered a separate species from *Podosphaera fusca* from Asteraceae and named *P. xanthii* (Braun & Takamatsu 2000; Braun & Cook 2012). A similar situation, with no genetic diversity shown in housekeeping genes, has been observed in *Fusarium oxysporum* f.sp. ciceris, where no variations were observed using five housekeeping gene markers (Jimenez-Gasco *et al.* 2002). An interpretation given by authors to explain the lack of variation is that this species is thought to derive from a small founder population that became pathogenic to *Cicer* spp. This could also be the case of *P. xanthii* that may be a pathogen of a recent speciation on cultivated cucurbits.

The identity of some AFLP bands with transposable elements (TEs) is another factor that should be considered in understanding the genetic structure of *P. xanthii*. The genomes of powdery mildews are full of TEs that, for example, account for 64 % of the genome size of *B. graminis*, which seem to be key elements for the evolutionary success of these pathogens (Spanu *et al.* 2010). Furthermore, the genomes of these obligate biotrophic pathogens are characterized by the deficiency in several classes of conserved primary and secondary metabolism genes such as the nitrate and sulfate assimilation pathways and plant cell wall hydrolytic enzymes (Spanu *et al.* 2012). These hallmarks may represent a trade-off between advantages of increased genetic variation independent of sexual recombination and irreversible deletion of genes dispensable for biotrophy (Spanu *et al.* 2010). Like *B. graminis*, it could be possible that the genomes of *P. xanthii* could be similar in size and that the genetic variation could be in part due to TEs. To support this hypothesis, three of the AFLP polymorphic fragments sequenced showed a high identity with transposons and polyproteins associated with TEs. Accordingly, two TEs have also been found to be associated with the only two full-length genes cloned so far from *P. xanthii*, the *CYP51* and *TUB2* genes (A.P., pers. comm.). Our hypothesis is that TEs could also be essential for the rapid adaptation of *P. xanthii* to overcome the deployment of new resistant cultivars.

Our results suggest that the populations of *P. xanthii* are likely to be predominantly clonal. From an epidemiological perspective, asexual reproduction is the most important system of reproduction and spread of the pathogen. In addition, producing a large amount of conidia and fungal biomass, represents an important source of inoculum for the dispersion of the pathogen that can be efficiently spread by the wind even over long distances (Bardin *et al.* 1997; Pérez-García *et al.* 2009; Miazzi *et al.* 2011).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.05.003.

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