Alma Mater Studiorum – Università degli Studi di Bologna

FACOLTÁ DI AGRARIA

Dipartimento di Scienze Agrarie (DipSA)

DOTTORATO DI RICERCA IN

ECOLOGIA MICROBICA E PATOLOGIA VEGETALE

Ciclo XXV°

Settore/i scientifico-disciplinare/i di afferenza:

AGR/12 PATOLOGIA VEGETALE

TITOLO TESI:

Epidemiology and population genetics of *Podosphaera fusca* and *Golovinomyces orontii*, causal agents of cucurbit powdery mildew

Presentata da: Alessandro Pirondi

Coordinatore dottorato

Prof. Paolo Bertolini

Tutor e relatore

Prof. Agostino Brunelli

Correlatore

Prof. Alejandro Pérez-García

Esame finale anno 2013

to *Beatrice* and *Nerina*

“I tell young people: Do not think of yourself, think of others. Think of the future that awaits you, think about what you can do and do not fear anything.”

Rita Levi Montalcini

TABLE OF CONTENTS

General introduction

1. Importance and origin of cultivated cucurbits

Cucurbits belong to the family *Cucurbitaceae* and consist of about 118 genera and 825 species, according to the last taxonomic treatment of Jeffrey (1990). Cucurbits are present in both the New and Old World and are among the most important plant families that supply human with edible products and useful fibers. Cucurbits are divided into five sub-families: *Fevilleae*, *Melothrieae*, *Cucurbitaceae*, *Sicyoideae*, and *Cyclanthereae*. The most important cultivated genera are *Cucurbita* L., *Cucumis* L., *Citrullus* L., *Lagenaria* L., and *Luffa* L., found in the sub-family *Cucurbitaceae*, and *Sechium* L., found in the sub-family *Sicyoideae* (Whitaker and Davis, 1962). Among the cucurbits, watermelon is the most popular in the world. The United Nations' Food and Agriculture Organization (FAO) estimated an average annual area of cultivation of 2.5 million ha and an annual production of 46.6 million tons of watermelon fruits between 1996 and 1998. Next in total world production were cucumber, melon, squash and pumpkins. In terms of countries, China is the leading producer of major cucurbit crops followed by Turkey, Iran and Ukraine. In the Americas, Argentina is an important producer of squash and pumpkins and the United States is an important producer of cucumber, melon and watermelon (FAO, 1998). The most important cucurbits in Brazil are squash, watermelon and melon, whose total production in 1995 was 535 million fruits harvested from an area of 206,000 ha (IBGE, 1996).

Although cultivated cucurbits are very similar in above ground development and root habit, they are extremely diverse for fruit characteristics. Fruits are eaten when immature (summer squash) or mature (watermelon). Fruits can be baked (squash), pickled (cucumber), candied (watermelon), or consumed fresh in salads (cucumber) or dessert (melon). Also, seeds, flowers (squash and pumpkins) and roots (chayote) are consumed by humans. Cucurbits are also produced for other uses than food. Fruits (bottle gourd) are used for storage, drinking

containers, bottles, utensils, smoking pipes, musical instruments, gourd craft decoration, masks, floats for fish net, and other items. The fiber of a mature loofah fruit can be used as a sponge for personal hygiene, household cleaning and various other purposes, including filtration. Seeds or fruit parts of some cucurbits are reported to possess purgatives, emetics and antihelmintics properties due to the secondary metabolite cucurbitacin content (Robinson and Decker-Walters, 1997). Therefore, cucurbits are among the largest and the most diverse plant families, have a large range of fruit characteristics, and are cultivated worldwide in a variety of environmental conditions (Bisognin, 2002).

Cucurbits are associated with the origin of agriculture and human civilizations and are also among the first plant species to be domesticated in both the Old and the New World. Archaeological records of the New World suggest that *Cucurbita* was one of the first plants to be domesticated (NEE, 1990). One of the first species to be domesticated in the New World was *C. pepo*. The origin and early spread of all *Cucurbita* species was in the Americas. According to a recent comprehensive biosystematic monograph of Kirkbride (1993), the genus *Cucumis* includes 32 annual and perennial species divided in two very distinct groups defined by geographic origin and chromosome number (African group $2n$ = 24 and Asiatic group $2n$ = 14 chromosomes): the African group includes melon (*C. melo*) and the Asiatic group includes cucumber (*C. sativus*). The genus *Citrullus* consists of eight species and sub-species. The most economically important *Citrullus lanatus* (watermelon) originated in Africa and India (Mallick and Masui, 1986).

2. Cucurbit powdery mildew disease

Within the over 200 disease affecting cucurbits (Zitter *et al.*, 1996), powdery mildew is considered the most important widespread disease limiting the cucurbit production. Even there are some indications of records of *Leveillula taurica* (Lév.) G. Arnaud (1921) as a causal species of cucurbit powdery mildew (El Ammari and Wajid Khan, 1983; Branzanti

and Brunelli, 1992; Vakalounakis *et al.*, 1994), the most common pathogens causing the disease are two obligate biotrophic ascomycetes fungi: *Podosphaera fusca* (syn. *Podosphaera xanthii*) and *Golovinomyces orontii* (syn. *Golovinomyces cichoracearum*). Symptoms induced by both pathogens are identical: a white powdery fungal mass composed by mycelia and conidia that appears generally first on lower and subsequently on upper leaf surface, petioles, stems (Figure 1) and rarely on fruits (Perez-García *et al.*, 2009). With favorable environmental conditions such dry weather, temperature of 20-27 °C, low light intensity, dense plant growth and high fertility (Hansen, 2009), the colonies grow and develop very rapidly and reduce photosynthesis, causing yellowing and sometimes death of plants (Pérez-García *et al.*, 2009).

Figure 1 Symptoms of powdery mildew infections on zucchini (a, c, d) and melon (b).

2.1 Taxonomy and host range

Both powdery mildew species belongs to Phylum *Ascomycotina*, Subdivision *Pezizomycotina*, Class *Leotiomycetes*, Order *Erysiphales* and Family *Erysiphaceae*. The nomenclature of both pathogens has been changed many times over the years.

Podosphaera fusca

P. fusca belongs to tribe Cystothecae and in the past was named *Sphaerotheca fuliginea* and *Sphaerotheca fusca* before the enclosing of the genus *Sphaerotheca* into *Podosphaera* on the basis of new molecular data (Braun and Takamatsu, 2000). Host range include families such *Asteraceae*, *Scrophulariaceae*, *Solanaceae*, *Lamiaceae*, *Verbenaceae* and *Cucurbitaceae* (Pérez-García *et al.*, 2009). Braun (2001) proposed a separation of *Podosphaera fusca* from *Podosphaera xanthii* based on morphological features of the teleomorph: on cucurbits it seems that the fungus has large ascomata (75-100 µm) and large oculus (15-30 µm) and thus the cucurbit pathogen has been named *P. xanthii*. However, some authors, retain that define a species based just on morphologically features is not satisfactory (Moncalvo, 2005). In the case of *P. xanthii*, to define the species based on features of the teleomorph stage it is simply not correct because the presence of chasmothecia is considered rare and they were never observed in many areas and also because ascospores from fruit bodies produced in laboratory were not able to infect cucurbits (McGrath, 1994). Furthermore, molecular data based only on internal transcribed spacer (ITS) sequences are not sufficient to support a species separation, since a multi-gene approach has to be considered for recognition a fungal species. Thus, in the absence of new molecular data and host range experiments, *P. xanthii* is considered as a synonymous with *P. fusca* for many authors (Pérez-García *et al.*, 2009).

Golovinomyces orontii

In the past, the pathogen was included in the genus *Erysiphe* and thus named *Erysiphe cichoracearum*. After that, the definition of the genus *Golovinomyces* as a section of the

genus *Erysiphe* named the pathogen *Golovinomyces cichoracearum*. Subsequently, based on anamorphic features and as a result of molecular phylogenetic studies the section was raised to the generic level (Braun, 1999) and *Golovinomyces* was moved to tribe Golovinomycetae (Braun and Takamatsu, 2000). The latter consist of three genera: *Golovinomyces*, *Neoerysiphe* and *Arthrocladiella*. Host range cover many families as *Asteraceae* (more than 1000 hosts), *Bolaginaceae*, *Scrophulariaceae*, *Solanaceae* and *Cucurbitaceae*. *Golovinomyces orontii* was distinguished from *G. cichoracearum* by the presence of curved foot cells at the base of the conidiophores (Cunnington *et al.*, 2009). However, the presence of these curved cells was described in *G. cichoracearum* (Braun, 1987). In fact some authors, like Shin (2000), did not recognize *G. orontii* as a species and continued to name the fungus *G. cichoracearum*. As the case of *P. xanthii*, morphological characters are not sufficient to discriminate at level of species and thus, the two names are considered as synonymous.

2.2 Biology

Both species, like other powdery mildews, are obligate biotrophic pathogens. The mycelium is totally epiphytic and is hyaline, septate and thin-walled. In particular the hyphae of *P. fusca* turn brown and become more or less thick-walled with age. From hyphae and at the end of the conidial germ tubes are formed structures called appressoria that allow mycelium to attach to the host. *P. fusca* has an indistinct appressorium characterized by a widening of the hyphae (Boeswinkel, 1977) that differ from that of *G. orontii* that is distinct and nipple-shaped. From the center of attachment of the appressorium, originates and arise the haustorium, a penetration hypha that enters the epidermal cells of host by enzymatic degradation of the cuticle and wall and mechanical penetration.

Asexual spores are called conidia. They are one-celled, uni-nucleate and vacuolated, and represent the anamorphic or imperfect stage of the fungi. They are produced singly or in

chains at the tip of structures called conidiophores that arise from superficial hyphae. The basal cell of conidiophores is called foot-cell.

Conidia of the two species differ in shape and size:

- *P. fusca*: elliptical or spherical and measure 25-37 × 14-25 µm (Figure 2a)

-*G. orontii*: oval or cylindrical and measure 25-45 × 14-26 µm (Figure 2b)

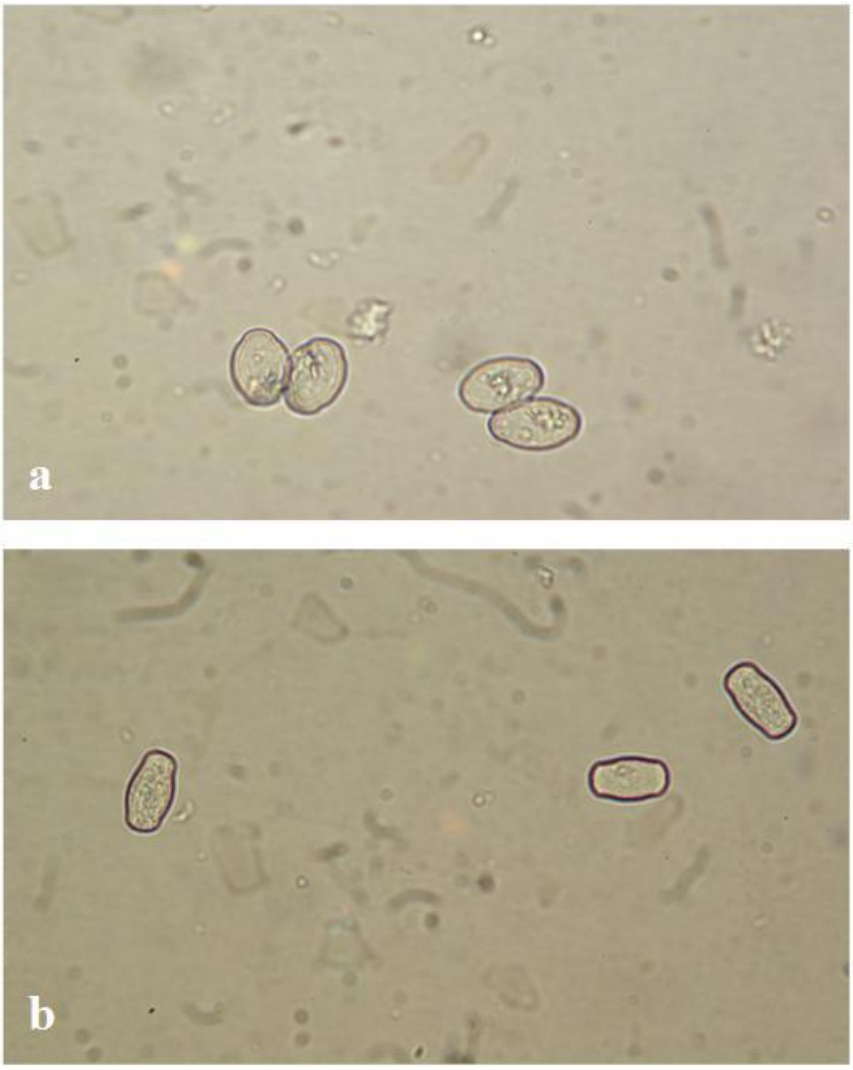

Figure 2 Conidia of the powdery mildew fungi *P. fusca* (a) and *G. orontii* (b).

Furthermore, conidia of *P. fusca* present peculiar cell inclusions called fibrosin bodies (Figure 3). These particles are made of lipids (Kiss *et al.*, 2011) and are refractive when

observed at light microscope in a 3% potassium hydroxide solution (Kable and Ballantyne, 1963). This characteristic is typical of the genera *Podosphaera*, *Cystotheca* and *Sawadaea*.

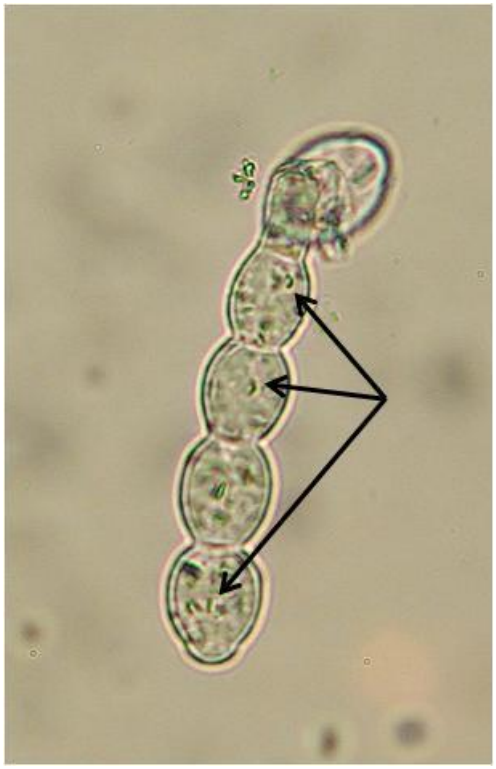

Figure 3 Presence of fibrosin bodies in conidia of *P. fusca*.

The two species differs also in the mode of conidia germination (Lebeda, 1983):

- *P. fusca*: short, often lateral forked germ tube without conspicuous appressoria (Figure 4a).

- *G. orontii*: single germ tube from the apical part of the conidia with a club-shaped appressoria (Figure 4b).

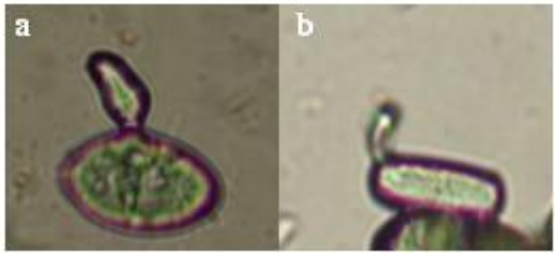

Figure 4 Conidial germination in *P. fusca* (a) and *G. orontii* (b).

Like the majority of all *Ascomycete* fungi, both powdery mildew species produce sexual spores (ascospores) that are contained in a sac called ascus that represents the teleomorph or perfect stage.

The asci of the major part of ascomycetes are produced in a fruit body called ascocarp, formed by an external wall (peridium) and by an internal layer (hymenia). Ascocarp of ascomycetes can be differentiated in:

- Apothecium: asci are produced in an open-, cup- or saucer-shaped ascocarp (*Discomycetes*).

- Pseudothecium: asci are formed directly within a stroma of mycelium (L*oculoascomycetes*).

- Perithecium: asci are contained in an ascocarp that is more or less close but at maturity has an opening (ostiole) to release the ascospores (*Pyrenomycetes*).

- Cleistothecium: the ascocarp is spherical and completely close having no predefined opening. The asci are irregular arranged and are discharged by the decay of the peridium.

In the case of the powdery mildews, the ascocarp is completely close without an ostiole but the internal structure is rather perithecium-like because asci are regularly arranged in hymenia fascicles. Furthermore, in powdery mildew fungi asci are not discharged by decay of the fruit body but the latter is ruptured by swelling asci that causes a peculiar dehiscence of the ascocarp by vertical slits. The name proposed for this particular kind of ascocarp is chasmothecium (Braun *et al.*, 2002).

Chasmothecia of the two cucurbit powdery mildew species differ in size and number of asci and ascospores (Lebeda, 1983):

- *P. fusca*: 65-98 µm in diameter and contains 1 ascus with 8 hyaline ascospores (Figure 5a).

- *G. orontii*: 80-140 µm in diameter with 10-15 asci containing 2-3 ascospores (Figure 5b).

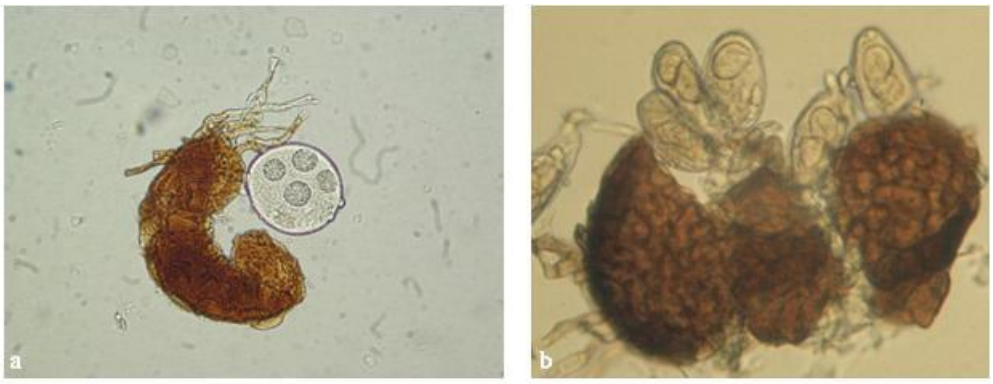

Figure 5 Chasmothecia of *P. fusca* (a) and *G. orontii* (b). Image "b" was taken from http://website.nbm-mnb.ca/mycologywebpages/NaturalHistoryOfFungi/DiscomycetousPlectomycetes.html.

Several studies conducted between the end of the 19th century and the middle of the 20th century suggested a great diversity of the sexual processes and attracted controversy over a long period (Braun *et al.*, 2002). Ali (1988), Dörfelt *et al.* (1989) and Dörfelt and Ali (1996) carrying out detailed examinations using both light (LM) and transmission electron (TEM) microscopy, provided comprehensive interpretations of the sexual reproduction and ascoma development of powdery mildews. In particular, Dörfelt and Ali (1996) recognized three main developmental types: sexual types (mono and polyascus types), pseudosexual types and non-sexual types. The results obtained by the same authors can be summarized as follows: the sexual processes and the ascoma development are generally uniform in all powdery mildews. Uninucleate, morphologically differentiated, "sexual organs" (gamocysts) are formed as lateral branchlets of the mycelium. The ascogonium (gymnogamocyst) and antheridium (androgamocyst) encircle each other or orient themselves closelly parallel. Cystogamy occurs and the nucleous of the androgamocyst migrates into the ascogonium which becomes and remain dicaryotic). Karyogamy have never been observed (the two nuclei are, however, always close to each other and simulate a single nucleus; this phenomenon is undoubtedly the reason for the report of karyogamy by numerous authors). Dikaryotization is followed by divisions of the nuclei (in *Podosphaera* sect. *Sphaerotheca*,

the dikaryon remains for a relatively long time; in other genera with numerous asci, the division of nuclei is immediately initiated after transfer of the nucleus into the ascogonium). Immediately following the division of the nuclei, surrounding hyphae arise from the haploid "stalk cells" of the ascosonium and initiate the peridium of the fruitbody. Later, the multinucleate ascogonium is divided into a variable number of cells that are irregularly arranged. A single dikaryotic cell of the secondary ascogonium (monoascus type) or several cells (polyascus type) develop into asci. After meiosis and mitosis, haploid nuclei surrounded by cytoplasm and enveloped by a wall inside the ascus are formed: the ascospores (Braun *et al.*, 2002).

2.3 Life cycle and epidemiology

Like other powdery mildew fungi, the two cucurbit powdery mildew species reproduce both sexually and asexually (Figure 6). The asexual cycle begins with the dispersion of conidia (usually by wind) from infected leaves. When conidia arrive on a susceptible host they produce a germ tube from which is differentiated a primary appressorium and a primary haustorium. A primary hypha is then formed from the primary appressorium (or from another pole of the conidium) and forms a secondary appressorium from which a secondary haustorium is formed. Subsequently, the primary hyphae originate the secondary hyphae from which arise the conidiophores with conidia at the tip. Secondary hyphae together with conidia form the white mycelia, typical of the powdery mildew fungi (Pérez-García *et al.*, 2009).

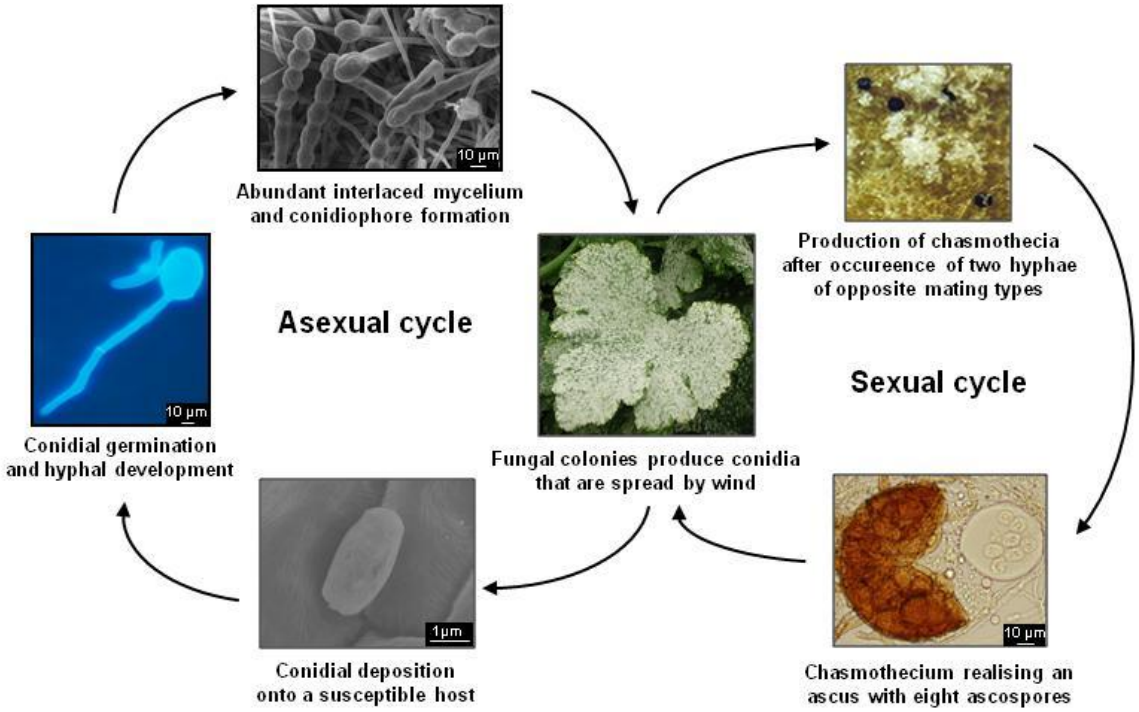

Figure 6 Life cycle of powdery mildew fungi (taken from Pérez-García *et al.*, 2009).

Regarding the sexual cycle, both species are heterothallic, forming chasmothecia when two hyphae of opposite mating type are encountered (McGrath, 1994). When chasmothecia releases either an ascus (*P. fusca*) or several asci (*G. orontii*) with ascospores inside, these are dispersed like conidia and can infect a susceptible host. Chasmothecia are produced on infected leaves or stems when the plants become senescent and usually represent the overwintering stage, with the ascospores being the cause of the primary infections. Then, these infections produce conidia that cause secondary infections during the growing season. However, the fungus may overwinter as mycelia or conidia. Chasmothecia of *P. fusca* have been observed in Germany (Ulbirch and Smolka, 1994), Hungary (Nagy, 1976), rarely in United States (McGrath, 1994), Greece (Valounakis and Klironomou, 1994) and Czech Republic (Kristková *et al.*, 2009). By contrast, they were never reported on cucurbits in Spain (Álvarez and Torés, 1995) and southern Italy (Miazzi *et al.*, 2011). However, in North of Italy, chasmothecia of *P. fusca* were found in abundance in the 80's as reported by Branzanti and Brunelli (1987). Chasmothecia of *G. orontii* are considered to be rare: they

were observed just in Germany (Ulbirch and Smolka, 1994), Hungary (Nagy, 1976) and Czech Republic (Křístková *et al.* 2009). These observations suggest that the asexual stage seems to predominate in both species and which can be the epidemiological relevance of the sexual stage remains unclear.

2.4 Ecological requirements

Nagy (1976) studied the effects of temperature and relative humidity on germination of conidia and described the requirements of the two species. He observed that germination optimum for *G. orontii* was about 25°C with the minimum of 10-20°C and the maximum of 30°C. The optimum of *P. fusca* was 22°C with a minimum of 20°C and maximum of 30°C. Below the minimum temperature and above 30°C germinating conidia of both pathogens were rarely found. On the basis of these observations, *G. orontii* seems to be the species with the widest range of temperature in which germination could be affordable. Considering that, in general, dry conditions are favoring the process of colonization, sporulation and dispersal of powdery mildew fungi (Butt, 1978); in the same study some differences were found in moisture requirements of the two species. *P. fusca* was found to be more sensitive to moisture than *G. orontii* because it requires 100% of relative humidity for conidia germination and tolerates higher moisture content than *G. orontii*. By contrat conidia from *G. orontii* germinates under relative lower moisture.

2.5 Races and pathotypes

The two cucurbit powdery mildew species are also highly variable in their pathogenicity and virulence, as evidenced by the existence of a large number of different pathotypes and races (Bertrand *et al.*, 1992; Lebeda *et al.*, 2004; Lebeda and Sedlàkovà, 2006; McCreight, 2006; Lebeda *et al.*, 2007). Pathotypes of a fungus can cause differential reactions on plant species and cultivars with different levels of resistance. Differences in pathotypes can influence a

screening towards resistant plants. It is this knowledge on differential reactions towards fungal isolates that can discriminate the existence of pathotypes (Leus *et al.*, 2002). Race can be defined as a population of individuals the members of which are similar to other such populations on morphological grounds but differ on physiological or pathological grounds. In pathogenic fungi, races are identified on the basis of infections on different species of the host.

Pathogenic specialization in cucurbit powdery mildew is well known. There is often a very clear expression of compatibility or incompatibility in host plant–powdery mildew interactions that allows for the classification of pathotypes and races based on the reaction patterns of compatible and incompatible reactions on the differential hosts species or genotypes (Lebeda *et al.*, 2008, 2011). A unified, objective system for the determination, denomination, and classification of pathotypes and races on cucurbits remains to be codified (Lebeda and Sedlàkovà, 2006; McCreight, 2006; Lebeda *et al.*, 2007, 2008). Cucurbit powdery mildew pathotypes are based on intergeneric and interspecific differences in host–pathogen interactions. For pathotype determination in *P. fusca*, two cultivars of the major cucurbit crops are normally used (Bertrand, 1991; del Pino *et al.*, 2002; Lebeda *et al.*, 2008). Most of the isolates are able to infect zucchini and melons cultivars, crop species that are very sensitive to powdery mildew. Differential responses are mostly associated with cucumber and watermelon cultivars, crop species that are either tolerant or traditionally resistant to the fungus, respectively (del Pino *et al.*, 2002).

Cucurbit powdery mildew races are characterized by the interactions of different isolates of a pathogen with different genotypes of a given host species (Bertrand 1991; Pitrat *et al.*, 1998; Bardin *et al.*, 1999; Lebeda and Sedlàkovà, 2010). Races of *G. orontii* and *P. fusca* have, to date, been differentiated only on melon (Lebeda *et al.*, 2011). The most frequently used sets of melon differential lines include 11 genotypes of *C. melo* (Iran H, Védrantais, Top Mark, PMR 45, PMR 5,

WMR 29, Edisto 47, PI 414723, MR-1, PI 124111, PI 124112) that can differentiate races originating from melon (McCreight, 2006) and other cucurbits (Lebeda *et al.*, 2004; Lebeda and Sedlàkovà, 2006; Lebeda *et al.* 2007; Lebeda *et al.*, 2008; Lebeda and Sedlàkovà, 2010). To date, 2 races of *G. orontii* and 25 of *P. fusca* have been identified (Pitrat *et al.*, 1998; Bardin *et al.*, 1999; Hosoya *et al.*, 2000; Bertrand, 2002; McCreight, 2006). This diversity of races is a serious problem for powdery mildew control because represents a serious limitation for the use of varieties only resistant to a limited number of races. In consequence, although many commercial varieties have been released with resistance to *P. fusca*, the development of new races of the pathogen hinders disease management through resistance breeding.

2.6 Control

According to current management practices, an integrated approach for management of powdery mildew fungi should include the use of the following components: powdery mildew-tolerant cultivars; biorational compounds, fungicides, biological agents, and chemical compounds that stimulate plant resistance. Cultivating plants with genetic resistance to powdery mildew is the best method of growing disease-free cucurbit crops. Consequently, an appropriate selection of tolerant or resistant cucurbit cultivars is the simplest way to deal with powdery mildew infections (Nuñez-Palenius *et al.*, 2009). Unfortunately, as mentioned above, there are several races of cucurbit powdery mildew fungi and some powdery mildew-resistant cultivars might be susceptible to a specific race (Zitter *et al.*, 1996). Although great efforts have been invested in plant breeding programs, growers still have important concerns about disease control and fungicides or other control agents must be employed to counteract the relentless cycles of a powdery-mildew disease in those tolerant or resistant cultivars. So, in practice, application of fungicides continues to be the principal tool for managing powdery mildew in most cucurbit crops (McGrath, 2001).

Table 1 Chemistries approved for cucurbit powdery mildew control in Italy in 2012 (Valmori, I.)

Chemical group	Common name	Mode of action	
		Target site	Inhibited function
Inorganic	Sulphur	Multi-site contact activity	
Dinitrophenoles	Meptyldinocap	Uncoupler of oxidative phosphorylation	ATP formation
DMI fungicides	Difenoconazole	C14α-demethylase	Sterol biosynthesis
	Bitertanole		
	Fenbuconazole		
	Myclobutanil		
	Penconazole		
	Propiconazole		
	Tetraconazole		
	Triadimenol		
	Tebuconazole		
Hydroxy-(2 amino-) pyrimidines	Bupirimate	Adenosin deaminase	Nucleic acids biosynthesis
QoI fungicides	Azoxystrobin	Cytochrome bc_1 complex at Q_o site	Respiration
	Kresoxim-methyl		
	Trifloxystrobin		
Pyridine-carboxamides	Boscalid	Complex II: succinate dehydrogenase	Spore germination and germ tube elongation
Quinolines	Quinoxyfen	G-proteins (proposed)	Signal transduction
Amidoximes	Cyflufenamid	Unknown	

Intensive fungicide development has resulted in a large number of different fungicides and formulations, although the number of active ingredients available to growers is progressively decreasing for reasons such as fungicide resistance development or environmental protection (Table 1). Within traditional molecules, sulphur-based fungicides still have a good control of the disease. However, on some crops as cucumber, they have to be applied with caution because of phytotoxicity risk, especially on protected crops, in particular on cucumber

(Brunelli and Gengotti, 2007). As shown in the table, many of the active ingredients that can be used to combat cucurbit powdery mildew belongs to the class of DMI (DeMethylation Inhibitors) fungicides, which include the chemical group of triazoles that were introduced in the 80' and 90' (difenoconazole, fenbuconazole, myclobutanil, penconazole, propiconazole, tebuconazole, tetraconazole, triadimenol and fenarimol). Another molecule introduced in the same period was the hydroxypirimidin bupirimate. From the end of the 90' several molecules were introduced: quinoxyfen (phenylquinoline group), the very popular among growers strobilurin fungicides (azoxystrobin, tryfloxystrobin, kresoxym-methyl) or QoI (Quinone Outside Inhibitors) and boscalid (carboxamide group). More recently, new molecules as cyflufenamid (amidoxime group) and pyriofenone (phenyl ketone group) have been introduced to improve the chemical control arsenal against powdery mildew (Collina *et al.*, 2012).

A good control strategy has to consider the different modes of action of these molecules and their persistency. Generally, application of fungicides is done at the appearance of the first symptoms but preventive application is recommended. Treatments with products based on molecules with specific mode of action have a good persistency and generally are sprayed in a 10 days interval. Because cucurbits are characterized by a scalar harvest, a critical point is represented by the pre-harvest interval (PHI) that must be respected after treatments (Brunelli and Gengotti, 2007). Products with the shortest PHI interval are QoI-based products with 3 days and the recent cyflufenamid based product with 1 day (Myrta *et al.*, 2012). Because of the potential of *P. fusca* for fungicide resistance development, strict anti-resistance strategies, including limiting the treatments and the use of mixtures and alternations, are highly recommended when using single-site systemic fungicides against cucurbit powdery mildew. Preferably, these fungicides should be combined or alternated with multi-site fungicides that have a low resistance risk (McGrath, 2001). In fact, resistance to some of the above mentioned fungicides has been documented: DMI (Brunelli *et al.*,

2010; López-Ruiz *et al.*, 2010), QoI (Collina *et al.*, 2006, Fernández-Ortuño *et al.*, 2006; Collina *et al.*, 2012;) and boscalid (Miyamoto *et al.*, 2010; Ishii *et al.*, 2011; Collina *et al.*, 2012). Nowadays fungicide resistance is an important factor to consider when planning an efficient control strategy against cucurbit powdery mildew.

3. Population genetics in plant pathogens and evolutionary potential

Genetic structure of a species is defined as the amount and distribution of genetic variation within and among populations of that species, which is determined by the evolutionary history of populations. Genetic structure is the result of the interactions between 5 factors affecting the evolution of populations: genetic drift, gene flow, mutation, modes of reproduction and selection (McDonald and Linde, 2002). Mutation is the fundamental source of genetic variation because it leads to the creation of new alleles in populations. Regarding plant pathogens, populations with more alleles have higher genetic diversity and, therefore, more likely to create strains capable of overcoming resistance genes or develop resistance to fungicides. The population size may affect the probability that a mutant is present and influence the genetic diversity of a population through a process called genetic drift. Mutation rates are relatively constant and almost always quite low, large populations typically have higher gene diversity (more mutant alleles) than small populations. Genetic drift occurs when a population is subjected to a bottle neck (catastrophic event causing a severe reduction in population size) or a founder effect (such as when a small population of the pathogen colonizes a new host population), circumstances in which the frequency of mutant alleles in the surviving populations or founders may differ significantly from the frequency of the original population (McDonald and Linde, 2002).

Gene flow is the process by which certain alleles (genes) or individuals (genotypes) are exchanged between geographically separated populations. Gene flow, therefore, can substantially increase the size of a population through the increase in size of the "genetic

neighborhood" through which genes and genotypes exchange and facilitate movement of mutant alleles between individual populations of the pathogen. Therefore, plant pathogens that have a high degree of gene flow have higher genetic diversity because they present higher population size and tend to be those who produce propagules with capacity of long-distance dispersal (McDonald and Linde, 2002).

The mode of reproduction affects the way in which genetic diversity is distributed within and among populations. Reproduction can be sexual, asexual, or mixed, as it is the case of many fungi presenting both sexual and asexual reproduction. Many of the most destructive and dangerous plant pathogens show a combination of sexual and asexual cycles, which can generate high levels of gene and genotypic diversity. During the sexual cycle many new combinations of alleles (genotypes) are generated that can be tested in different environments, such as the presence of new gene combinations that confer resistance to fungicides. During the asexual phase most suitable genotypes are maintained through a clonal reproduction and may even increase their frequency. Temporal and spatial distribution of clones or clonal lines within or between populations will mainly depend on the capabilities of dispersal and survival of the asexual propagules. If spores or asexual propagules can disperse over long distances, then the clone with a higher capacity for survival may be widely distributed through a relatively quickly genotypic flow, causing an epidemic (McDonald and Linde, 2002)..

Finally, selection is the main force driving the changes in frequency of mutant alleles. For example, there is a strong directional selection with the intensive use of a new resistance gene or a new fungicide, which leads to an increase in the frequency of mutants fungicide-resistant or virulent (that have lost the elicitor complementary to the resistance gene). There are many examples of overcoming plant resistance genes and development of resistance to fungicides that demonstrate that selection is an effective evolutionary mechanism in the

majority of modern agro-systems that are based on the genetic uniformity of monocultures and intensive use of plant protection products (McDonald and Linde, 2002).

3.1. Risk assessment: Evolutionary potential of powdery mildew fungi

According to this approach, the pathogens of highest risk for agriculture are those presenting a higher evolutionary potential (higher genetic diversity). Once evaluated the evolutionary potential of a particular pathogen, disease management programs should be developed and designed to reduce the genetic diversity of the pathogen through the maintenance of low levels of pathogen populations; limiting the movement of genes and genotypes among populations; limiting the occurrence of sexual reproduction or the persistence and distribution of asexual propagules; using cultivars carrying various resistance genes or by the alternation of cultivars carrying different resistance genes; and above all diversifying the use of fungicides. Among the plant pathogens with the highest evolutionary risk, the powdery mildews can be highlighted because in them, the 5 evolutionary forces are set out clearly (McDonald and Linde, 2002). During most of the growing seasons they reproduce asexually in a prolific manner through the formation of conidia that are dispersed by the wind over long distances (Bardin *et al.*, 1997; Pérez-García *et al.*, 2009; Miazzi *et al.*, 2011). They also have a sexual stage (teleomorph) which leads to the formation of chasmothecia, structures containing ascospores and ultimately responsible for genetic variability. Since disease control is practically reduced to the employment of resistant cultivars and of repeated application of fungicides (McGrath, 2001; Brunelli and Gengotti, 2007; Nuñez-Palenius *et al.*, 2009 Pérez-García *et al.*, 2009), they are subject to a strong selection. Finally, although mutation rates are unknown, it is known that, in many cases, fungicide resistance is linked to mutations in target genes (Bartlett et al., 2002; Miyamoto *et al.*, 2010).

3.2. Tools and techniques of population genetics

In many areas of investigation, the precise identification, discrimination and characterization of fungal species and populations are of relevant importance. However, sometimes this is difficult to achieve because this characterization is done mainly on the basis of morphological and biochemical criteria. Molecular markers have become part of a repertoire of tools needed to assess the amount of genetic variation within populations (Rotondo, 2011). In recent years, molecular markers and especially DNA-based markers, have been extensively used in many areas such as gene mapping and tagging (Karp and Edwards, 1997; Kliebenstein *et al.*, 2001), characterisation of sex (Flachowsky *et al.*, 2001), analysis of genetic diversity (Godt and Hamrick, 1999; Lerceteau and Szmidt, 1999; Martinez-Palacios *et al.*, 1999; Erschadi *et al.*, 2000) or genetic relatedness (Brookfield, 1992; Roa *et al.*, 1997;).

According to Stansfield (1986), the term "marker" is usually used for "locus marker". Each gene has a particular place along the chromosome called "locus". Due to mutations, genes can be modified in several forms mutually exclusives called "alleles" (or allelic forms). All allelic forms of a gene occur at the same locus on homologous chromosomes. When allelic forms of one locus are identical, the genotype is called "homozygote" (at this locus), whereas different allelic forms constitute a "heterozygote". In diploid organisms, the genotype is constituted by the two allelic forms of the homologous chromosomes. Thus, molecular markers are all loci markers related to DNA (markers can also be biochemical, or morphological).

According to Solè (2003), a good molecular marker should be/have:

1- Mendelian inheritance: transmit from one generation to another.

2- Polymorphic: present several alleles at the locus investigated (multiallelic).

3- Codominant: allow the discrimination between homo and heterozygotes.

4- Neutral: all alleles have the same fitness.

5- Not epistatic: one can determine the genotype of a phenotype irrespective of the genotype of the other loci.

6- Independent of environment: no phenotypic plasticity.

7- Frequent occurrence in the genome.

8- Even distribution throughout the genome.

9- Highly reproducible.

An outstanding advantage of this molecular approach is the immense amount of potential data that markers provide (Avise, 1994). Moreover, rates of evolution of different parts of the genome are extremely variable, allowing molecular data to be applicable at any taxonomic level. Both dominantly (e.g. AFLP, RAPD, and ISSR) and codominantly inherited markers (e.g. allozymes, microsatellites) have been used to study population genetics and life history traits in many species. Among these, polymerase chain reaction (PCR)-derived markers obtained with non-species specific primers have become exceedingly popular since they do not request sequence information for the target species (Rotondo, 2011).

The first and so far most commonly used method in this group of techniques is called RAPD (Random Amplification of Polymorphic DNA) and was introduced in 1990 (Weir *et al.*, 1998, Pryor and Gilbertson, 2000; Roberts *et al.*, 2000; Pryor and Michailides, 2002). A few years later, the relatively similar techniques designated ISSR (Intersimple Sequence Repeats) (Hong et al., 2006; Park *et al.*, 2008) and AFLP (Amplified Fragment Length Polymorphism) (Vos *et al.*, 1995) were introduced. However, despite of the obvious advantages of these methods related to the efficient and quick PCR amplification of polymorphic DNA fragments starting from small amounts of template, they have a number of limitations in the interpretation of the multi-band profiles produced. For example, heterozygotes cannot be detected because of their dominant nature and homology of

comigrating bands cannot be assigned certainly. From a technical point of view, competitive priming and the occurrence of artefactual bands produced by nested primer annealing or interactions within and between DNA strands during PCR, still remain potential problems (Halldén *et al.*, 1996; Rabouam *et al.*, 1999). The difficulty of achieving robust profiles, particularly in RAPDs, may make the reliability of these markers somehow questionable, but the reproducibility of RAPD analysis can be enhanced through improved laboratory techniques and band scoring procedures (Hansen *et al.*, 1998), meanwhile AFLP and ISSR are less affected by the problem of reliability than RAPD because longer primers and higher annealing temperatures are employed (Zietkiewicz *et al*., 1994; Vos *et al.*, 1995). In data compilations, estimates of genetic variation obtained with different types of dominant markers (AFLP, RAPD, ISSR) proved to be quite similar in magnitude, both for within and among populations (Meng and Chen, 2001).

3.2.1. Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a relatively fast, cheap, easy, and reliable method to generate hundreds of informative genetic markers (Vos *et al.*, 1995; 1997). The method is based on the observation of DNA polymorphisms. These polymorphisms are a result of point mutation or rearrangements (insertions, deletions, etc) in the DNA and are detected by scoring band presence versus absence in banding patterns. Because of that multiple loci can be analyzed in one experiment, AFLP can only be used to study dominant genetic markers. However, the main advantage of AFLP technique is its capacity of analyzing simultaneously many DNA regions distributed randomly throughout the genome. To achieve high reliability of the screen, genomic DNA is prepared in a way that combines the strengths of two methods: the repeatability of restriction fragment analysis and the power of the PCR (Vos *et al.*, 1995; 1997). AFLP is also a very reliable and robust

technique, which is unaffected by small variations in amplification parameters (e.g. thermal cyclers, template concentration, PCR cycle profile).

AFLP markers can be generated from DNA of any organism, and no initial investment in sequence analysis is required. Small amounts of DNA (~500 ng) are digested with a combination of one rare cutter and frequent cutter enzymes. Then, the adaptors, designed on the base of restriction site blunt ends, are ligated and so, the restriction sites are not reconstituted. Two subsequent PCR reactions are performed (pre-amplification PCR and selective PCR). The first is performed with no extension or a single-bp extension, followed by a more selective primer with up to a 3-bp extension (Figure 7). Generally, the band profiles are separated using polyacrylamide gel electrophoresis.

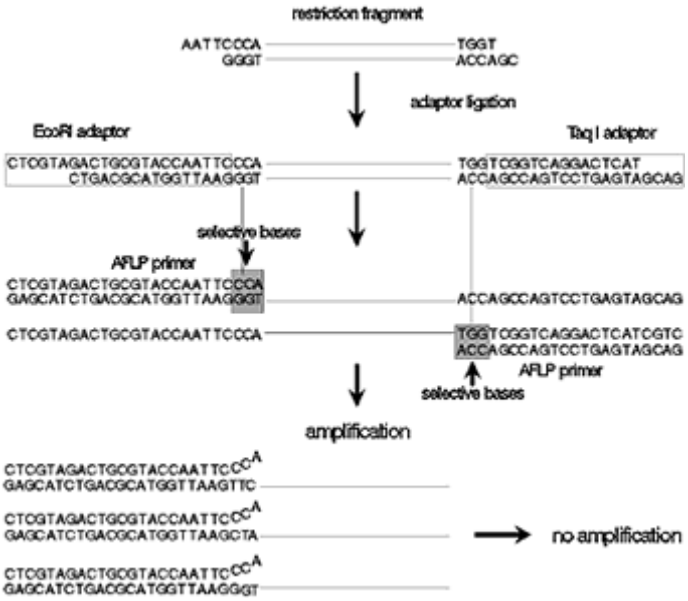

Figure 7 The selective principle of the AFLP technology. Image was taken from Keygene (www.keygene.com).

AFLP markers have been proved to be useful for assessing genetic differences among individuals, populations and independently evolving lineages, such as species. For a wide range of taxa, including fungi, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species that had been impossible to resolve with morphological or other molecular systematic characters (Huys *et al.*, 1996; Janssen *et al.*, 1997). AFLP markers have found the widest application in analyses of genetic variation

below the species level, particularly in investigations of population structure and differentiation. The high resolution of AFLP markers also enables testing for clonal identity between individuals (i.e. absence of recombination), and thus permits inferences about sexual versus asexual modes of reproduction (Majer *et al.*, 1996; Rosendahl and Taylor, 1997; Majer *et al.*, 1998). AFLP markers have the potential to resolve genetic differences at the level of DNA fingerprints for individual identification and parentage analysis.

3.2.2. Multilocus Sequence Typing (MLST)

Another method to study population genetics is represented by Multilocus Sequence Typing or MLST analysis. MLST, used first to study bacterial populations (Taylor, 2003), is a PCR-based method that involves the amplification of DNA fragments of several housekeeping genes. After DNA sequencing and sequence alignment, sequence variations in the introns or exons can be observed among the isolates and haplotype could be differentiated. Because of that, this technique allows analyzing only a locus per experiment and of that locus it is possible to distinguish the allelic variation. MLST is considered to be a co-dominant marker. MLST, compared with other methods, such RAPD and RFLP, is highly reproducible and produces unambiguous and suitable data for epidemiological and population studies (Ahmed *et al.*, 2006). However, due to DNA sequencing of several loci of a large number of isolates, MLST is expensive and require previous sequence information to design the corresponding MLST primers. This technique has been used successfully to study population genetics in powdery mildew species such as *Erysiphe necator* (Brewer and Milgroom, 2010) and *Blumeria graminis* (Inuma *et al.*, 2007), the powdery mildews of grape and barley, respectively, and could represent an optimal approach to study population genetics of other powdery mildews.

4. Aim of the thesis

Cucurbit powdery mildew is a major limitation for cucurbit production in Northern Italy. In order to achieve an effective disease control, a good understanding of the disease causal agent is needed. In the North of Italy, the presence of both main causal agents of cucurbit powdery mildew, *G. orontii* and *P. fusca*, has been documented. In order to determine the epidemiological relevance of both powdery mildew species in the most important cucurbit production areas of Northern Italy, detailed information about the occurrence and distribution of these powdery mildew species during the growing season is needed. This data would allow a more effective use of the control tools available for these pathogens. In the same sense, information about the evolutionary potential of cucurbit powdery mildew fungi would be also very relevant for disease management. Information on genetic diversity of pathogen populations would allow, for example, predicting the durability of new resistance cultivars or novel chemical practices, this way leading to a more efficient and rational use of these control means.

Considering the stated above, the aim of this thesis could be summarized in the following two particular objectives:

1. To determine the occurrence and distribution of the cucurbit powdery mildew species during the cucurbit growing season in Northern Italy.
2. To analyse the genetic diversity of the *Podosphaera fusca* populations, the main causal agent of cucurbit powdery mildew.

Part one: Epidemiology

Species replacement of cucurbit powdery mildew-causing fungi in Northern Italy

1. Introduction

Cucurbit powdery mildew is probably the most important disease affecting cucurbitaceous plants worldwide. Like other powdery mildews, disease symptoms consist of a white powdery fungal mass that covers the entire surface of plants reducing the photosynthesis and thus reducing the yield and quality of crops (Pérez-García *et al.*, 2009). Although great efforts have been invested in plant breeding programmes, growers still have important concerns about disease control, and the application of fungicides continues to be the principal practice for the management of powdery mildew in most cucurbit crops (McGrath, 2001; Pérez-García *et al.*, 2009).

In Italy, cucurbits are cultivated from the North to the South of the peninsula. Only in the northern part of the country, 240.675 ha are cultivated representing the 26% of the total Italian cucurbit cultivated area of 910.583 ha (Istat, 2012). In particular, in the North cucurbits are mainly cultivated in Lombardia and Emilia-Romagna regions. Data on area of cultivated land and production of cucurbit crops in Italy are presented in Table 2. In Italy, like in many other countries of the Mediterranean basin, cucurbit powdery mildew is a major problem for these crops.

Table 2 Cucurbit production in Italy. Data on surface cultivated (ha) and production yields (t) corresponding to 2011 are given. Data were taken from Istat (2012).

Crop	**Melon**		**Watermelon**		**Zucchini**		**Cucumber**	
	Field	Tunnel	Field	Tunnel	Field	Tunnel	Field	Tunnel
Surface	23.615	269.037	10.719*	135.061	14.199	391.728	1.430*	64.794
Total	**292.652**		**145.780**		**405.927**		**66.224**	
Production	545.620	94.842	433.668*	76.510	359.320	179.657	30.538*	36.345
Total	**640.462**		**510.178**		**538.977**		**66.883**	

The disease in cucurbits can be caused either by the Ascomycetes fungi *Podosphaera fusca* (Fr.) Braun and Shishkoff (2000) and *Golovinomyces orontii* (Castagne) V.P. Heluta (1988). *G. orontii* is stably present in some temperate European countries like Northern Italy (Branzanti and Brunelli, 1987), Germany (Ulbirch and Smolka, 1994), Czech Republic (Lebeda, 1983), Hungary (Nagy and Kiss, 2006), France (Bertrand *et al.*, 1992), Bulgaria (Velkov and Masheva, 2002), Switzerland (Corbaz *et al.*, 1992) and Ukraine (Tomason and Gibson, 2006) while *P. fusca* is considered the predominant species in the Mediterranean basin (Bardin, 1997).

In particular, *P. fusca* is the only species infecting cucurbits in Spain (del Pino *et al.*, 2002; Fernández-Ortuño *et al.*, 2006), Israel, Turkey (Kristkova *et al.*, 2009), Greece (Vakalounakis and Krilomonou, 1994) and Morocco (Endo *et al.*, 2012). Furthermore it is widespread distributed in Germany, Czech Republic (Lebeda, 1983), Italy (Branzanti and Brunelli, 1987), Bulgaria (Velkov and Masheva, 2002), Hungary (Nagy and Kiss, 2006) and Ukraine (Tomason and Gibson, 2006). Besides, it is also present in the United States of America (McGrath *et al.*, 1996), China (Liu *et al.*, 2011) and all the tropical and sub-tropical areas as Brazil (Reifschneider *et al.* 1985; de Melo Aguiar *et al.*, 2012), Mexico (Felix-Gastelum *et al.*, 2005, Bojorquez-Ramos *et al.*, 2012), Iraq (Ibrahim *et al.*, 1985), Sudan (Mohamed *et al.*, 1995), India (Gupta and Sharma, 2012) and Australia (Letham and Priest, 1989). Furthermore, mixed infections of the two species have been recorded in the North of Italy (Branzanti and Brunelli, 1987), Czech Republic, Netherlands, Great Britain, Germany (Kristkova *et al*, 2009), Bulgaria (Velkov and Masheva, 2002), Hungary (Nagy, 1976) and rarely in France (Bertrand, 1992).

As mentioned above, the occurrence of the two powdery mildew species has been documented in Northern Italy (Branzanti and Brunelli, 1987). In this area, understanding the epidemiology of the two species could be very useful to plan an efficient control strategy against cucurbit powdery mildew, especially considering that previous studies have

demonstrated a different sensitivity of these species to some fungicides (Bertrand, 1992; Sedláková and Lebeda, 2008). The aim of this work was to monitor the occurrence and distribution of the two cucurbit powdery mildew species in distinct locations in North of Italy during different growing seasons. In this way, relevant epidemiological data should be obtained which can be subsequently applied to the rational design of disease management programmes.

2. Material and methods

2.1. Sampling

Samples were collected during the cucurbit-growing seasons 2010, 2011 and 2012 from different cucurbit crops and either from field or plastic tunnels, in farms located in the provinces of Bologna and Mantua, important areas of cucurbit production in the North of Italy (Figure 8). Host plants covered all of the major cultivated cucurbit crops: zucchini (*Cucurbita pepo*), melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and pumpkin (*Cucurbita maxima*). Samples from watermelon (*Citrullus lanatus*) were not included because of the lower sensitivity of this species to cucurbit powdery mildew under field conditions. Location of farms subjected to sampling and the corresponding host plants are shown in Table 3.

Each season, sampling started when the first powdery mildew infections were observed, that in the North of Italy usually happens at the end of May, and continued till the end of the crop season, usually at October-November. If possible, both infected leaves and chasmothecia were collected. At least 15 to 20 leaves showing typical powdery mildew symptoms were randomly collected in either field or plastic tunnel crops. In order to monitor the powdery mildew species composition during the crop season, sampling was repeated each 15-18 days on the same plants or, when a crop cycle ended, on plants of surrounding crops in the same

farms. From August till October, both infected senescent leaves and soil under the plants were collected to find chasmothecia.

2.2. Isolation of chasmothecia

Chasmothecia were extracted using a modification of the methods used by Pearson and Gadoury (1987), Cortesi *et al*. (1995) and Portillo (2010). About 50 g of leaves were placed in 500 ml flasks and double distilled water was added to cover completely them. After that, flasks were manually shacked for 3 min and the resulting solution was than filtered through a column of 4 test sieves (Retsch®, Haan, Germany) measuring 10, 30, 60 and 170 mesh corresponding to 2000, 600, 250 and 90 µm. To better clean chasmothecia and to facilitate the movement of them through the last two sieves, the column was washed under running water. The last two sieves were then washed with double distilled water and liquid suspension with chasmothecia was placed on filter paper (ultra-rapid). After filtration, filter papers with chasmothecia were placed in a 90 mm Petri dish, air-dried for at least 24 h and conserved at 4°C. For extraction of chasmothecia from soil the protocol was essentially the same as described above. The only difference was that 10 g of soil collected under infected senescent leaves were placed directly on the column and washed with running water. Presence of chasmothecia was finally verified under the stereo microscope.

Table 3 Locations and crops subjected to sampling in the provinces of Bologna (BO) and Mantua (MN).

Farm code	**Location**	**Coordinates**	**Host**	**Crop cultivation**
BO1	Bologna	44°31'26.68"N 11°23'3.90"E	*C. pepo*/*C. melo*	Tunnel
BO2	Bologna	44°31'11.74"N 11°23'27.40"E	*C. pepo*	Tunnel
BO4	Granarolo	44°32'39.49"N 11°25'2.63"E	*C. pepo*	Tunnel/Field
BO6	Granarolo	44°32'35.39"N 11°24'30.19"E	*C. pepo*	Tunnel/Field

BO7	Altedo	44°38'56.99"N 11°29'45.45"E	*C. pepo*	Field
MN1a	Moglia	44°56'19.39"N 10°55'58.81"E	*C. pepo*	Field
MN1b	Moglia	44°56'19.39"N 10°55'58.81"E	*C. sativus*	Field
MN2a	Sermide	44°57'34.35"N 11°15'23.32"E	*C. melo*	Tunnel
MN2b	Sermide	44°57'34.35"N 11°15'23.32"E	*C. maxima*	Tunnel
MN3	Sermide	44°59'11.30"N 11°14'4.42"E	*C. melo*	Tunnel/Field
MN4	Sermide	44°57'55.67"N 11°13'42.76"E	*C. melo*	Tunnel/Field

Figure 8 Sampling area of powdery mildew infected cucurbit plants in Northern Italy. Images were obtained from Google Earth software 6.1.0.5001 (Google Inc., Mountain View, California, USA).

2.3. Species identification

To verify the occurrence of the two powdery mildew species in a given sample, a precise identification of the two fungal pathogens was imperative. Species identification was carried

out both morphologically, by observation of conidial shape and germination under light microscopy, and molecularly, by means of a Multiplex-PCR reaction.

2.3.1. Morphological identification

The mode of germination is specific for a particular taxon and represents a useful diagnostic tool for the taxonomy of *Erysiphaceae* (Braun *et al.*, 2002). Thus, the mode of germination was used to morphologically identify the two cucurbit powdery mildew species and to assess the percentage of them in each sample. Germination was assessed by taking from the pool of 20 collected leaves, 5 groups of 4 leaves that were manually shaken on three dry well slides, in order to have repetitions of each count. The slides were supported by two toothpicks above a wet paper tissue in a Petri dish and incubated for 24 h at 25°C to induce germination (Zacarovitis, 1965). After that, well slides were observed under a light microscope (20×) and the percentage of conidia belonging to both species was calculated based on the total number of germinated conidia. For each well slide, 100 germinated conidia were counted and from this count, the percentage of conidia belonging to both species was assessed. Species were identified according to germination criteria described by Nagy (1976) and Lebeda (1983). Barrel-shaped conidia with single germ tubes produced apically were identified as belonging to *G. orontii,* whereas elliptical conidia with germ tubes usually forked and produced laterally were identified as belonging to *P. fusca*.

Morphological features of chasmothecia were observed by placing a single chasmothecium in a glass slide with a water drop. After breaking it with a yellow pippette tip, examination was undertaken by light microscope. According to Lebeda (1983), the two species can be identified also on the basis of morphological differences of the teleomorphs: chasmothecia of *P. fusca* measure 65-98 μm in diameter and contains 1 ascus with 8 hyaline ascospores while

those of *G. orontii* measure 80-140 µm in diameter with 10-15 asci containing 2-3 ascospores.

2.3.2. Molecular identification

For molecular identification of *G. orontii* and *P. fusca*, pieces of the same infected leaves used for morphological identifications were used to corroborate the results. DNA was extracted using a modification of the protocol of Doyle and Doyle (1987). Fungal biomass was recovered from infected leaf material by washing with 2 ml of CTAB-0.04% β-mercaptoethanol solution, previously heated at 65°C for 1 h. After that, 1 ml of the conidial suspension was deposited in a 2 ml eppendorf tube, vortexed for 20-30 s and 2.5 µl of proteinase-K (10 mg ml^{-1}) was added. The suspension was then heated at 65°C for 2 h. Subsequently, 1 ml of a mixture of chloroform-octanol (24:1) was added to the solution and centrifuged for 5 min at 8000 rpm. After the addition of 5 µl of RNAase (10 mg ml^{-1}) to the supernatant, 1 ml of chloroform-octanol was added again and the solution was centrifuged as described above. DNA precipitation was carried out by adding 0.7-0.8 volumes of isopropanol to the supernatant and centrifuging for 20 min at 14000 rpm. After that, the pellet was dried under vacuum, washed with 500 µl of 70% ethanol and centrifuged for 5 min at 12000 rpm. Finally, the pellet was air dried and re-suspended in 20-50 µl of sterile double distilled water. DNA concentration and ratio A260/A280 were assessed using an Infinite 200 NanoQuant spectrophotometer (Tecan® Group Ltd., Grödig, Austria) while quality was verified by running 5 µl of DNA on 0.8% agarose gels in 0.5×TAE buffer.

Molecular identification of the two pathogens was carried out by Multiplex-PCR using the ITS regions of ribosomal DNA of both species as target DNA. Amplification of the ITS regions was carried out using a modification of primers S1/S2 and G1/G2 previously described (Chen *et al.*, 2008). After amplification and sequencing of the ITS regions of both

species with the universal primer pair ITS1F/ITS4, the reverse primers S2mod (5'-CGCCACTCTGTCGCGAGATACA-3') and G2mod (5'-CAACACCAAGCCACACACACGGCG-3') were designed. Primer pairs S1/S2mod and G1/G2mod were used in a Multiplex-PCR reaction for molecular identification of *P. fusca* and *G. orontii*, respectively. PCR reactions were carried out in 25 µl using 2.5 µl of TaKaRa 10×buffer, 1.5 µl of 25 mM $MgCl_2$, 1.5 µl of 10 mM dNTPs, 1 µl of 10 mM solution of each primers, 0.125 µl of TaKaRa Taq polymerase (5 U/ml) (Takara® Bio Inc., Otsu, Japan), 14.375 µl of double distilled water and 1 µl of genomic DNA (20-300 ng μl^{-1}). PCR programme consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 40 s, 62°C for 60 s and 72°C for 1 min and 30 s, and a final extension step of 5 min at 72°C (Chen *et al.*, 2008). To verify reactions, 10 µl of each PCR product were separated on a 2% agarose gels in 0.5×TAE buffer, stained with ethidium bromide and visualized and photographed under UV light.

Specificity of the primer pairs designed for diagnostic purposes was tested on DNA of the following phytopathogenic fungi and oomycetes that affect cucurbitaceous plants: *Alternaria alternata*, *Dydimella bryoniae*, *Fusarium oxysporum*, *Pseudoperonospora cubensis* and *Sclerotinia sclerotiorum*.

2.4. Mating type identification

Because only chasmothecia of *P. fusca* were found, molecular identification of the mating types was only planned for this species. From each location, several monoconidial isolates of *P. fusca* were obtained and subjected for molecular analysis. DNA isolation was carried out as described above. Subsequently, based on sequences of both mating types identified in *P. fusca* by Brewer *et al.* (2011), a Multiplex-PCR reaction to rapidly identify the allele at the locus *MAT* was developed. Based on sequences of *P. fusca* genes *MAT 1-1-1* (GeneBank

HQ171903.1) and *MAT 1-2-1* (GeneBank HQ171900.1), primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 were designed (Table 4). PCR reactions were conducted in a volume of 25 µl using GoTaq DNA polymerase (Promega®, Fitchburg, Wisconsin, USA). PCR conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 30 s, 52°C for 30 s at and 72°C for 60 s, and a final extension step at 72°C for 5 min. After reaction, PCR products were directly used or stored at 4°C. PCR products were separated on 1.5% agarose gels in 0.5×TAE buffer, stained with ethidium bromide and visualized under UV light.

Table 4 Specific primers designed to amplify *MAT 1-1-1* and *MAT 1-2-1* gene fragments of *P. fusca*. Fragment size is referred to that observed after sequencing.

Allele	***MAT 1-1-1***	***MAT 1-2-1***
Primer pairs	aboxF2 5'-GGCTTCAGAAGTATGTCATG-3' aboxR2 5'-CCGCAGAAATTATAGACCAC-3'	hmgF2 5'-AAGGCTAAGCATGGAGAAAC-3' hmgR2 5'-CCTGTAACGATAACCTGGAT-3'
Fragment size	111 bp	216 bp

2.5. Statistical analysis

The chi-squared (χ^2) non parametrical statistical inference test was used to test the null hypothesis that the frequencies of *P. fusca* mating types were equal. Chi-squared test was performed using GraphPad Prism 6.01 software (GraphPad Software Inc., La Jolla, California, USA).

2.6. Collection of powdery mildew fungi from weeds, spontaneous plants and non-cucurbitaceous crops

To verify if spontaneous plants and non cucurbitaceous crops could represent alternative hosts for *P. fusca* and *G. orontii*, several samples of spontaneous species and non cucurbitaceous crops showing powdery mildew symptoms were collected at the same

locations during the 2012 growing season. After collection, powdery mildew species were identified by observations on the conidia germination (Zacarovitis, 1965). Only in the case of the powdery mildews collected on *Convolvolus arvensis* and *Cichorium intybus* molecular identification was carried out by amplification and sequencing of ITS regions of the ribosomal DNA using the universal primers ITS1F/ITS4. Furthermore, pathogenicity on cucurbits of the different powdery mildew species collected was tested by inoculation on zucchini cotyledons cv. Giambo.

2.7. Climate data

Climate data of temperature, relative humidity and rainfalls of Mantua sampling area was obtained from the database of the "Agenzia Regionale Prevenzione e Ambiente – ARPA" of Lombardia region (http://ita.arpalombardia.it/meteo/meteo.asp). Temperature and rainfalls data were recorded in weather stations n° 816 Sermide (Id sensors respectively n° 8223 and n° 8222) and n° 110 in Gonzaga (Id sensors n° 2134 and n° 2140), while relative humidity data were only available in the Gonzaga station n° 110 (Id sensor n° 2135). For Bologna area, data were obtained from the weather station of the experimental farm located in Altedo, managed by University of Bologna.

3. Results

3.1. Occurrence and distribution of cucurbit powdery mildew species

3.1.1. Morphological identification

Samples were taken during the 2010–2012 growing seasons from powdery mildew diseased cucumber, melon, pumpkin and zucchini plants collected in the provinces of Bologna and Mantua in Northern Italy. In most cases the two more important cucurbit powdery mildew pathogens *G. orontii* (barrel-shaped conidia without fibrosin bodies and apical germination) and *P. fusca* (elliptical conidia with fibrosin bodies and lateral germination, in some cases,

with forked germ tubes) were found. The graphics that illustrate the presence of the two powdery mildew pathogens during the crop season based on the percentage of germinated conidia belonging to both species are the following:

Bologna

During the three years of sampling, the first powdery mildew infections were recorded between the last week of May and the first half of June, with the only exception of farm BO7, where crops were grown in field conditions and powdery mildew symptom appeared during the second half of July (Figure 9Figure 10Figure 11). With the exception of farms BO1 and BO2 where in 2011 it was recorded a 5% of conidia belonged to *P. fusca* (Figure 10), the only species causing the earlier infections was *G. orontii*. From then second half of June, in all farms it was observed a progressive increase of conidia belonging to *P. fusca* and a decrease of those of *G. orontii*. Conidia of the two species were found in the same proportions from the second to the third week of July in 2010 (Figure 9), from the first to the second week of July in 2011 (Figure 10) and from the last week of June to the first week of July in 2012 (Figure 11), only for farms BO1, BO2 and BO4. After those periods the predominant conidia found on infected leaves were those of *P. fusca* that became progressively the only species infecting cucurbits from the second week of August until the end of the crop season (September for BO1 and BO2 and October for BO4 and BO6).

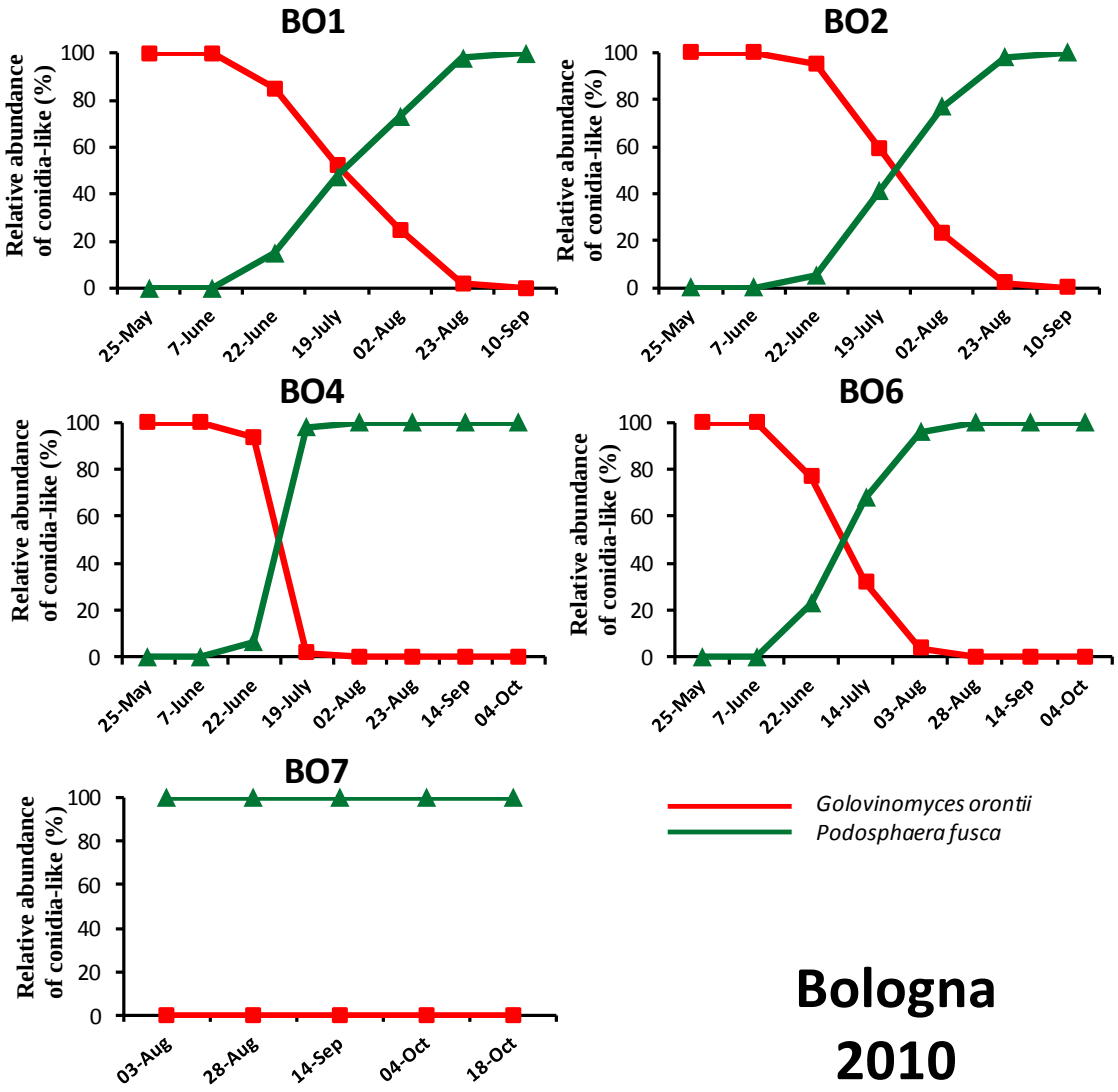

Figure 9 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Bologna farms during the growing season 2010.

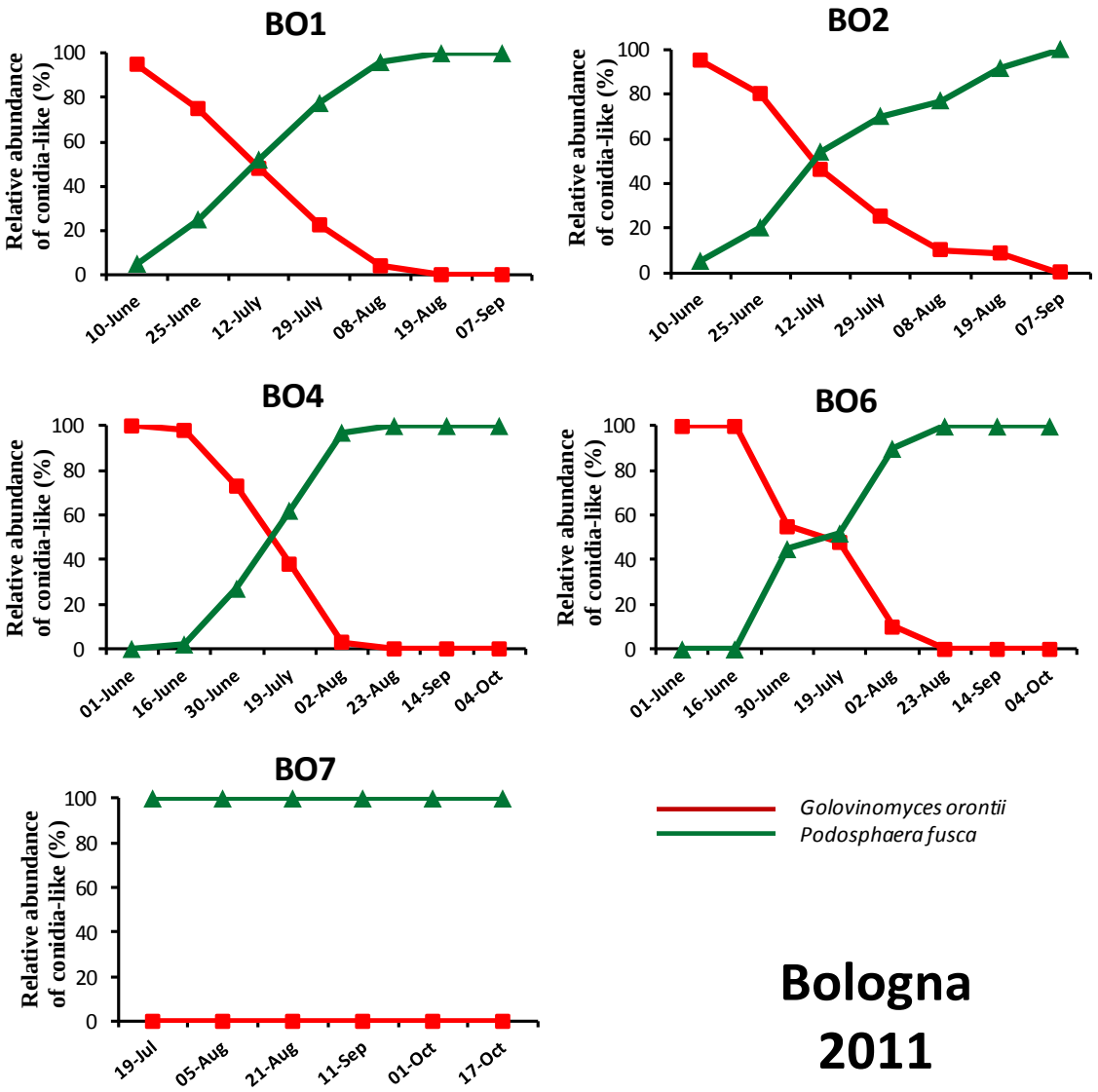

Figure 10 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Bologna farms during the growing season 2011.

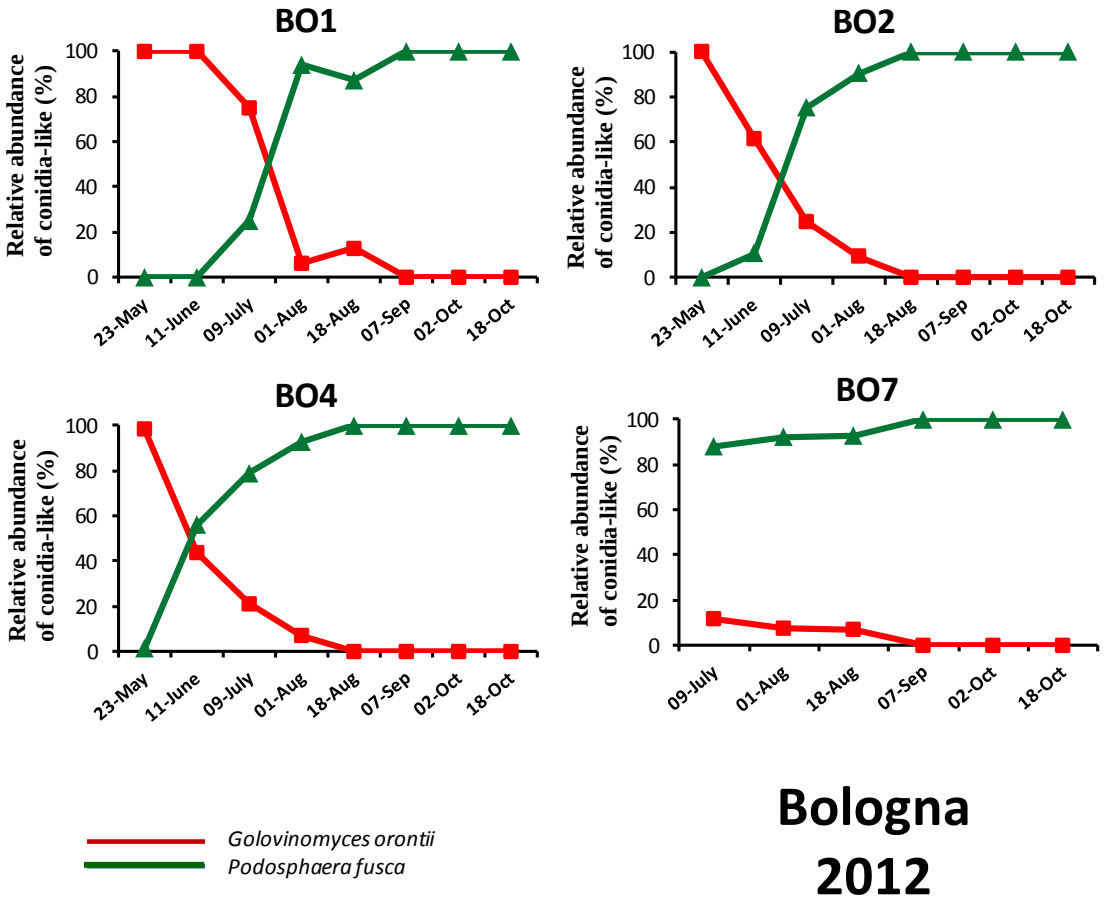

Figure 11 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Bologna farms during the growing season 2012.

Mantua

During the three years of sampling, the first powdery mildew infections appeared at the end of May in farms MN2, MN3 and MN4 with crops under plastic tunnels (Figure 12, Figure 13 and Figure 14). Only in the case of farms MN1a and MN1b, where crops were cultivated only in field conditions, the first powdery mildew symptoms were observed during the last week of June and the first half of July for *C. sativus* and *C. pepo*, respectively. Except for farm MN1, where the predominant powdery mildew species during all growing seasons was *P. fusca*, in all the farms conidia observation indicated that the first infections were caused mainly by *G. orontii* that was the predominant species infecting crops until a period that

goes from the last half of June to the first half of July. After that and until the end of the crop season, the percentage of conidia belonging to *P. fusca* progressively increased, becoming the only species infecting cucurbits from the last week of July until the end of the crop season (September-October) with a 100% of conidia belonging to this species.

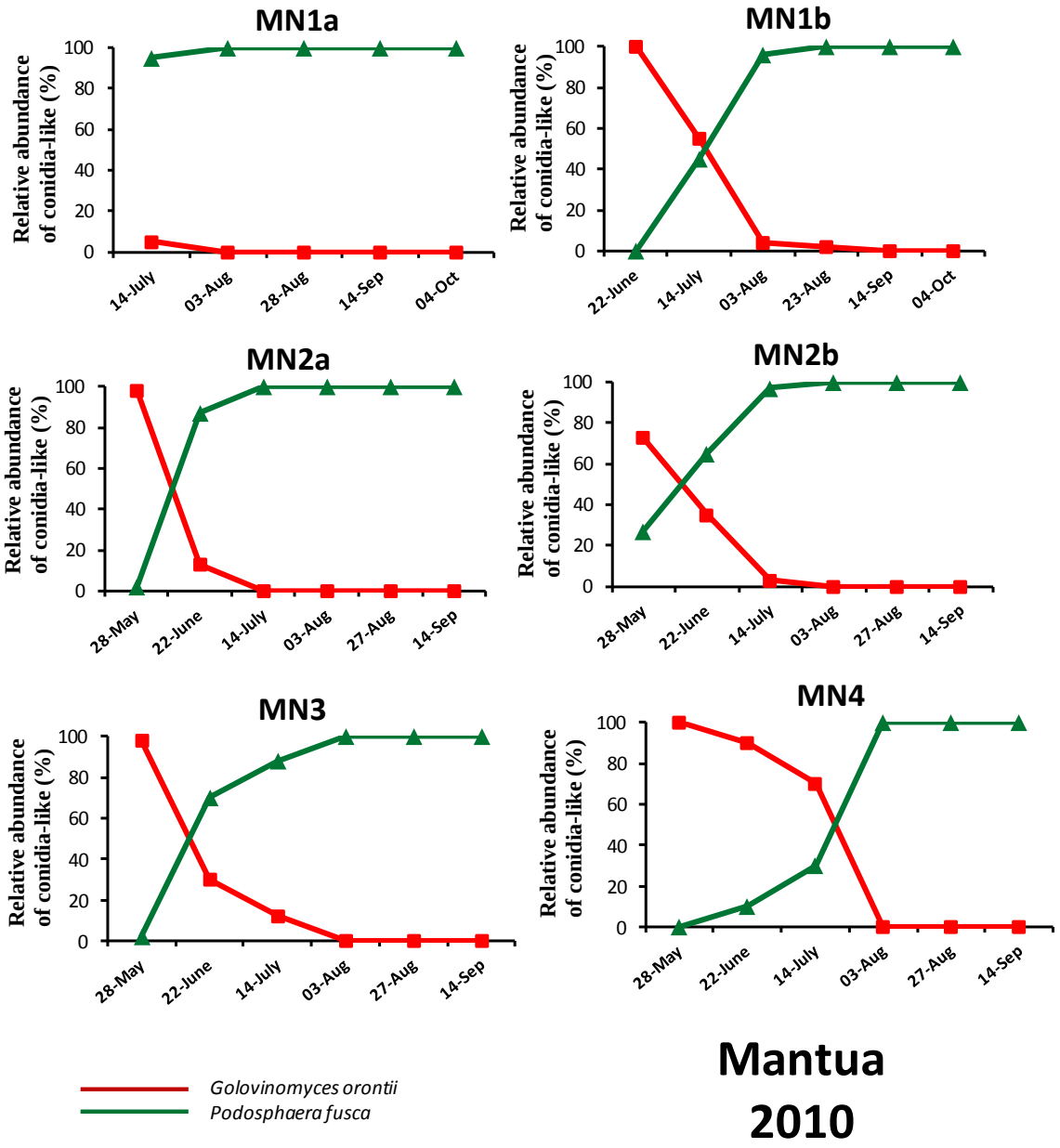

Figure 12 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Mantua farms during the growing season 2010.

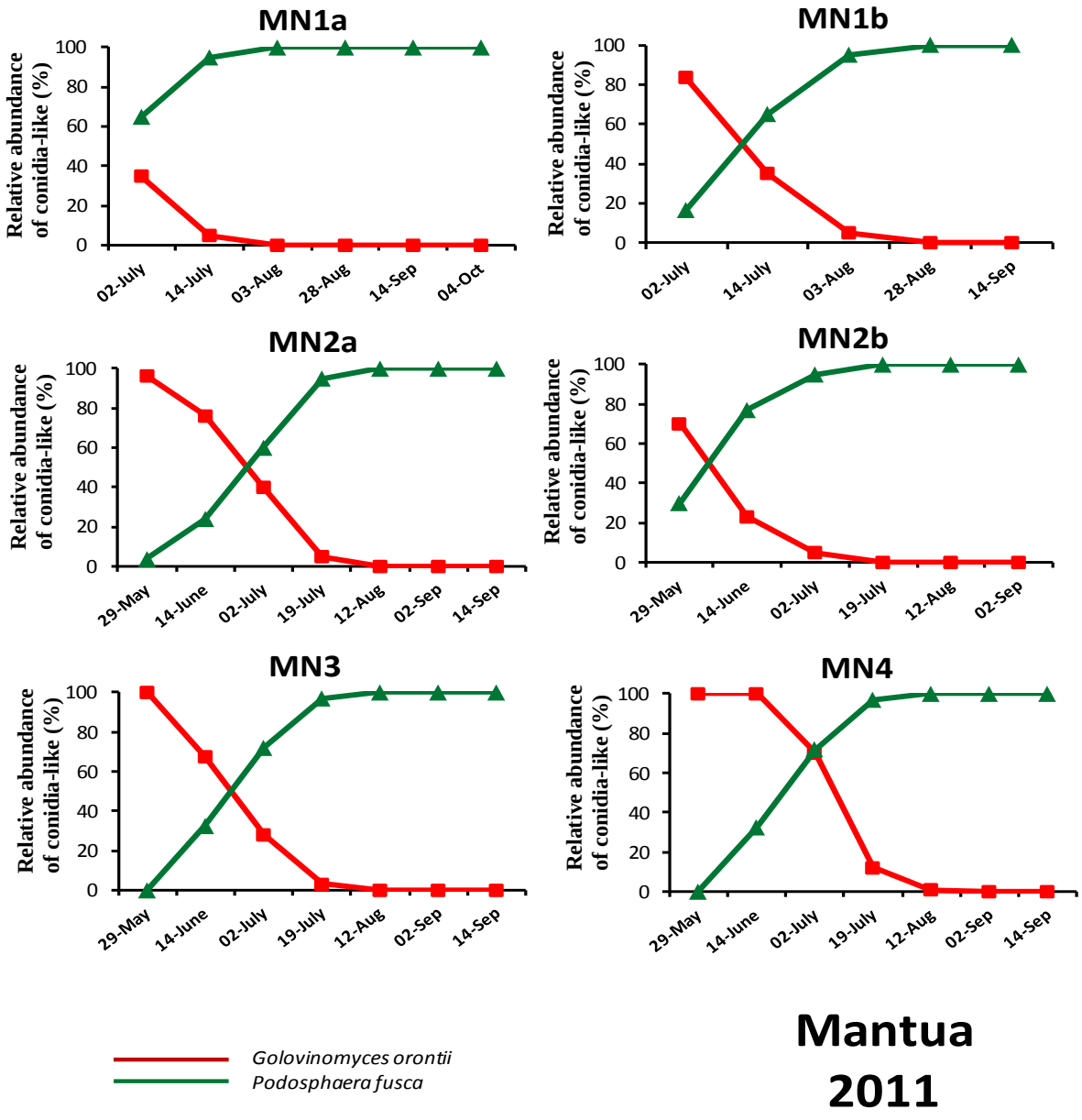

Figure 13 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Mantua farms during the growing season 2011.

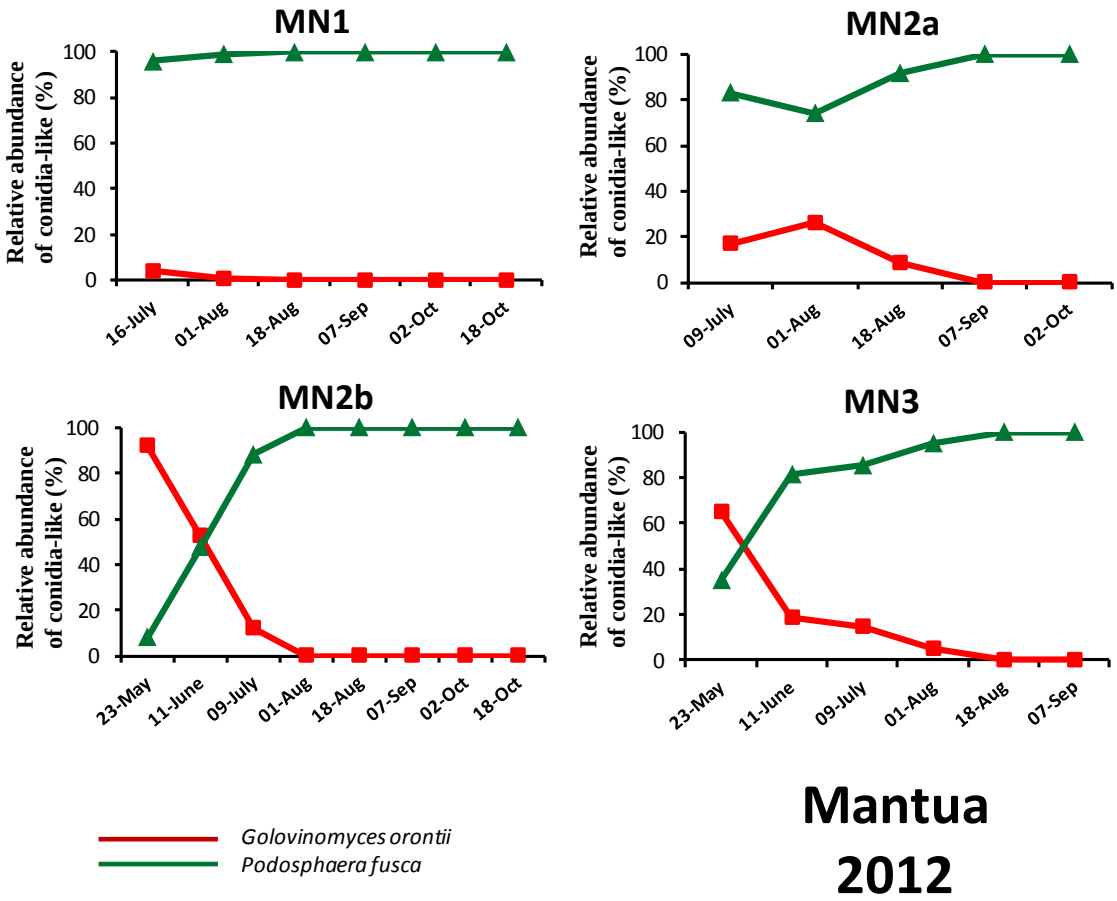

Figure 14 Presence of the two cucurbit powdery mildew pathogens assessed as the percentage of germinated conidia belonging to both species in Mantua farms during the growing season 2012.

3.1.2. Molecular identification

A multiplex-PCR assay was designed to detect the presence of both cucurbit powdery mildew species and molecularly confirm the results obtained by microscopic analysis. ITS regions of ribosomal DNA were used as target DNA. By this assay, a difference of 40 bp in size of amplified bands allowed us to directly identify the two species. Furthermore, primers S1/S2mod and G1/G2mod did not amplify any DNA of the cucurbit pathogens *A. alternata*, *D. bryoniae*, *F. oxysporum*, *P. cubensis* and *S. sclerotiorum* (data not shown), confirming the specificity for the ITS region of the two powdery mildew species and the robustness of the assay.

The results confirmed the same seasonal trend of the two species observed by conidia germination trials. An example is given in Figure 15 for farm BO6 for the three years of sampling. Double bands that represent the simultaneous presence of the two species, where obtained in a period that goes from the last half of June until the second/third weeks of July that corresponds, in the morphological observations, to about 40-60 % of conidia belonging to both species.

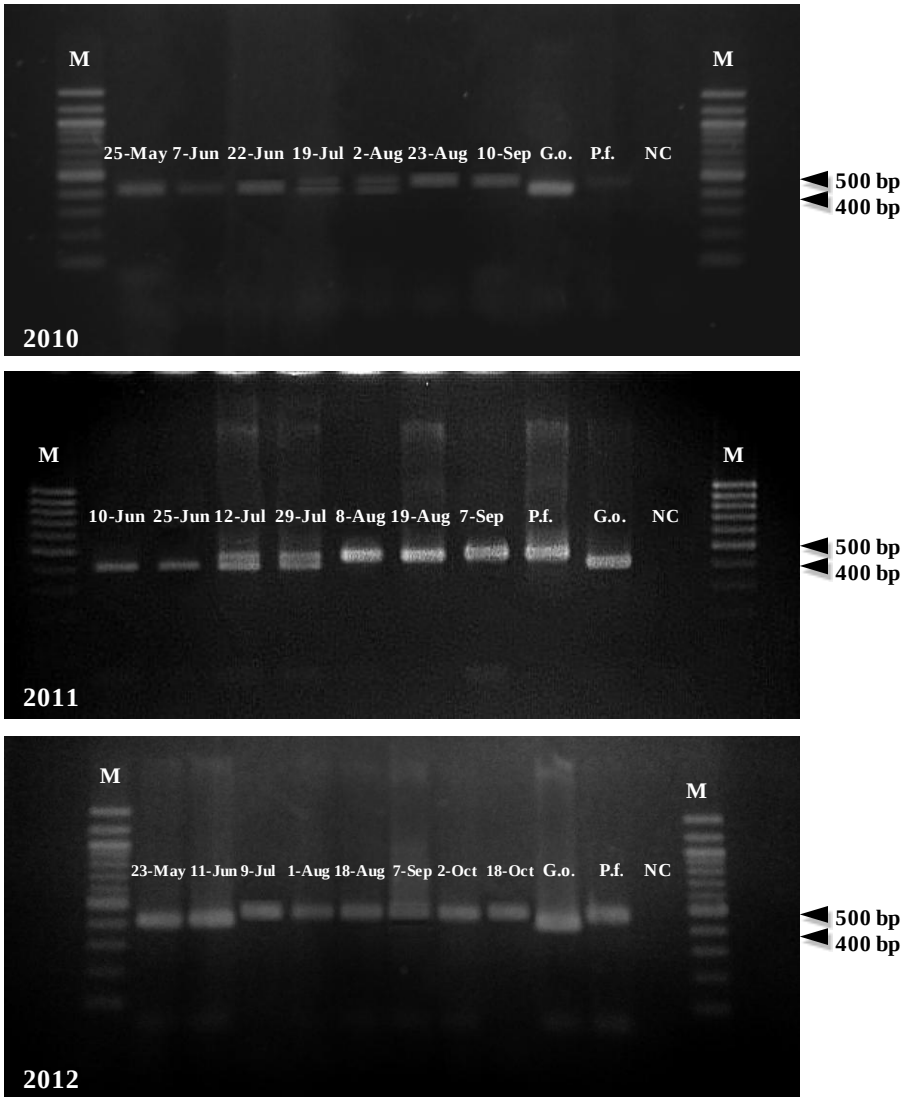

Figure 15 Molecular detection of *P. fusca* and *G. orontii* by multiplex-PCR. Presence of the two cucurbit powdery mildew species was investigated by multiplex-PCR in all farms sampled during the crop seasons 2010, 2011 and 2012. Representative pictures of farm BO2 are given. DNA was isolated from diseased plants and amplified with the primers S1/S2mod and G1/G2mod as described in Materials and methods. PCR products were fractionated in 2% agarose gels. Genomic DNA from *P. fusca* (P.f.) and *G. orontii* (G.o.) were used as positive controls. M is the molecular size marker 100 bp DNA ladder (New England BioLabs®, Ipswich, UK) for 2010 and 2012 and MassRuler Low Range DNA ladder (Thermo Scientific®, Waltham, Massachusetts, USA) for 2011, and NC is the negative control (no DNA was added).

3.2. Chasmothecia collection and mating type identification

Chasmothecia were found from the middle of August till the end of October only on senescent leaves of crop plants and mainly in October in the soil under the plants (Figure 16a). No asci were found inside chasmothecia during the years 2010 and 2011 (Figure 16b). This is more likely due to the fact that, at the moment of collection, chasmothecia were not completely mature as they were light/dark brown. Considering that chasmothecia were found in infected plants or soil where only *P. fusca* was present, it was assumed that they belonged to this species. In 2012 chasmothecia were collected mature as they were already black when observed under the stereo microscope. Only one ascus containing eight ascospores was found inside (Figure 16d), a morphological feature that corresponds to chasmothecia from *P. fusca*.

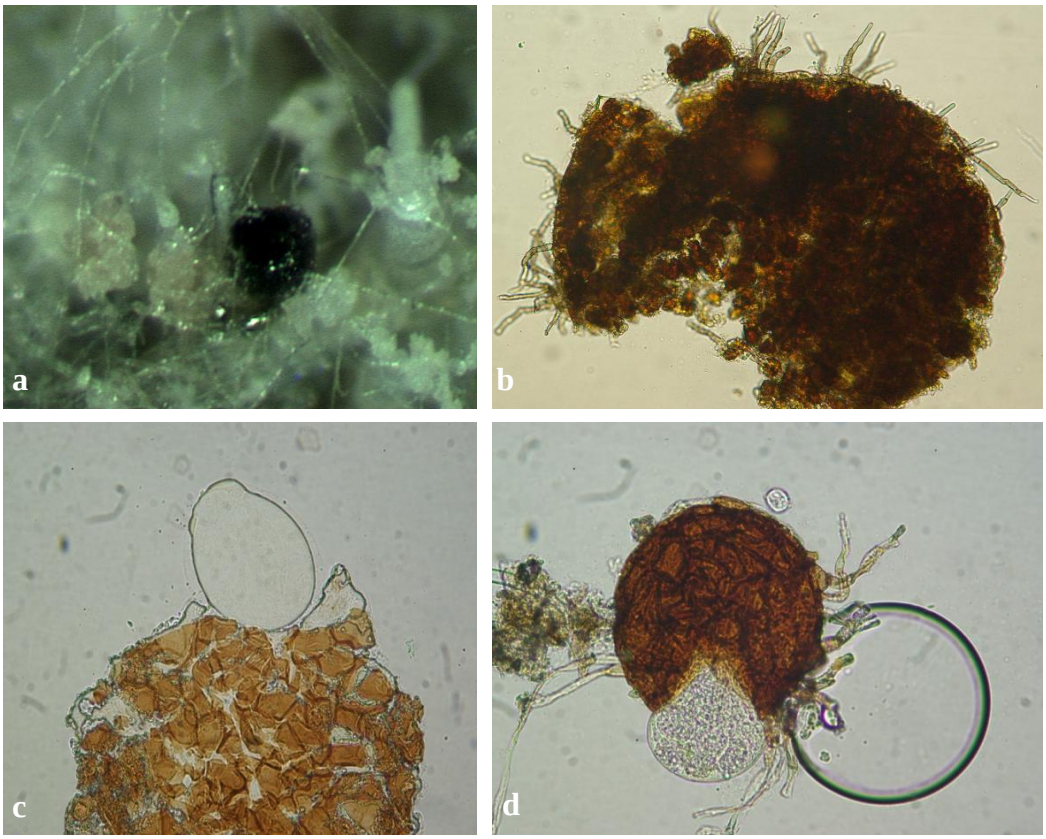

Figure 16 Chasmothecia from *P. fusca*. A dark mature chasmothecium formed on a zucchini leaf (a), empty chasmothecium lacking ascus (b), chasmothecium bearing one ascus with ascospores in formation (c), and mature chasmothecium bearing one ascus containing eight ascospores (d).

Considering that possibly only chasmothecia from *P. fusca* were found, a multiplex-PCR assay was designed to molecularly determine the frequency of mating types in a collection of monoconidial isolates of *P. fusca* and study the impact of sexual reproduction in the populations of Northern Italy. The primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 were used to successfully amplify PCR products of 111 and 216 bp (length after sequencing), corresponding to the alleles *MAT 1-1-1* and *MAT 1-2-1*, respectively (Figure 17). This difference of 105 bp in size of the amplified bands allowed an easy discrimination of the two alleles.

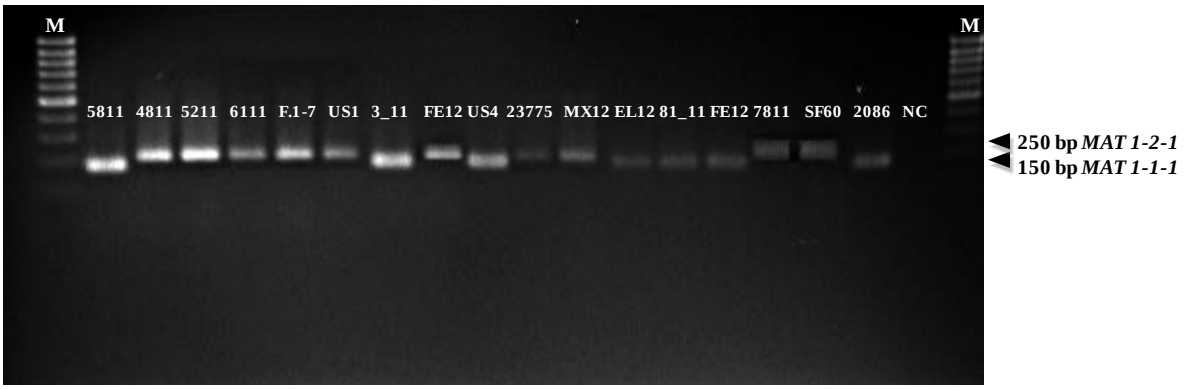

Figure 17 Molecular detection of mating type alleles in *P. fusca* isolates. DNA was amplified by primers aboxF2/aboxR2 and hmgF2/hmgR2 designed and combined in a Multiplex-PCR reaction that allowed to detect the corresponding allele at the MAT locus. PCR products were separated on 1.5% agarose gels in 0.5×TAE buffer, stained with ethidium bromide and visualized under UV light. M is the molecular size marker MassRuler Low Range DNA ladder (Thermo Scientific®, Waltham, Massachusetts U.S.A.) and NC is the negative control (no DNA was added). Molecular size (bp) of amplified fragments is indicated on the right.

This multiplex-PCR assay was used to evaluate the frequency of the two alleles in the areas under investigation (Figure 18). Results obtained from Multiplex-PCR indicated that the mating type ratio tended to be 1:1. To verify this tendency, mating type frequencies shown in Table 5 were analysed using the non parametric statistical chi-squared test (χ^2), the null hypothesis being that frequencies of *MAT 1-1-1* and *MAT 1-2-1* alleles were equal. The results of the test were a χ^2 value of 2.032 and a P value of 0.3621. According to the statistical table of chi-squared test, the corresponding critic χ^2 value with 2 degree of freedom and $\alpha<0.05$ is 5.99. This way, according to the test, the null hypothesis is accepted and the

differences observed between the frequencies of the mating types are not statistically significant for P<0.05. In other words, the results observed indicated that the *P. fusca* populations of Northern Italy seem to be actively mating and, in consequence, recombination may occur.

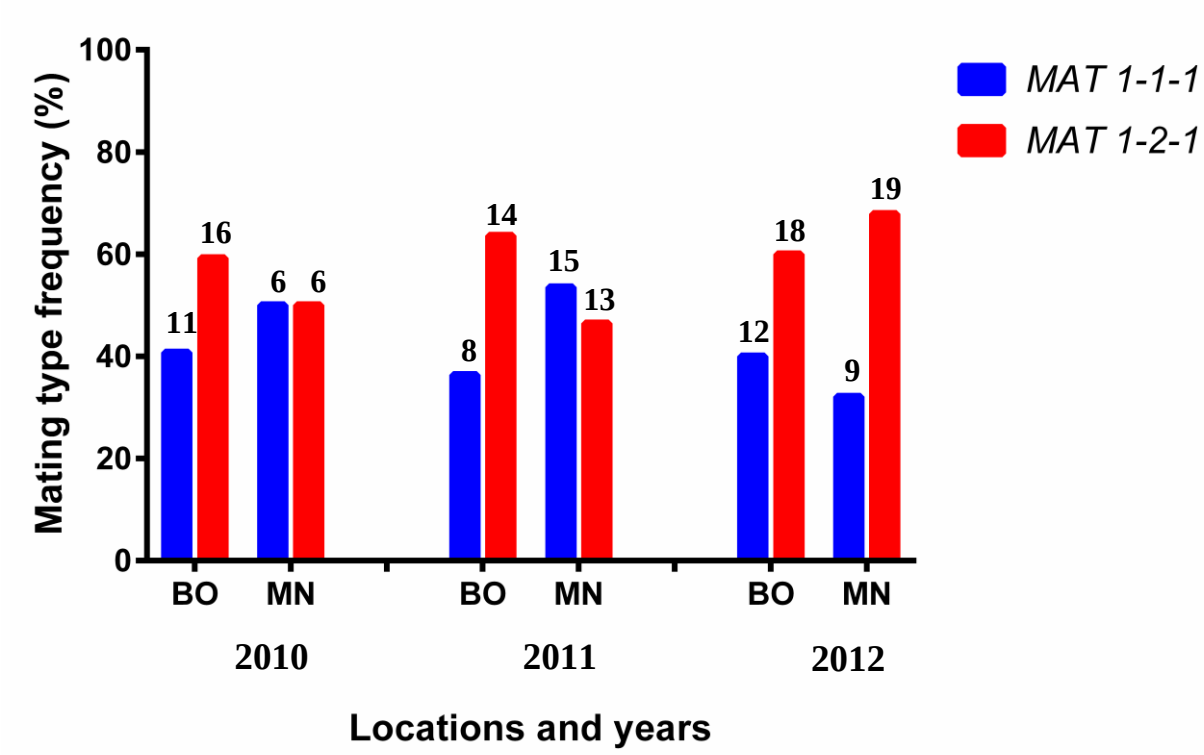

Figure 18. Frequency (%) of *P. fusca* mating types in Northern Italy. The mating type of monoconidial isolates of *P. fusca* was determined by PCR detection of alleles *MAT 1-1-1* or *MAT 1-2-1*. Frequency of mating types was evaluated separately in isolates obtained from Bologna and Mantua farms during the crop seasons 2010, 2011 and 2012. The number of isolates belonging to each mating type that were analyzed are indicated above each column. BO, Bologna; MN, Mantua.

Table 5 Frequencies (%) of alleles *MAT 1-1-1* and *MAT 1-2-1* in *P. fusca* populations of Northern Italy during 2010, 2011 and 2012 growing seasons. The total number of isolates identified for each *MAT* allele in each year of sampling is indicated in brackets.

***MAT* allele**	Year of sampling		
	2010	**2011**	**2012**
MAT 1-1-1	43.6 (17)	46.0 (23)	36.2 (21)
MAT 1-2-1	56.4 (22)	54.0 (27)	63.8 (37)

3.3. Powdery mildew species in alternative hosts

During the three years of sampling, powdery mildew symptoms were observed in a number of host plants in the surroundings of the studied farms (Table 6). In order to determine whether these plants could act as reservoirs for the cucurbit powdery mildew species, conidia were collected from those plants, identified on the basis of criteria of conidial stage and inoculated on zucchini cotyledons from cv. Giambo maintained in Petri dishes. Conidia observation of powdery mildews collected on non cucurbitaceous hosts revealed that only the ones obtained from *Taraxacum officinale* showed elliptical conidia with a lateral forked germ tube and presence of fibrosin bodies, all of them characteristics of the genus *Podosphaera*. The rest of powdery mildews showed barrel-shaped conidia with an apical germ tube and lacked fibrosin bodies, which are typical features of genera *Golovinomyces* and *Erysiphe*. In addition, after performing BLASTn algorithm, ITS sequences obtained from powdery mildew collected on *Convolvolus arvensis* and *Cichorium intybus* showed an identity of 93% with *Erysiphe cruciferarum* (GeneBank AF031283.1) and 99% with *Golovinomyces cichoracearum* (GeneBank AF031282.1), respectively. Regarding pathogenicity for cucurbits, none of them could reproduce powdery mildew symptoms after inoculation on zucchini cotyledons, indicating that they did not belong to any of the major cucurbit powdery mildew species and that those host plants were not alternative hosts for these cucurbit pathogens.

Table 6 Morphological features and pathogenicity of powdery mildew isolates collected from non cucurbitaceous plants.

Observation	Host species							
	Taraxacum officinale	*Convol-volus arvensis*	*Amaran-thus retroflexus*	*Helian-tus tubero-sus*	*Chenopo-dium album*	*Panicu-lum avicular e*	*Cichorium intybus*	*Lactu-ca sativa*
Number of samples	2	4	2	1	1	1	2	2
Fibrosin bodies	+	-	-	-	-	-	-	-
Germ tube	lateral	apical	apical	apical	apical	apical	Apical	apical
Pathogenicity on zucchini	-	-	-	-	-	-	-	-
Genus/ species	*Podosphaera*	*Erysiphe*	*Erysiphe*	*Erysiphe*	*Erysiphe*	*Erysiphe*	*G. cichoracea-rum*	*Erysiphe*

3.4. Climate data

In order to find correlations between the presence of the cucurbit powdery mildew species in the North of Italy and the environmental conditions during the sampling periods, climate data of temperature (ºC), relative humidity (%) and rainfalls (mm) were recorded in Bologna and Mantua areas during the crop seasons 2010, 2011 and 2012 (Figure 19 and Figure 20).

Year 2010 was characterized by frequent precipitations in both Bologna and Mantua provinces, especially during June and August. In the same year, the lowest value of humidity and the highest value of temperature were recorded in July. In 2011 precipitations were lower than 2010, in particular in the area of Mantua precipitations were higher than in Bologna especially in June and October. In Bologna area, minimum value of relative humidity were observed in August while in Mantua humidity tended to have lower value with a minimum recorded in May. In both areas temperature was the same, with a maximum recorded in August. In 2012 year, precipitations have low values like in 2011 and also in this year the lowest value of relative humidity and the highest of temperature were recorded in August in both provinces.

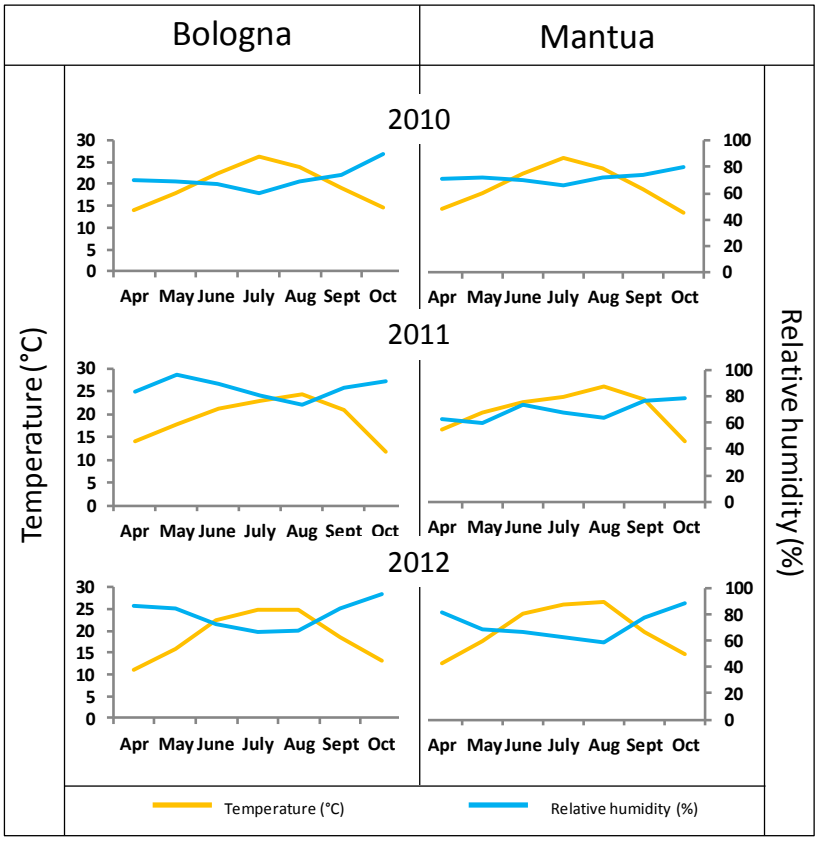

Figure 19 Environmental conditions**,** temperature (ºC) and relative humidity (%), recorded in Bologna and Mantua provinces during the periods of samplig in the crop seasons 2010, 2011 and 2012.

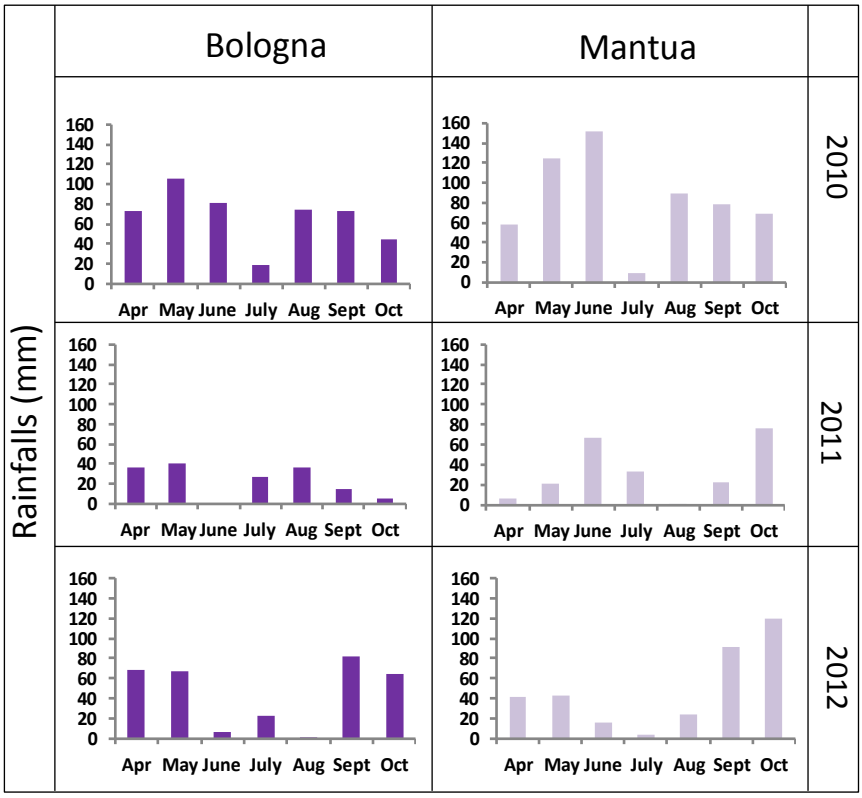

Figure 20 Rainfalls (mm) recorded in Bologna and Mantua provinces during the periods of samplig in the crop seasons 2010, 2011 and 2012.

4. Discussion

From morphological and molecular observations of powdery mildew diseased plants collected from cucurbit farms located in Bologna and Mantua provinces during the crop seasons 2010, 2011 and 2012, temporal variations in the cucurbit powdery mildew species composition were observed. These seasonal variations seem to be independent of the host plant but dependent on environmental conditions. In particular, *G. orontii* was the only species found in the earlier infections in all locations investigated and remained as the main species infecting cucurbits until a period that generally goes from the second half of June till the third week of July when *P. fusca* started to appear. After that time, *P. fusca* progressively became the only species causing powdery mildew till the end of the crop season in September-October. This peculiar behaviour could be influenced by different factors. The most obvious factor that could be considered is represented by the different ecological requirements of the two species in terms of temperature and relative humidity. According to Nagy (1976), optimum germination for *G. orontii* is about 25°C with a minimum of 10-20°C and a maximum of 30°C while that of *P. fusca* is 22°C with a minimum of 20°C and maximum of 30°C. From the same study, *P. fusca* was found to be more sensitive to moisture than *G. orontii* because it requires 100% of relative humidity for conidia germination and tolerates higher moisture content than *G. orontii*. Considering these differences, for example, *P. fusca* should appear in the second half of July because it requires higher temperature and relative humidity than *G. orontii*. However, as already hypothesized by Branzanti and Brunelli (1992), this should be in contrast to the fact that this pathogen is not found in the earlier infection that occurs when all crops are cultivated under plastic tunnels where the values of temperature and relative humidity are higher than in field conditions. The wide range of *G. orontii* in terms of temperature could explain why the species is the first to appear in May but not the fact that the pathogen was never found in the

new infections on crops in field conditions that usually occurs from the third week of July, normally the warmest period of the year.

Moreover, comparing the climate graphics with those that illustrate the presence of the two species in farms in both provinces (Figure 9 and Figure 12) it is interesting to note that *P. fusca* tended to be the prevalent species from the middle of July that was the month with the lower value of relative humidity (RH) and the higher value of temperature (t). Normally this happen because in the same farms the crop cycle switch to field conditions and there, *P. fusca* was found to be the predominant species (for example as observed in farms BO7 and MN1a/b). However, the same behavior of species variation is observed in farms where the entire crop cycle is conducted under tunnel conditions that have higher values of t and RH than in field conditions. Comparing graphics of 2011 climate data with those of the two species (Figure 10 and Figure 13), it can be observed that species variation does not seem to correlate with any parameter related to climatic conditions because, like in the previous year, most part of crops were under the microclimatic conditions of plastic tunnel. Also, in farms where crop cycle switched to field conditions (June-July), values of t where lower than 2010 and those of RH were higher but the species behavior observed was the same of 2010. So, the seasonal behaviour of cucurbit powdery mildew species appears not to be well correlated with the ecological requirements of both species.

It is interesting to note that under plastic tunnels *G. orontii* was progressively replaced by *P. fusca* but, in the period that the crop cycles switch to field conditions (end of July-beginning of August), the first symptoms were caused by *P. fusca*. This could be observed in locations BO7 and MN1a where crops were always under field conditions. Only in case of farm MN1b, the first symptoms observed were induced by *G. orontii*. However, due to the use of a variety of early cucumber, this was the only location in which a crop was cultivated from May in field conditions, a period where only infection of *G. orontii* were observed in other farms.

These temporal variations in the cucurbit powdery mildew species composition seem to be clearly influenced also by the cultivation systems. As already mentioned, crops under plastic tunnels may represent an opportunity to *G. orontii* to start its life cycle on cucurbits. The fact that this species will be progressively replaced should be due by the major aggressiveness of *P. fusca* (Chat-Locussol and Lavoy, 1990; McGrath, 2011) but also because *G. orontii* could slack its life cycle and progressively produce latent mycelia. The fact that cultivation of cucurbits under field conditions starts in the middle summer affects also the powdery mildew species composition. From these observations we conclude that, in these areas, *P. fusca* easily infects cucurbits under field conditions. An explanation for this could be that chasmothecia of *P. fusca*, that overwinter in soil and crop residues, could play a significant role in initiating these field infections. In fact, an important factor to consider that could explain this temporal variation is represented by the different overwintering strategies of the two species. As indicated previously, only chasmothecia of *P. fusca* were collected. The lacking of chasmothecia of *G. orontii* suggests that this species should overwinter in form of mycelium on alternative hosts. In particular, the Bologna area is characterized by the cultivation in October-December, in the same farms monitored, of *Lactuca serriola* that could act as a secondary host for *G. orontii*. The same could happen with spontaneous plants and weeds. However, no symptoms were observed on zucchini cotyledons inoculated with powdery mildew isolates collected from non-cucurbitaceous species. The lack of pathogenicity to zucchini should be due to the particular artificial conditions of the experiment. In literature, however, pathogenicity of *G. orontii* on cucurbits was observed for isolates collected from *Picridium vulgare* and *Senecio vulgaris* (Álvarez and Torés, 1995) and *Cichorium pumilum*, *Nicotiana tabacum* and *Lactuca serriola* (Cohen and Eyal, 1988). The overwintering of *G. orontii* as mycelium, together with the fact that the pathogen is adapted to survive to a wide range of temperatures and relative humidities, could made that this species is ready to infect cucurbits in spring.

On the contrary *P. fusca*, overwintering as chasmothecia at least in the north of Italy, should infect crops more slowly because it starts from ascospore infections. Also, the fact that in the first part of the crop season (March-July) the crops are all under plastic tunnel, can negatively influence the preservation of chasmothecia over the next winter because plants are cultivated on black plastic material that is removed together with crop residues at the end of each season. Furthermore, the preservation of chasmothecia is facilitated in soil and plant material and could explain the fact that *P. fusca* predominate in the infections that occurs on field crops.

However, chasmothecia should not be considered the only source of primary inoculum of *P. fusca* in the north of Italy. This species produce abundant fungal mass and, like other powdery mildew fungi, conidia can be efficiently disseminated by wind even to very long distances (Bardin *et al.*, 1997; Pérez-García *et al.*, 2009; Miazzi *et al.*, 2011). Cucurbit cultivations in the southern part of Europe could represent an important source of primary inoculum. In South of Italy, especially in Apulia and Sicily regions, due to the different climatic conditions, cucurbit cultivation starts earlier in the season (first transplanting from January on plastic tunnel and from April-May on field) than in Lombardia and Emilia-Romagna regions and also powdery mildew infections appear early. In this area, Sirocco wind that blow in spring-summer from South-East could transport powdery mildew conidia to the northern part of the peninsula. Moreover, in southern Spain, especially in the regions of Andalusia and Murcia, the most important areas of cucurbit production in Europe, cucurbit cultivation never stops during the year, and therefore, they could be responsible of a continuous source of powdery mildew inoculum. In these areas South-Western winds like Libeccio (Lebeche in Spanish) could play a significant role in transporting powdery mildew conidia to the North of Italy. Genetic diversity analysis of *P. fusca* populations from Northern Italy and Southern could contribute to answer these questions.

A similar variation in the occurrence of the two cucurbit powdery mildew species, although not supported by molecular data, was observed in the same area in the middle of 1980s (Branzanti and Brunelli, 1992). In the same years, a very similar behaviour was reported in France. In open field cultivations in the South of France, *G. orontii* is more frequent in May-June but *P fusca* is the predominant species in summer. From punctual observations, the arrival of *P. fusca* in open field cultivations is delayed in the North of the country compared with the South (Bertrand *et al.*, 1992). However, mixed infections of the two species were recorded very rare in this country (Křístková *et al.*, 2009). In Czech Republic, where *G. orontii* is considered the predominant species, observations carried out during 5 years indicated that, from the beginning, infections were caused by either *G. orontii* alone or mixed with *P. fusca* without any temporal succession. Only in Olomouc-Holice (Central Moravia) *G. orontii* appeared first and later it was followed by *P. fusca*, but both pathogens persisted until the end of the growing period (Křístková *et al*., 2009).

The different period of appearance of the two cucurbit powdery mildew species could be of great interest in the management of cucurbit powdery mildew in Northern Italy. Differences between the two species in sensitivity to benomyl, bupirimate, and DMI fungicides (triadimefon, fenarimol and bitertanol) were observed in France (Bertrand *et al.*, 1992). Similarly, Sedláková and Lebeda (2008) observed differences in sensitivity to fenarimol, dinocap and benomyl fungicides in 108 powdery mildew isolates tested. The results obtained in this work could be of great relevance if differences between both species could be found in the sensitivity to modern fungicides. Considering the actual critical situation in fungicide efficacy against cucurbit powdery mildew and the upcoming new limitations on fungicide applications in 2014 in the European Union, research on sensitivity to QoI, DMI, boscalid, cyflufenamid and quinoxyfen based fungicides in the cucurbit powdery mildew populations from Northern Italy must be carried out, in order to obtain information on fungicide

resistance. This information should allow designing new disease management programmes and improving the control efficacy.

Part two: Population genetics

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

1. Introduction

As illustrated above, in the North of Italy like in many areas of the world, *P. fusca* is the predominant species causing cucurbit powdery mildew. Furthermore, in this area, an abundant production of chasmothecia has been confirmed, since they can be easily collected from senescent leaves and soil. This is a very interesting observation from the epidemiological point of view especially since the epidemiological relevance of the sexual reproduction in *P. fusca* is still uncertain (Pérez-García *et al.*, 2009).

In literature, sexual stage of *P. fusca* has been found in Italy (Marras and Corda, 1977; Branzanti and Brunelli, 1987), Germany (Ulbirch and Smolka, 1994), Hungary (Nagy, 1976), Bulgaria (Velkov and Masheva, 2002), United States (McGrath, 1994), Greece (Vakalounakis and Klironomou, 1995), France (Bardin *et al.*, 1997) and Morocco (Endo *et al.* 2012) but never observed in Spain (Álvarez and Torés, 1995) nor in the South of Italy (Miazzi *et al.*, 2011). Sexual stage of *G. orontii* was never found in the area of study, according to the fact that chasmothecia of this species are considered to be rare. In fact, they were observed just in Germany (Ulbirch and Smolka, 1994), Hungary (Nagy, 1976) and Bulgaria (Velkov and Masheva, 2002). Considering that the epidemiological study conducted in North of Italy (previous chapter) evidenced the presence of chasmothecia only of *P. fusca* and that this is considered to be the most important and widespread species causing powdery mildew on cucurbits we asked whether sexual reproduction in *P. fusca* populations of North of Italy would have a significant impact on the genetic diversity of this pathogen.

As illustrated above, there are some geographical differences in chasmothecia production of *P. fusca*; so, it could be of great interest to investigate the genetic structure of *P. fusca* populations to determine if there is any evidence of recombination in the areas where chasmothecia were found and also to understand the role that sexual reproduction and recombination could play in the life cycle of this species. Information regarding the genetic structure within *P. fusca* populations is very scarce. Bardin and coworkers (1997), performing RAPD (Random Amplification of Polymorphic DNA) analysis, revealed a low degree of polymorphism in 28 isolates of *P. fusca* from different countries, and cluster analysis did not separate any group. In contrast, RAPD analysis performed by Miazzi and coworkers (2011) revealed high genetic variation within 82 isolates of *P. fusca* from Apulia (southern Italy) but, also in this case, cluster analysis did not separate groups and no markers could be associated with host plants and geographical origin of the isolates. Similarly, RAPD analysis conducted on *P. fusca* isolates from southern Spain revealed no variations among the isolates (Pérez-García, pers. comm.).

Fernández-Ortuño (2007) within a study on strobilurin resistance in *P. fusca* populations from southern Spain sequenced ITS regions from 25 isolates; only one nucleotide substitution was found in two isolates and in different positions. An AFLP (Amplified Fragment Length Polymorphism) study conducted by Naruzawa *et al*. (2011) evidenced high variability within 22 isolates of *P. fusca* from Brazil with a Jaccard similarity coefficient ranging between 0.23 and 0.69. However, the dendrogram obtained evidenced 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 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.

Control strategies must target a population instead of an individual if they are to be effective (McDonald, 1997). The cucurbit powdery mildew fungus *P. fusca* is pathogen of difficult control (McGrath, 2001; Pérez-García *et al.*, 2009). Given the versatility exhibited by the pathogen to adapt not only to environmental conditions but also to overcome different control strategies, understanding the genetics of *P. fusca* populations is a crucial aspect to anticipate how populations will evolve in response to new control strategies. With the fragmentary information about the genetic diversity of *P. fusca* populations, we considered to examine this question by different approaches. In order to shed some light in this fundamental aspect of *P. fusca* biology, in this chapter a population genetics study on *P. fusca* has been addressed using both MLST (Multilocus Sequence Typing) and AFLP techniques.

2. Material and methods

2.1. Fungal collection, maintenance and conservation

Podosphaera fusca isolates were obtained from powdery mildew infected plants from cultivated cucurbits in North of Italy in Bologna and Mantua provinces. Furthermore, to expand the area of study, isolates from South of Italy, South-Central Spain, France, Czech Republic, Bulgaria, United States, Canada and Central-South America were gently supplied by collaborators who sent powdery mildew strains as infected leaves or extracted DNA. Isolates of *P. fusca* used in the work are listed in Table 7.

Table 7 Isolates of *P. fusca* used in this study.

Isolate	Location	Year of collection	Host
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*
FE12	Molveno (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)	2008	*Cucurbita pepo*
311251	Murcia (Spain)	2008	*Cucumis melo*
311271	Murcia (Spain)	2008	*Cucumis melo*
811301	Badajoz (Spain)	2008	*Cucumis melo*
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*
31784	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*

Fungal material was collected from crops in fields or plastic tunnels. From all material collected, monoconidial isolates were obtained. With an ethanol disinfected eyelash one conidia chain was taken from an infected leaf and inoculated on zucchini cotyledons cv. Giambo maintained *in vitro* at 22°C. Cotyledons were previously disinfected in 0.1% $HgCl_2$, rinsed twice in sterile distilled water, air dried in a laminar flow cabinet and kept in 60 mm diameter Petri dishes containing 40 g l^{-1} of saccharose, 10 g l^{-1} of agar-agar and 30 mg l^{-1} of benzimidazole (Álvarez and Torés, 1997). Isolates were initially maintained by transferring every 7 days a single conidia chain to a fresh cotyledon. The process was repeated three times to obtain a pure monoconidial colony. After that, isolates were maintained by transferring conidia every 2-3 weeks to fresh cotyledons as described above. Finally, the fungal biomass was in part used for DNA extraction and in part preserved at -80°C using silica gel as described by Pérez-García *et al.* (2006).

2.2 Morphological identification

To confirm that we were working with strains of *P. fusca*, from each monoconidial colony conidia were taken and examined by light microscopy to determine the lateral position of

germ tubes and the presence of fibrosin bodies as typical identifiying features of *P. fusca* conidia (Kable and Ballantyne, 1963; Zacarovitis, 1965).

2.3. DNA isolation

DNA extraction was carried out as described in the previous chapter. The only difference was that biomass of the powdery mildew isolates was collected 15-20 days after inoculation and deposited in a 2 ml eppendorf tube and freezed at -80°C overnight. After that, CTAB buffer-0.04% β-mercaptoethanol solution (previously heated at 65°C for 1 h) and 2.5 µl of proteinase K 10 (mg ml^{-1}) were added to fungal mass. The protocol of extraction and quantification of DNA followed the steps previously described. To get higher DNA yields, several samples were whole genome amplified by the Multiple Displacement Amplification method using the protocol of "Illustra GenomiPhi" kit (GE Healthcare Bioscience, Piscataway, USA) as described by Fernandez-Ortuño *et al.* (2007).

2.4 Primers design for Multilocus Sequence Typing (MLST) analysis

To address fungal diversity by a MLST approach, we planned to sequence the following gene regions: α-tubulin (*tub-1*), chitin synthase I (*csI*), intergenic spacer (*IGS*), translation elongation factor α (*tef-1α*), and mitochondrial small subunit of ribosomal DNA (*mt SSU rDNA*). To isolate fragments from the genes described above, primers were designed on conserved gene regions by comparing sequences of other powdery mildew fungi that are phylogenetically close to *P. fusca* using Clone Manager Professional Suite 7.11 software. These powdery mildew species were *Blumeria graminis* (barley powdery mildew), *Golovinomyces orontii* (*Arabidopsis* powdery mildew) and *Erysiphe pisi* (pea powdery mildew). The genomes of these species were recently obtained (Spanu *et al.*, 2010) and sequences are available at www.blugen.org for *B. graminis* and on on-line databases of the Max Planck Institute for Plant Breeding Research at Cologne (Germany)

(http://www.mpipz.mpg.de/14157/fungal_genomes) for *G. orontii* and *E. pisi*. Sequences of other Ascomycete fungi obtained from NCBI database (GeneBank) were also aligned for primer design. Sequence accession numbers and genome contigs of species used for primer design are shown in Table 9. Only in case of translation elongation factor 1-α, the gene fragment was obtained using the degenerated primers 983F/EF-gr (Rehner, 2001).

Table 9 Accession numbers and sequence information used for primer design. *B. graminis* contigs are available on-line at www.blugen.org while those of *G. orontii* and *E. pisi* can be found at http://www.mpipz.mpg.de/14157/fungal_genomes.

Species	***tub-1***	***csI***	***IGS***	***mt SSU rDNA***
B. graminis	contig_006388	AF188934.1	HM538452.1	-
G. orontii	Go_V1_Contig9705.1	Go_V1_Contig1307.1	Go_V1_Contig33130.1	Go_V1_Contig155831.1
E. pisi	Ep_V2_Contig02881	-	-	-
E. necator	-	-	GQ255476.1	-
S. sclerotiorum	XM001597669.1	-		-

Oligonucleotides were synthesized by Invitrogen® (Life Technologies, Carlsbad, California, USA). All PCR reactions were conducted in a volume of 25µl using 5 µl of Promega® GoTaq Green Buffer 5×, 1 µl of 10 mM dNTPs, 2 µl of 25 mM $MgCl_2$, 1 µl of each primer (10mM) , 0.125-0.25 µl of GoTaq flexi DNA polymerase (5 u/µl) Promega® (Madison, Wisconsin, USA), 13,875-13,75 µl of double distilled water and 1 µl of DNA sample. PCR reactions were conducted in Bio-Rad® T100™ Thermal Cycler (Hercules, California, USA). PCR conditions were as follows: an initial denaturation step of 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55-60°C (depending on primer sets) for 30 s, and 72°C for 1 min, and a final extension step of 72°C for 5 min. To improve the specificity of primers and to

increase the yield of PCR products, the amplifications of *mt SSU rDNA* and *tef-1α* were carried out using a touchdown-PCR protocol that consisted of an initial denaturation at 95°C for 3 min, 10 cycles of 30 s at 95°C, 30 s at annealing temperature of 60°C decreasing each cycle 1°C in order to reach a final temperature of 50 °C for the remaining 35 cycles, and an extension step at 72°C for 60 s for each cycle, with a final extension step at 72ºC for 5 min (Rehner, 2001). PCR products were separated on 1.5 % agarose gel in 0.5× TAE buffer, stained with ethidium bromide and visualized under UV illumination. PCR products were purified using GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare Bioscience, Piscataway, New Jersey, USA). Purified PCR products were sequenced at Macrogen Europe (Amsterdam, The Netherlands). All sequences obtained from sequencing were corrected and assembled using Contig Express software (Vector NTI Advance 10.3.0) and aligned with ClustalW2 Multiple Alignment Software at http://www.ebi.ac.uk/Tools/msa/clustalw2.

In addition to *tub-1*, *csI*, *IGS*, *TEF1-α* and *MTS-rDNA* genes, for MLST analysis the following genes were also amplified and sequenced: Internal Transcribed Spacer (*ITS*) using universal primers PN23/PN34 (Mouyna and Brygoom 1993), sterol 14-α-demethylase (*cyp51*) with primers Cyp51-F/Cyp51-R (López-Ruiz, unpublished), and β-tubulin (*tub-2*) with primers Ibtub1-F/Ibtub-R (Vela-Corcía, unpublished).

2.5. Mating type identification

To identify the mating type of each *P. fusca* isolate, the primer sets aboxF2/aboxR2 and hmgF2/hmgR2 described in the previous chapter were used to amplify the alleles *MAT 1-1-1* and *MAT 1-2-1*, respectively.

2.6. AFLP (Amplified Fragment Length Polymorphism) analysis

AFLP analysis was performed following the method described by Vos *et al.* (1995) with minor modifications. Genomic DNA was digested using two restriction enzymes: Tr1I (MseI) (frequent cut) and EcoRI (rare cut) supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). Each digestion reaction was carried out in a volume of 40 µl: 25 µl of double distilled water, 8 µl of 5× RL buffer, 0.5 µl of EcoRI (15 u/µl), 0.5 µl of Tr1I (10 u/ µl) and 6 µl of genomic DNA (50 ng μl^{-1}). Digestion was obtained by incubation at 37°C for 1 hour. After digestion, MSE and ECO adapters were ligated to digested genomic DNA in a reaction mixture composed by 2 µl of 5× RL buffer, 1 µl of each adapter (10 mM), 1 µl of 10 mM ATP and 0.2 µl of T4 DNA ligase (1 unit/ µl) supplied by Invitrogen® (Life Technologies, Carlsbad, California, USA) in a final volume of 10 µl that were added to the 40 µl of digestion reaction. Ligation was carried out by incubation at 37°C for 3 h, and after that, 450 µl of double distilled water were added to each ligation mixture.

Following, the pre-amplification reaction with primers M01 (Eco RI + A) and M02 (MseI + C) was performed in a total volume of 20 µl: 4 µl of Promega® 5× colorless buffer, 0.4 µl of 10mM dNTPs, 1.2 µl 25mM $MgCl_2$, 1 µl of each primer (10mM), 0.08 µl of GoTaq Promega® DNA polymerase (5 u/ µl), 7.32 µl of double distilled water and 5 µl of diluted ligation mixture. PCR protocol consisted of an initial denaturation at 94°C for 1 min followed by 40 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, with a final extension step of 72°C for 5 min. PCR reactions were performed in Bio-Rad® T100™ Thermal Cycler. PCR products were stored at 4°C or used immediately for the selective amplification reaction.

Before to proceed to the selective amplification, PCR products from pre-amplification reaction were diluted at different dilution factors (1:5, 1:10, 1:20, 1:30, 1:40, 1:50) and amplified by selective amplification primers. Each amplification reaction was conducted in a

total volume of 20 µl: 4 µl of Promega® 5× colorless buffer, 0.4 µl of 10mM dNTPs, 1.2 µl of 25mM $MgCl_2$, 1 µl of each primer (10mM), 0.08 µl of GoTaq Promega® DNA polymerase (5 u/µl), 7.32 µl of double distilled water and 5 µl of diluted pre-amplification product. PCR protocol consisted of an initial denaturation at 94°C for 2 min followed by a cycle of 94°C for 1 min, 65°C for 30 sec and 72°C for 1 min. After, 12 cycles of 94°C for 30 sec, 64.3°C for 30 sec with a decrease in temperature of 0.7°C in each cycle, and 72°C for 1 min were performed. Subsequently, 12 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min were performed. Final extension consisted of 72°C for 7 min. PCR products were conserved at 4°C. The optimal dilution factor obtained after selective amplification reaction (described below) was 1:5. After that, a preliminary screening using 16 primer combinations was performed on 8 isolates in order to select the appropriate primers for selective amplification reactions. Primers tested had three or two more nucleotides at the 3' end of the adapter sequences. Primers used for screening and number of polymorphisms evidenced for each primer combination are illustrated in Table 8. Primer names were taken from universal codes at www.keygene.com.

Table 8 Primers used for preliminary screening and number of polymorphisms observed on 8 isolates tested.

Primer names and extensions		**MseI primers**		
EcoRI primers		**M47** MseI + CAA	**M48** MseI + CAC	**M50** MseI + CAT
E31	EcoRI + AAA	0	**4**	0
E32	EcoRI + AAC	-	**4**	2
E14	EcoRI + AT	3	1	0
E24	EcoRI + TC	**5**	**6**	**4**
E20	EcoRI + GC	2	2	**5**
E11	EcoRI + AA	-	1	0

On the basis of this screening, only primer combinations that showed at least 4 polymorphisms were chosen for selective amplification reactions: M48+E31, M48+E32, M47+E24, M48+E24, M50+E24, M50+E20. Selective amplification reactions and protocol used were the same described above. Separation of amplified fragments was carried out by electrophoresis on 5% polyacrylamide gels containing 54 ml of TBE urea (42 g of urea, 20 ml of 5× TBE and 34 ml of double distilled H_2O), 8 ml of 40% acrylamide, 8 ml of 2% bis-acrylamide, 45 µl of TEMED (Sigma-Aldrich®, St. Louis, Missouri, USA) and 300 µl of 10% APS (ammonium persulphate) solution. A pre-run was performed for about 30 min at 65W and 45°C. Before gel loading, each PCR product was denaturated by adding 10 µl of denaturing solution (1 mg ml^{-1} bromophenole blue, 1 mg ml^{-1} xylene cianol, 98% formamide and 0.5 M EDTA), then heated for 3 min at 95°C and cooled in ice. After, 4.5 µl of each denaturated PCR product was loaded on gel and run was performed for 2 h and 45 min in the same pre-running conditions. After electrophoresis, gels were fixed in about 2 l of 10 % glacial acetic acid solution for 30 min in constant agitation and washed twice in 2 l of double distilled water. After fixation, gels were silver stained in 1.5 l solution of 1 g l^{-1} $AgNO_3$ and 2.2 ml of 37 % formaldehyde for 1 h in agitation and then washed with 2 l of double distilled water for about 20 s. Gel developing was done in 2 l of sodium carbonate (30 g l^{-1}) solution prior adding 3 ml of 37 % formaldehyde and 400 µl of sodium thiosulphate (10 mg ml^{-1}).

Each of the 6 AFLP gels was manually analyzed by giving score of 1 for the presence of a common band and 0 for a band absence. Resulting data from all primer combinations were combined in a binary matrix. After the conversion in a distance matrix, cluster analysis was performed both by unweighted pair group method with arithmetic mean (UPGMA) method based on simple matching similarity coefficient with NTSYS software, and by neighbor-joining algorithm using MEGA 5 software. Finally, a dendrogram from UPGMA analysis and a phylogenetic tree from neighbour-joining analysis were generated using the same software.

2.7. Cloning of AFLP polymorphic bands

AFLP polymorphic bands were selected, manually excised from gels and re-amplified with the corresponding selective primers. After that each PCR product was cloned in a plasmid using the pGEM-T Vector System kit (Promega®). Each ligation reaction consisted of 2.5 µl of T4 DNA ligase buffer with 2×ATP, 0.5 µl of plasmid pGEM, 05 µl of DNA ligase units (1 u/µl) and 1.5 µl of PCR product. Reaction was carried out at 4°C overnight. Transformation was performed using the heat shock method. For each transformation reaction, 100 µl of *E. coli* DH5α competent cells and 2 µl of ligation reaction were mixed in a 1.5 ml eppendorf tube, and incubated for 30 min in ice. Subsequently, eppendorf tubes were incubated at 42°C for 45 s and rapidly transferred back to ice for 2 min. After that 900 µl of SOC medium were added to each tube and cell suspension was incubated at 37°C for 1 h. Finally, 0.1 ml of each cell suspension were inoculated on Petri dishes containing LB medium supplemented with ampicillin (50 µg ml^{-1}), IPGT (24 mg ml^{-1}) and X-Gal (20 mg ml^{-1}). Petri dishes were incubated at 37°C for 24 h. After incubation, white colonies were selected and transferred to new Petri dishes with LB and ampicillin (50 µg ml^{-1}) medium and incubated at 37°C for 8 h. Subsequently, single colonies were transferred to a sterile 15 ml propylene tube containing 5 ml of LB and 5 µl of ampicillin (50 µg ml^{-1}) and incubated at 37°C overnight. Plasmid extraction was carried out by the following protocol. Cells were centrifuged at 3000 rpm for 10 min and pellet was resuspended with 0.2 ml of A solution (50 mM glucose, 10 mM EDTA and 25mM Tris-HCl pH 8) and transferred to ice. After, 0.4 ml of 0.2 N NaOH and 1% SDS solution were added, and tubes were incubated in ice for 5 min and mixed by inversion. Subsequently, 0.3 ml of NaOAc 3M (pH 5.2) were added to each tube, gently mixed and centrifuged at maximum speed for 10 min. After centrifugation, 0.85 ml of each solution was transferred to new tubes and 0.6 ml of cold isopropanol was added. Tubes were then gently mixed and left for precipitation for 10 min. A maximum speed centrifugation was performed for 20 min and then 2 volumes of cold 100% ethanol were

added for precipitation. Finally, pellets were air dried and re-suspended in 50 µl of sterile double distilled water. When necessary, incubation at 65ºC for 10 min was carried out to facilitate pellet re-suspension. Plasmid extraction efficacy was verified by electrophoresis on 1% agarose gels in 0.5× TAE buffer. Plasmid inserts were sequenced with universal primers SP6 and T7 at Macrogen Europe (Amsterdam, The Netherlands).

3. Results

3.1. Isolation of housekeeping gene fragments

All degenerated primers designed and primers 983F/EF-gr successfully amplified the corresponding gene fragments in all isolates tested of *P. fusca*. A total of 5 different housekeeping gene fragments were amplified. For *tub-1*, *csI* and *tef1-α* genes it was necessary to design new specific primers on the sequences obtained with degenerated or semi-degenerated primers, to improve the specificity and yield of the PCR amplification. With semi-degenerated primers atuba1 5'- GGCCAAGGMAAATAYGTACC-3' and atubb2 5'- CAGATGGTGAAATGCGATCC-3' a fragment 700 bp of *tub-1* was amplified, then specific primers abtubbs/abtubas were designed. For *csI*, degenerated primers cs1f 5'-CGAACTCGAGCMGTYCTAGC-3' and cs1r 5'-GGMAGCTTCTCRGCYTTRTC-3' amplified a fragment of 1500 bp, then specific primers csIs-a/csIs-b were designed. Degenerated primers 983F/EF-gr (Rehner, 2001) successfully amplified a 900 bp fragment of *tef1-α* containing an intron, then specific primers efspf1/efspf2 were designed. Gel pictures showing the PCR products obtained for each marker gene are illustrated in Figure 21. After sequencing of each gene fragment, useful sequence information was reduced in about 50 to 100 bp due to sequencing reaction, so the final lengths were reduced compared to those observed in gel. Features of the new primers designed and others used for MLST analysis are showed in Table 9, while gene fragment structures are showed in Figure 22.

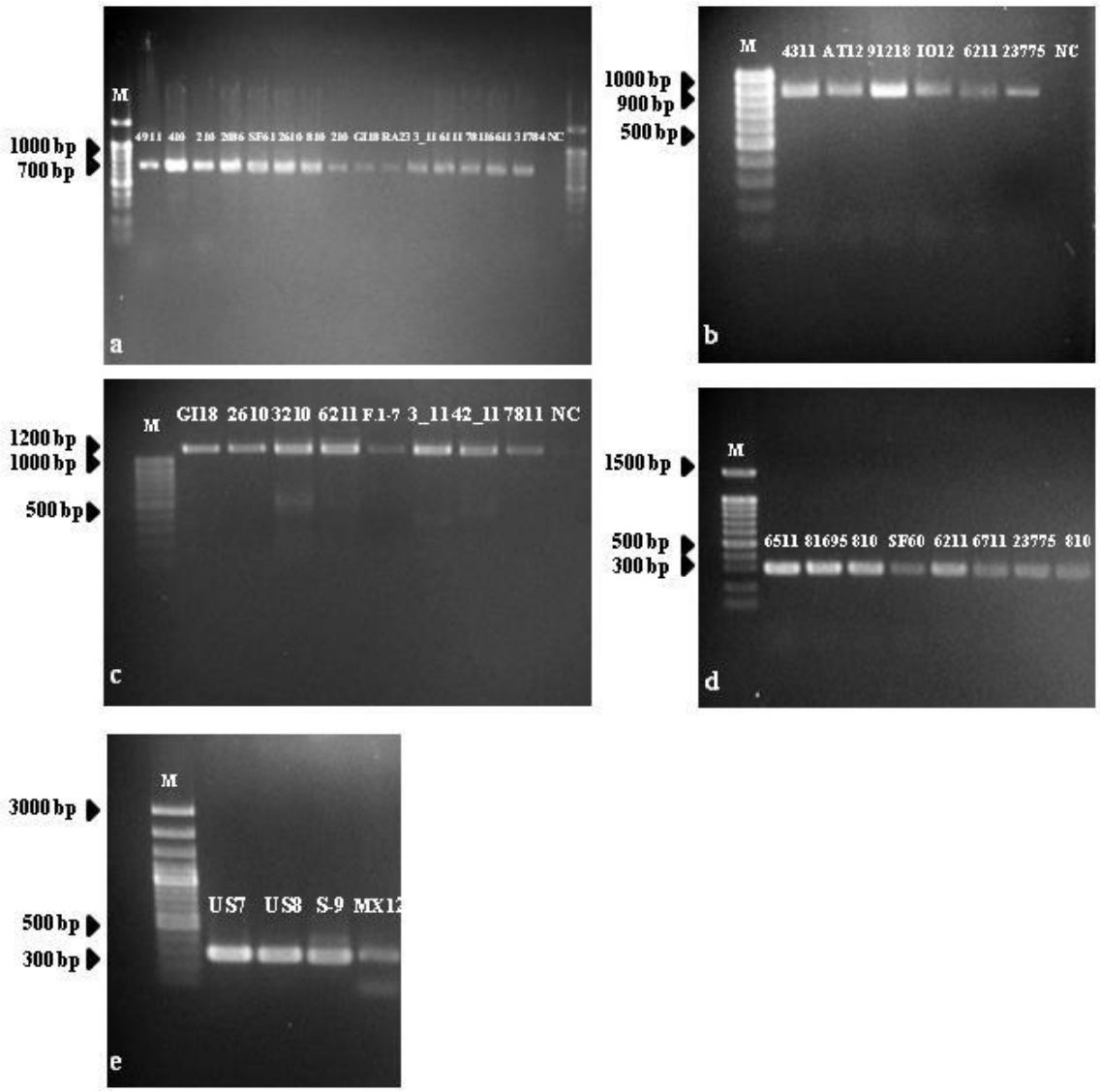

Figure 21 Amplification of MLST markers in *P. fusca* isolates: *tub1* (a), *tef1-α* (b), *csI* (c), *mt SSU rDNA* (d) and *IGS* (e). Different molecular size markers (M) were used: 100 bp DNA ladder Promega® (a and d), MassRuler™ Low Range DNA ladder (b and c) and GeneRuler™ 100bp Plus DNA ladder (e), both supplied by Fermentas®. NC is the negative control (no DNA was loaded).

Table 9 Gene fragmentes used for MLST analysis in *P. fusca*.

Targe tgene	Primer names	Primer sequences (5'-3')	Annealing tempera-ture (C°)	Fragmen t[1] size (bp)	Intron size (bp)	Identity [2] (%)
tub1	abtubbs abtubas	ATTGCGACCTTGAGCCCAAC GTTAGCCCTGCGAAAGCTTC	60	650	79	89
csI	csIs-a csIs-b	GAGTATACGACCCAGGTCAG ACATATGCCACGGATCGAAG	60	1100	43	95
tef1-α	efspf1 efspf2	TATCATCGCTGCTGGAACTG CGATAACCTGTGCCATGAAG	52	700	50	91
IGS	igs1 igs2	GGAAAGCCACYACTSRTAGC GGSWGRRTCAMCCAGGTAAC	55	290	-	82
mt SSU rDNA	mtssu1 mtssu2	TGCCAGCAGTCGCGGTAATC TGTTCGCTACCCGAGCCTTC	50*	250	-	88
ITS	PN23 PN34	CACCGCCCGTCGCTACTACCG TTGCCGCTTCACTCGCCGTT	60	800	-	Mouyna and Brygoo, 1993
cyp51	cyp51-F cyp51-R	CTTTCTCAGAGGCGCGATGG CGGATCTTCCTCGCCTCACA	62	1000	52	López-Ruiz, unpublished
tub2	Ibtub1-F Ibtub1-R	GGGCGCCAAGCCTTCACTCG AAAGGGACCAGCGCGAACAG	55	1200	20-62	Vela-Corcía, unpublished

[1] Fragment size referred to those of useful sequence obtained after sequencing.

[2] Identity shows the result after using Blastx (for *tub-1*, *csI* and *tef1-α*) or Blastn (for *IGS* and *mt SSu rDNA*) tools at http://blast.ncbi.nlm.nih.gov/Blast.cgi

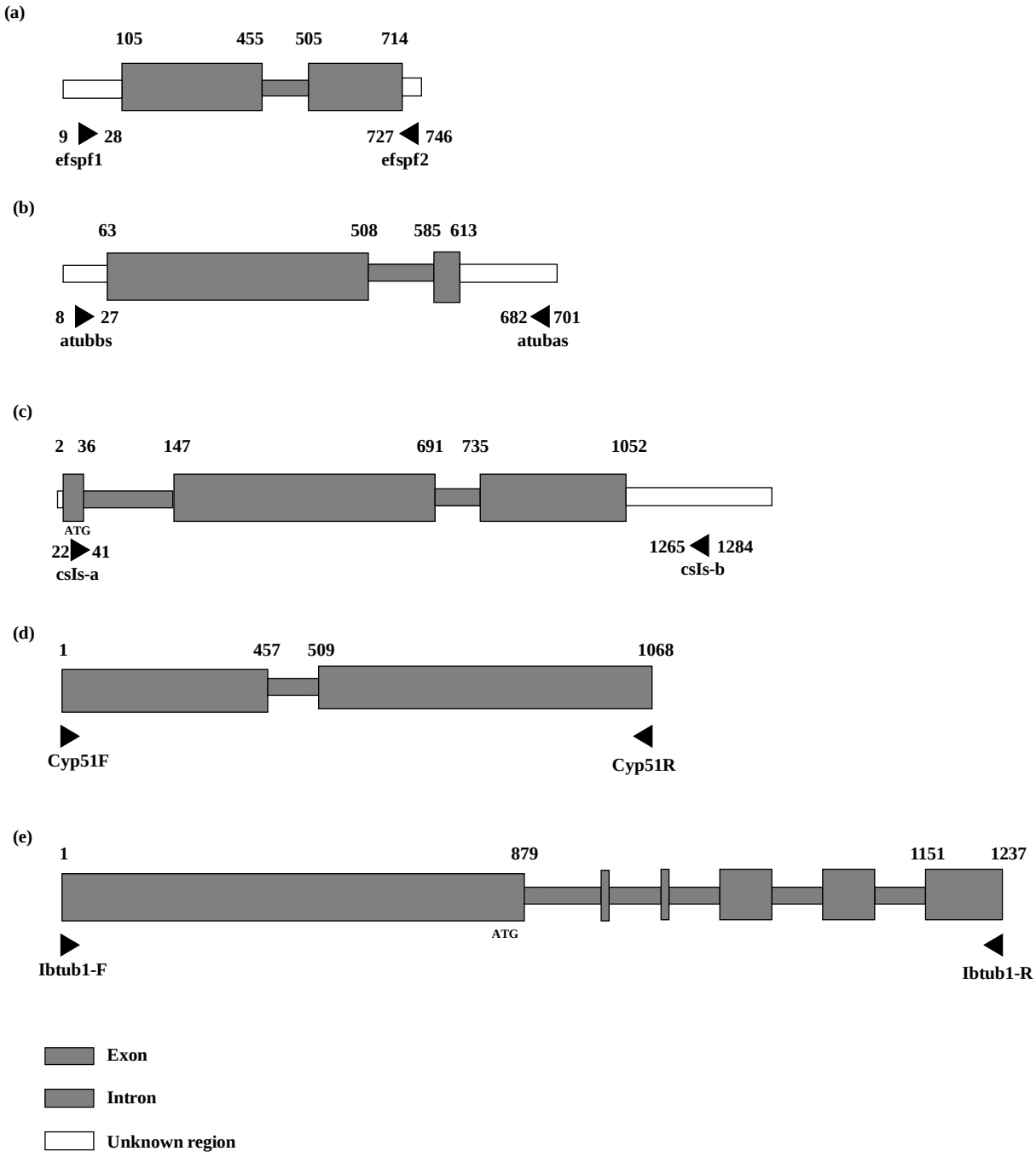

Figure 22 Gene fragment structures of *tef1-α* (a), *tub1* (b) , *csI* (c), *cyp51* (d) and *tub2* (e) and sites where specific primers were designed. Prediction of gene structure of *tef1-α*, *tub1* and *csI* was obtained by comparing sequences with genome of *S. sclerotiorum* using HMM-based gene structure prediction tool at http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind while cyp51 and tub2 were obtained from Lopez-Ruiz (unpublished) and Vela-Corcia (unpublished).

3.2. Genetic diversity analysis of *P. fusca* populations by MLST method

Once the specific primers of the new marker genes were designed and tested, a systematic MLST analysis of *P. fusca* isolates was undertaken. From the different isolates, each marker was sequenced three times from three independent PCR amplifications. After sequencing, consensus sequences were obtained for each isolate and marker. Subsequently, sequences from the same marker were aligned in order to find genetic variation. As shown in Table 10, no variations were observed among the isolates; only 1 allele was found for each of the eight marker genes analysed.

Table 10 MLST analysis in *P. fusca*. Number of isolates sequenced and alleles observed for each marker.

MLST marker	***ITS***	***IGS***	***tub1***	***tub2***	***csI***	***tef-1α***	***mt SSU rDNA***	***cyp51***
Sequence length (bp)	800	290	650	1200	1100	700	250	1000
Isolates sequenced	70	68	67	81	53	72	78	42
Number of alleles	1	1	1	1	1	1	1	1

3.3. Frequencies of *P. fusca* mating types

Primer pairs aboxF2/aboxR2 and hmgF2/hmgR2 were used to molecularly characterised the mating type (*MAT 1-1-1* or *MAT 1-2-1*, respectively) of the different *P. fusca* isolates and this way estimate the mating type frequencies in relation to the geographical origin of the isolates (Figure 23). As shown in the figure, ratio of *MAT* frequencies of isolates from Northern Italy tended to be 1:1 as already observed above in "Part one: Epidemiology". A similar tendency was observed in other countries such Czech Republic, Greece and Bulgaria, although it is important to note that only a small number of isolates was tested. By contrast, *P. fusca* populations from Spain, France and from the American continent showed an

unbalanced ratio, where the proportion of isolates belonging to *MAT 1-2-1* allele was prevalent.

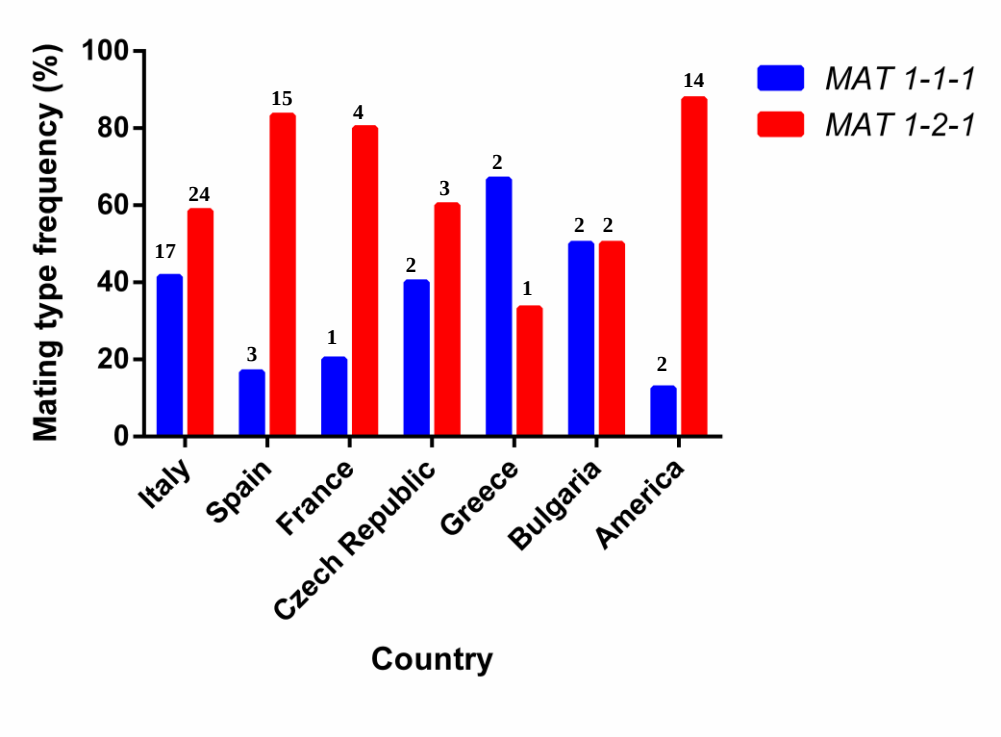

Figure 23 Frequencies of mating type alleles in *P. fusca* populations from European countries and American continent. Mating type alleles were molecularly identified by a Multiplex-PCR using primer pairs aboxF2/aboxR2 and hmgF2/hmgR2. Numbers above columns indicate the number of isolates identified for each allele in each country.

3.3. AFLP fingerprinting

Using the six primer pairs described previously for the selective amplification reaction, presence and absence of polymorphisms and common main bands were observed in each AFLP gel and scored for the binary matrix construction. AFLP gels obtained can be visualized in Annexes, while markers selected from each primer combination and relative percentage of polymorphism are illustrated in Table 11.

Table 11 AFLP analysis in *P. fusca*. 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**

Afterwards, cluster analysis with UPGMA method using simple matching similarity coefficient was performed and a dendrogram showing genetic similarity was obtained (Figure 24).

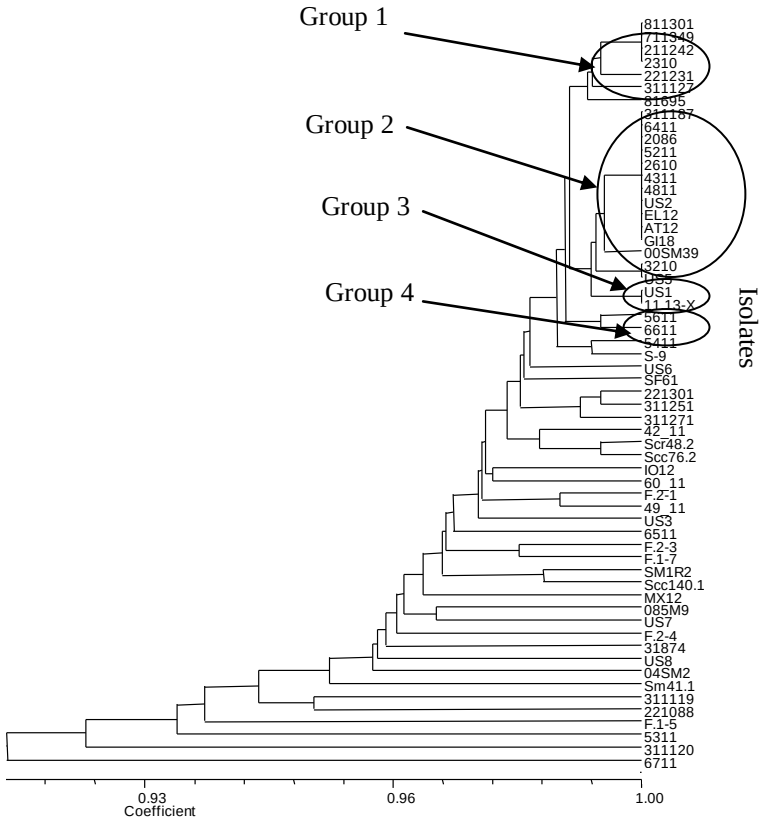

Figure 24 Dendrogram obtained from cluster analysis showing genetic diversity of *P. fusca* isolates. The binary matrix was converted to a distance matrix by NTSYS software and then cluster analysis was performed using UPGMA algorithm with simple matching similarity coefficient.

As shown in the dendrogram, genetic similarity was very high as it ranged from 0.91 and 1.00. Isolates did not cluster in groups in function of geography, host plant, years 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 is composed by isolates 811301, 711349 and 211242 (all from Spain), the second group by isolates 311287 (Spain), 2086 (Greece), 5211,6411, 2610, 4311, 4811, EL12, AT12, GI18 (all from North of Italy) and US2 (U.S.A.), the third group by isolates 3210 (North of Italy) and US5 (U.S.A.) and the fourth group by isolates US1 and 11.13-X (all from U.S.A.).

Subsequently, with the same distance matrix a neighbor-joining tree was constructed (Figure 25). Even if two groups are clearly separate, tree is not informative as isolates did not separate in function of geography, host plant, years of collection or mating types.

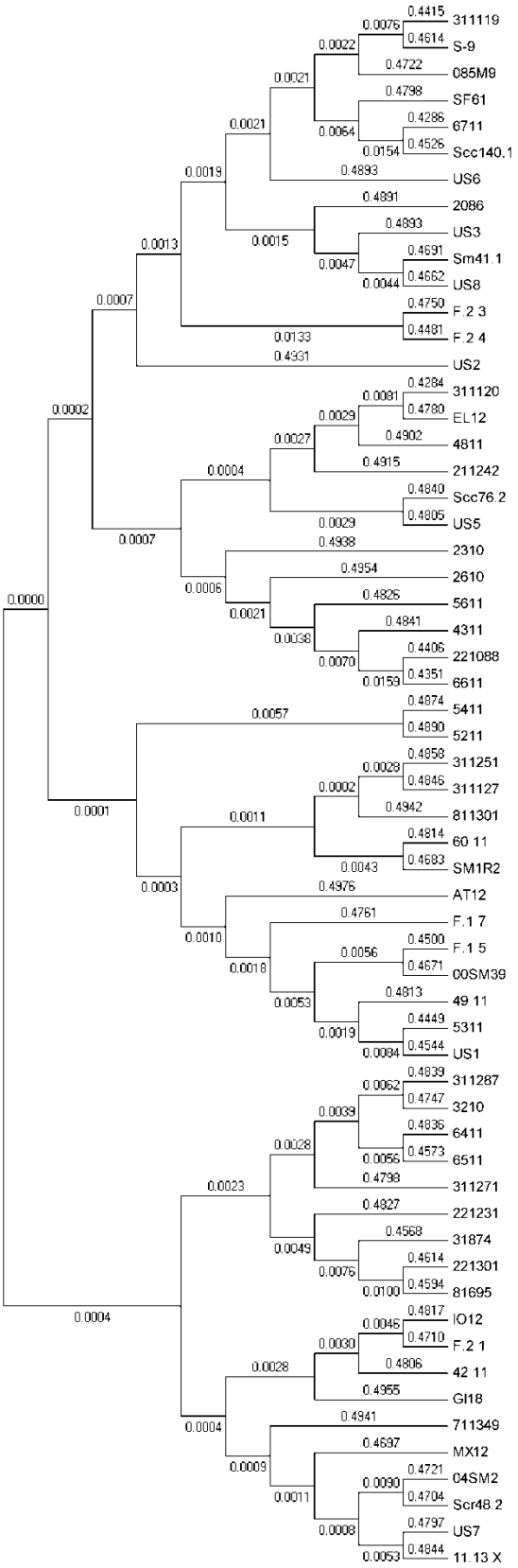

Figure 25 Neighbor-joining tree of 59 isolates of *P. fusca*. Tree was performed on distance matrix using MEGA 5 software. The distances between nodes in the tree are represented as different lengths of the branches connecting the nodes. The greater the length of the branch that joins two nodes, the greater the distance between these nodes.

3.4. Cloning of polymorphic bands

Although, in general, a low degree of genetic diversity was observed, AFLP analysis could reveal some polymorphisms among *P. fusca* isolates. In order to get insights into the DNA sequences that were apparently subjected to variation, 12 of these polymorphic bands were isolated from AFLP gels, cloned into plasmids and sequenced. After sequencing, Blastn and Blastx algorithms were performed in order to find similarity to sequences deposited on databases. The results of this analysis are illustrated in Table 12. As shown in the table, polymorphisms from isolates 8511, US6 and 311271 showed similarity to microsatellite sequences, from 31119 and 5311 to a polyprotein associated to a Long Terminal Repeat (LTR) transposable element, from 31784 to a transposon, from 41.1 with the sub-unit 1 of citochrome *c* oxidase. The rest of isolates showed similarities to different proteins. In particular, polymorphisms from isolate 085M9 showed simililarity to an ABC transporter protein, from isolate 31119 to a guanylate binding protein and to nitrite reductase and finally, from isolate 8511 to alcohol dehydrogenase II and to RNA polymerase II mediator complex.

Table 12 Isolation and sequencing of some polymorphisms revealed by AFLP analysis of *P. fusca* isolates. Only the results of the best hit of Blastn or Blastx analysis are shown.

GEL	Isolate number	Progressive number in polyacrilamide gel	Polymorphism number on gel	Accession number	Putative function	Identity (%)
2 (M47-E24)	311119	7	34	AAZ28935.1	polyprotein associated to a LTR transposon	47
2 (M47-E24)	5311	20	34	AAZ28935.1	polyprotein associated to a LTR transposon	47
2 (M47- E24)	8511	34	32	HQ885810.1	microsatellite	100
3 (M50-E20)	085M9	41	24	CCD54073.1	ABC transporter	80
3 (M50-E20)	US6	54	19	HQ884420.1	microsatellite	100
4 (M50-E24)	41.1	49	23	AB070473.1	sub-unit 1 of citochrome *c* oxidase	91
5 (M48-E31)	311271	6	22	HQ888161.1	microsatellite	89
5 (M48-E31)	311119	7	19	EGG23654.1	guanylate binding protein 1	38
5 (M48-E31)	31874	8	21	XP_002478092.1	transposon	53
5 (M48-E31)	8511	34	20	XP_001396235.1	alcohol deydrogenase 2	70
5 (M48-E31)	8511	34	23	EFY89126.1	RNA polymerase II mediator complex component	51
6 (M48-E32)	311119	7	24	BAH95949.1	nitrite reductase	81

4. Discussion

In this study, fragments of five housekeeping genes from *P. fusca* were isolated and used for MLST analysis, demonstrating that designing primers on conserved sequences of phylogenetically close related species is a good way to find genes in a species of unknown genome. Those markers together with *ITS, tub-2* and *cyp51* markers were used to investigate the genetic variability in *P. fusca*. Some of these markers have been successfully use in population genetics studies in other powdery mildew species such as *E. necator* (Brewer and Milgroom, 2010) and *B. graminis* (Inuma *et al.*, 2007). However, in *P. fusca*, they did not reveal genetic variation despite the fact that isolates from geographical origins very distant were included in the analysis. Using these markers, this species seems to be a clonal population. This result is in contrast with the occurrence of chasmothecia and the mating type ratio 1:1 observed, for example, in the North of Italy, that suggests the existence of actively mating populations. Under this circumstances, recombination should occur, this way introducing some degree of genetic variability at least in Italian populations of *P. fusca*. During sexual reproduction, meiosis results in independent assortment of chromosomes and recombination within chromosomes. This has two major effects on population structure: relatively high levels of genotypic diversity and random association between alleles at different loci, such that genotype frequencies can be predicted from the allele frequencies at each locus. These two characteristic of population structure distinguish sexual from most asexual populations. Also, the first major effect of sexual reproduction at the population level is the production of recombinant genotypes (Milgroom, 1996). This seems not to be the case for *P. fusca*. Even if sexual stage is easy to find in the North of Italy, the lack of genetic variation arises an important question: what is the relevance and the role of the sexual stage in the life cycle of *P. fusca*?

It is known that one of the advantages of recombination is to produce novel genotypes that may allow organisms to adapt quickly to changing environments (Milgroom, 1996).

Although in literature the appearance of sexual stage of *P. fusca* is considered rare or was never observed in some areas (McGrath, 1994), in the North of Italy the fungus seems to overwinter as chasmothecia because of the absence of host plants during the autumn and winter seasons and this is also supported by the fact that an equilibrium between the frequencies of both *MAT* alleles was observed. On the contrary, in the South of Spain, where cucurbits are cultivated during all year, chasmothecia have never been found (Álvarez and Torés, 1995) and as shown by this study, *MAT* genes seems to be under a selection that tends to favour the *MAT 1-2-1*. However, despite this difference in mating type frequencies, no genetic variation was found between Italian, Spanish and other European isolates of *P. fusca*. We expected to find, however, at least some geographical differences between European and American isolates. Population genetic studies of grape powdery mildew (*E. necator*) evidenced greater haplotype richness and nucleotide diversity within Eastern U.S. populations with respect to European populations (Brewer and Milgroom, 2010). This is because of the pathogen originated from the Eastern part of the U.S. country and only two genetic groups were separately introduced in Europe. Because of the Central-South American origin of some cultivated cucurbits, in particular of the genus *Cucurbita* (Bisognin, 2002), this should be also the case of *P. fusca*. However, using MLST markers this was not the case, since no differences were observed between European and American isolates. A similar situation has been observed in *Fusarium oxysporum* f.sp. ciceris where no variations were observed using EF1-α, β-tubulin, histone H3, actin and calmodulin 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 be also the case of *P. fusca* that could be a pathogen of a recent speciation on cultivated cucurbits and that the differentiation in pathotypes and races should be due to relatively recent and minor genetic changes.

Given the absence of genetic variation observed in the populations of *P. fusca* by MLST, genetic diversity was addressed by the AFLP technique. Using 59 of the 92 isolates analysed by MLST, genetic similarity resulted to be again very high as it ranged between 0.91 and 1.00. Unlike the AFLP study of Naruzawa *et al.* (2011) performed only on Brazilian strains, the isolates analysed in this study showed a higher similarity. Both dendrogram and neighbour-joining tree did not group isolates in function of geographical origin, host plants, climate areas, cultivation systems or mating types. In particular, dendrogram looks like a random mating population. Considering the epidemiology of this pathogen and the role that winds may play in the spread of this species, the absence of geographical groups separated could make sense. Accordingly, differences among isolates are very little as they are all distributed in a very little range of similarity coefficient.

To explain these little differences among isolates several factors can be considered. One of these factors could be found in the genomes of these obligate biotrophic pathogens. Recent genome sequencing of phylogenetically close related powdery mildew species such *B. graminis*, *E. pisi* and *G. orontii* revealed that genomes of these species are 120, 151 and 160 Mb in size, respectively, which means that they are four times larger than the median of other ascomycete fungi. It is interesting to note that because of the obligate biotrophism, these genomes are characterized by the deficiency in several classes of conserved primary and secondary metabolism genes. These include the nitrate and sulfate assimilation pathways and plant cell wall hydrolytic enzymes. In addition, a massive proliferation of transposable elements (TEs) was found. In *B. graminis*, where TEs account for 64% of the genome size, the most abundant families comprise non–long terminal repeat (LTR) retrotransposons lacking LTRs. According to the authors of this study, these hallmarks may represent a tradeoff between advantages of increased genetic variation independent of sexual recombination and irreversible deletion of genes dispensable for biotrophy (Spanu *et al.*, 2010). A similar genome is also presented in the ectomicorrhizal symbiont ascomycete

Tuber melanosporum that is 125 Mb in size and TEs account for approximately 58% of the genome (Martin *et al.*, 2010). Like those powdery mildew fungi, it could be possible that genome of *P. fusca* could be similar in size and that the genetic variation could be mostly due to TEs. To support this hypothesis, three of the AFLP polymorphic fragment sequenced showed a high identity with transposons and polyproteins associated to LTR TEs. In accordance, two TEs have been also found associated with the only two full-length genes cloned so far from *P. fusca*, the *cyp51* and *tub2* genes (Pérez-García, pers. comm.).

According to MLST results, no polymorphic AFLP fragments belonging to housekeeping genes were identified. This suggest that such genes are very much conserved in *P. fusca* and that are not suitable for MLST analysis. As illustrated in Table 4, sequencing of AFLP polymorphic bands showed high identities with other interesting genes that could explain, at least in part, the differences observed among isolates in the AFLP dendrogram. So, the second factor to consider is the nature of the different selection pressures that could act on each individual. The application of fungicides is the principal tool in most cucurbit crops for managing powdery mildew disease (Pérez-García *et al.*, 2009) and, therefore, most of the fungal populations must be submitted to a continuous selection. Accordingly, *P. fusca* has shown a high potential to develop fungicide resistance (McGrath, 2001). Thus, in response to the different modes of action of fungicides, the interactions with natural enemies or antagonists such as the hyperparasite fungus *Ampelomyces quisqualis* or the antagonistic bacterium *Bacillus subtilis*, or even the action of host plant defence compounds, selection could account for little changes in the genotype (point mutations, small insertions, deletions, etc) to cope with these factors. Such factors could select molecular changes in the individuals that are not detected as genetic variation using housekeeping marker genes.

Supporting this hypothesis, two AFLP fragments showed high similarities with two interesting proteins: an ABC transporter and a guanylate binding protein. ABC transporter proteins are thought to contribute to fungicide resistance in plant-pathogenic fungi by

helping cell detoxification (del Sorbo *et al.*, 2000; Stergiopoulos *et al.*, 2002). In particular, they have been extensively studied in *Botrytis cinerea*. It was found that ABC transporter proteins played a role in fungal protection against the plant defence compounds phytoalexins such as resveratrol, and fungicides such as fenpiclonil and fludioxonil (Schoonbeek *et al.*, 2001; Vermeulen *et al.*, 2001). Moreover, Pane *et al.* (2008) observed that an ABC protein is immediately activated to defend *B. cinerea* against H_2O_2 produced by plants in the earlier stage of infection. Similarly, Vermeulen *et al.* (2001) affirmed that other fungicides such as the azole fungicide tebuconazole and the strobilurin fungicide trifloxystrobin also induced transcription of some of the ABC transporter genes in the same pathogen. The same authors proposed that several ABC transporters possibly work in protection of the fungus against fungicides and could be responsible of multi-drug resistance development.

Studying hypovirulence of strains of the chestnut blight fungus *Cryphonectria parasitica* harboring RNA viruses of the genus *Hypovirus*, a guanylate binding protein was found to be involved in a virus-mediated attenuation of fungal virulence (Choi *et al.*, 1995). Considerations must be done about the perspectives resulting from the finding of these two proteins associated with AFLP polymorphism in *P. fusca*. It is interesting to think that ABC transporters and guanylate binding proteins could probably exert some influence on control strategies and virulence processes, respectively. In the case of *P. fusca* ABC transporters could be very important in fungicide resistance, for example in relation to the yet not clear mechanism of resistance to QoI fungicides (Fernandez-Ortuño *et al.*, 2008), since a similar efflux-transporter-mediated mechanism of resistance to QoI fungicides has been reported in field isolates of *Pyrenophora triticirepentis* (Died) Dreschsler (Reimann and Deising, 2005).

Another AFLP polymorphism resulted to be the sub-unit 1 of the mitochondrial cytochrome *c* oxidase. Cytochrome ***c*** oxidase subunit I (*cox1*) is a mitochondrial encoded gene, which is recognized as an extremely useful DNA barcode capable of accurate species identification in a very broad range of eukaryotic life forms (Hebert *et al.*, 2004; Ward *et al.* 2005;

Hajibabaei *et al*,. 2006; Seifert *et al.*, 2007). *Cox* is the default DNA barcode approved by GeneBank and the Consortium for the Barcode of Life (CBOL) and has proven to be very useful in phylogenetic studies of the oomycete genus *Phytophthora* (Martin and Tooley 2003; Kroon *et al.*, 2004) and in the barcoding of red algae (Saunders, 2005). Robideau *et al.* (2011) studying the utility of *cox* and ITS for phylogeny of oomycetes, concluded that *cox* sequencing is a very useful addition to the oomycete molecular toolbox and that the use of both ITS and *cox* rather than one or the other, is recommended for taxonomic identification of oomycetes. Considering the lack of variation in the ITS region in *P. fusca*, further studies using the citochrome *c* oxydase subunit 1 marker gene must be conducted.

Together with fungicide resistance, the other phenotype that could be analyzed in *P. fusca* is virulence, for example in terms of race or pathotype identification. It is important to note that information about race or pathotype identification in the collection of isolates used in the present study is very scarce and fragmentary. This makes impossible to observe any grouping in the AFLP dendrogram and tree based on these elements. In previous studies, it was reported a noticeable variation both in virulence and fungicide resistance in the populations of *P. fusca* of south-central Spain (de Pino *et al.*, 2002; Fernández-Ortuño *et al.*, 2006; López-Ruiz *et al.*, 2010). Although genetic diversity studies should be focused in DNA sequences not subjected to selection pressure (Robles et *al.*, 2004), it would interesting to know whether AFLP polymorphisms *in P. fusca* could be indeed associated with phenotypes of different virulence (race/pathotype) or fungicide sensitivity.

Considering the results of genetic diversity in *P. fusca* obtained by Naruzawa *et al.* (2011) and that in this study only three isolates from Central-South America (Argentina, Martinique and Mexico) were included, to better investigate the genetic diversity in this pathogen further studies should be conducted with more isolates from this part of the American continent and also with samples obtained from Africa and Asia continents, where the other genera of cucurbits are originated (Bisognin, 2002). In any case, our results suggest that the

populations of *P. fusca* are likely to be a clonal population, with some differences among isolates probably due to agricultural practices such as fungicides treatments and cultivated hosts. In addition, even when the sexual stage could be found in the North of Italy or in other few countries, the impact of sexual reproduction on disease epidemiology seems to be minor, although this aspect should be studied further. Thus far, with the data we have in hand, the asexual cycle, with the production of a lot of fungal biomass and thousands of conidia, appears to be the most common way to the spread and colonization of this pathogen and it should be the target of the different control approaches to combat the disease.

General discussion

Although the occurrence of *G. orontii* and *P. fusca* had been previously documented in the North of Italy (Branzanti and Brunelli, 1992), in this study we described for the first time the population dynamics of both pathogens in the Bologna and Mantua areas during three consecutive growing seasons. After three years of survey, our results unequivocally showed that *G. orontii* was the first species to appear, in most cases around May. The other species, *P. fusca*, usually appeared in middle June and, after a short period of coexistence of the two species, rapidly become the only agent causing powdery in cucurbits during the second part of the growing season. In other words, our data clearly illustrated a replacement of species causing powdery mildew in cucurbits during the growing season. However, considering the incidence of the disease and severity of the symptoms, the prevalence during the growing season and the final impact on crop productivity, it is clear to us that *P. fusca* is the most important causal agent of cucurbit powdery mildew in Northern Italy and, in consequence, this species should be considered the main target of disease management practices.

According to Gause (1934), species sharing the same resource cannot stably co-exist and this behavior could be influenced by several factors. Giving the lack of correlation observed between occurrence of these species and climate conditions (the behaviour was the same in farms with plastic tunnel/field cultivation and in farms with only the micro-climatic conditions of plastic tunnels), there are no obvious climatic reasons that could explain the seasonal behaviour observed for both species. What are then the factors that govern the species dynamics observed in the North of Italy? A similar temporal succession, where epidemiology is influenced by the different overwintering strategies of the two species, has been observed in France for *Erysiphe quercicola* and *Erysiphe alphitoides*, the causal agents of oak powdery mildew (Feau, 2012). Perhaps a close examination of the perennation

strategies of the cucurbit powdery mildew pathogens could add some clues to answer the question.

Perennation is the process of bridging a period of restricted activity (overwintering or oversummering). Because powdery mildews are obligate parasites, they must be able to survive during seasons when susceptible host tissue is unavailable for infection. Knowledge of how perennation proceeds in a given powdery mildew/host/environment system is useful in devising effective control strategies (Glawe, 2008). There are three primary means of perennation in powdery mildews. Production of chasmothecia, which are the structures resulting from sexual reproduction in powdery mildews. Chasmothecia are well-adapted to serve as resistant structures in regions with cold winter temperatures and also provide a means of surviving hot, dry summers. Bud perennation that occurs when the fungus overwinters within dormant buds. Infected buds can contain hyphae with haustoria, conidiophores, and conidia. After breaking dormancy, infected buds give rise to "flag shoots" that can be covered with profusely sporulating mycelia, supplying the primary inoculum to initiate the disease cycle. The third kind of perennation involves mycelia that persist through unfavorable conditions, either the winter on hosts with persistent leaves or when high temperatures suppress growth and sporulation (Glawe, 2008).

In the area of sampling, chasmothecia of *P. fusca* were easily collected from senescent leaves and soil, indicating that this species goes through the sexual stage in the North of Italy. By contrast, chasmothecia of *G. orontii* were never found. Chasmothecia of *P. fusca* were never or rarely found is many cucurbit production areas of several countries (McGrath, 1994; Álvarez and Torés, 1995; Bardin *et al.*, 1997; Miazzi *et al.*, 2011) and more importantly, even when chasmothecia production has been obtained in laboratory conditions (McGrath, 1994), successful ascospore infections have been never reported. These are the main reasons why the epidemiological role of the sexual cycle of *P. fusca* has yet to be determined. Interestingly, the occurrence of chasmothecia was also supported by an

estimated ratio for mating types of 1:1, indicating the existence of populations of the pathogen actively mating and suggesting that *P. fusca*, could undergo sexual recombination at least in the North of Italy.

The occurrence of chasmothecia from only one of the two species observed in the North of Italy clearly indicates the different perennation strategies of both pathogens. Although not shown in this work, *G. orontii* could survive the winter on alternative host as previously described (http://triscience.com/Plant/Crops/alternate-hosts-of-cucumber-powdery-mildew/doculite_view; Sharma, 1989). This finding together with its wider adaptation to environmental conditions, could explain why this species is responsible of the first powdery mildew symptoms observed in the growing season. In *P. fusca*, however, overwintering should be due to chasmothecia, like other powdery mildew fungi such as *Erysiphe necator* (Gadoury *et al*., 2012). In this case, only after breaking these structures ascospores are released to act as primary inoculum and initiate the disease cycle. The optimal conditions for breaking chasmothecia seem to occur in middle spring, which may explain the delay in the appearance of the pathogen on cucurbit crops.

However, for powdery mildews, the production of huge numbers of spores, which are wind dispersed from one susceptible host to another, is essential for reproduction and survival because these pathogens are completely dependent on living host tissue for survival (Brown and Hovmøller, 2002). In this sense, long-distance dispersal of cucurbit powdery mildew spores could also and additional be source of primary inoculum especially for *P. fusca*, which is the predominant species in the Mediterranean basin where cucurbits are grown year around and the pathogen is always present either on protected crops or open fields (Bardin *et al.*, 1997; Miazzi *et al.*, 2011). If this was case, an unbalanced ratio of mating types should have been observed, considering the fact that, as shown in Chapter 3, in Spain the most important country for cucurbit production, a preferential selection for *MAT 1-2-1* is

observed. In Figure 26 we present a model summarizing the epidemiology of cucurbit powdery mildew disease in Northern Italy.

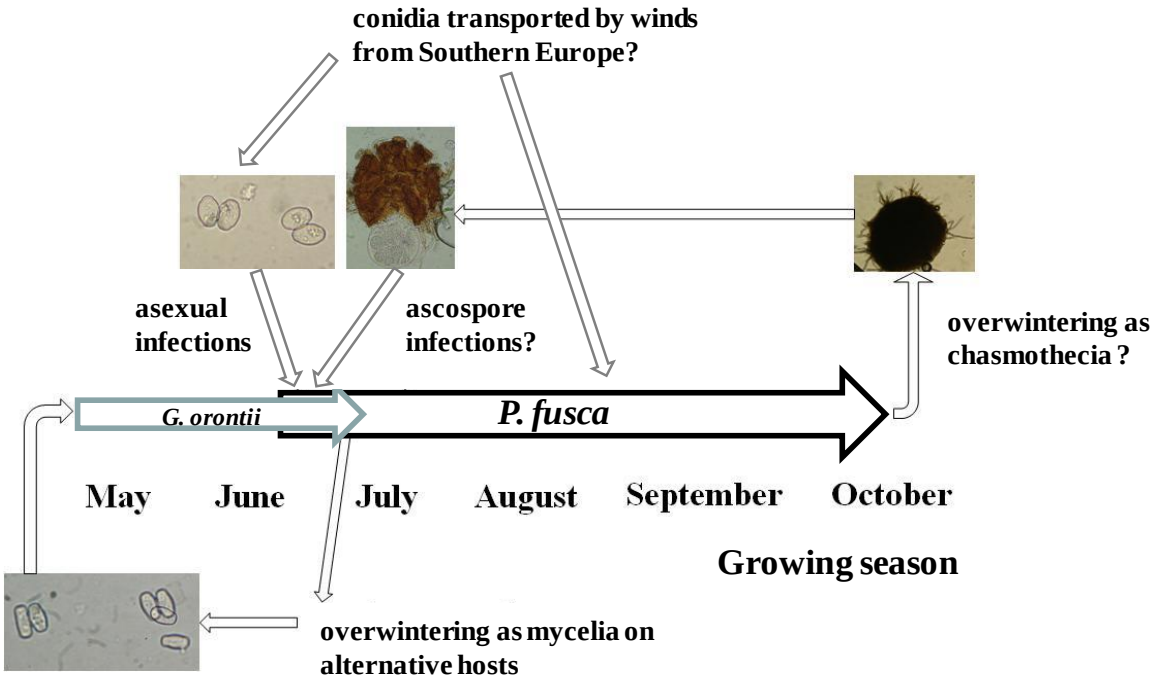

Figure 26 Epidemiology of cucurbit powdery mildew disease in Northern Italy during the growing season. The two pathogens may have different overwintering strategies. As indicated by question marks, there are a number of questions that remain to be answered.

The different occurrence of the two species during the growing season could be very important to plan new control strategies against the disease, which are mainly based on fungicide applications and the use of resistant or tolerant cultivars (McGrath, 2001; Brunelli and Gengotti, 2007; Pérez-García *et al.*, 2009). Regarding fungicide use, some differences in fungicide sensitivity have been found (Bertrand *et al.*, 1992; Sedláková and Lebeda, 2008) but further studies on sensitivity to modern molecules such as boscalid, quinoxyfen and cyflufenamid must be carried out. The lack of information makes difficult to plan a differential strategy for a chemical-based control of the two species. Regarding cultivar use, as already mentioned, although great efforts have been made in plant breeding programmes, the great variety of races especially in the case of *P. fusca* (Pitrat *et al.*, 1998; Bardin *et al.*,1999; Hosoya *et al.*, 2000; Bertrand, 2002; McCreight 2006, Lebeda *et al.*, 2011) makes

really difficult to select cultivars with resistance against the various races that can be present in a given area and that can be different over the growing season.

The occurrence of chasmothecia only from *P. fusca* is an interesting finding that arises two important questions: what are the importance and the role of the chasmothecia on the epidemiology of the disease? How chasmothecia production can influence the life cycle of this pathogen? To answer these questions we planned to investigated the genetic structure of *P. fusca*, using for that purpose isolates not only from North of Italy but also from the Southern part of the peninsula and from other countries where the disease is present: Spain, France, Czech Republic, Greece and from American continent. We hypothesized that because no chasmothecia are found in some of the above mentioned countries and that some geographical barriers may exist to avoid contact between populations, at least some genetic diversity should be evidenced among isolates. To our surprise, using fragments of 8 housekeeping genes as molecular markers for a Multilocus Sequence Typing (MLST) scheme, no differences were found within the isolates in neither intronic nor coding regions, suggesting that *P. fusca* was a clonal population.

These results are in clear contrast to the results obtained in other powdery mildew fungi. In particular, in a MLST study on *B. graminis* (barley powdery mildew), *csI* and *tub-2* provided high levels of phylogenetic signals especially in the intronic regions and at the third base position of exons, and were found to be more informative than ITS and 28S regions (Inuma *et al.*, 2007). Moreover, ITS/IGS regions of nuclear rDNA, *tef1-α* and *tub-2* were used successfully in a phylogeography and population structure study of *E. necator* (grape powdery mildew). Also in this case all of the polymorphisms in protein-coding genes were found in introns or as synonymous substitutions in coding regions (Brewer and Milgroom, 2010). According to similar data, several authors affirm that in the Erysiphales, the evolution rates of protein-coding genes are faster than those of non-coding rDNA regions (Wyand and Brown, 2003; Inuma *et al.*, 2007). This seems not to be the case for *P. fusca*, as no variations

were observed in both introns and exons of protein-coding genes and in non-coding rDNA regions.

Resident pathogen populations are expected to be more diverse than introduced populations because introduced populations have smaller effective population sizes due to losses in genetic diversity from population bottleneck and genetic drift associated with small founder population sizes (Nei *et al.*, 1975; Dlugosch *et al.*, 2008; Brewer and Milgroom, 2010). In addition, for sexually reproducing organisms, recombination from sexual reproduction may be more prevalent in resident or native populations, whereas clonal reproduction may dominate in introduced or marginal populations since multiple mating types necessary for sexual reproduction may not be present (Goodwin *et al.*, 1994; Milgroom *et al.*, 2008; Brewer and Milgroom, 2010). This could be the case for *P. fusca*. It could be possible that all the isolates analyzed may represent a single population and, although both mating types and chasmothecia were found in North of Italy, clonal reproduction appears to predominate in this species. Moreover, the pathogen could be of a recent speciation on cultivated cucurbits. Fungi of the *Erysiphaceae* family probably originated during the Cretaceous (Mori *et al.* 2000, Takamatsu and 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.*, 2000). Multiple host shifts from trees to herbs may have then occurred during the Tertiary (Takamatsu, 2004). Also, as suggested by some authors, the species infecting cucurbits could be a separate species distinct from *P. fusca* and already namely *P. xanthii* (Braun *et al.*, 2000). Alternatively, housekeeping genes are maybe highly conserved and thus unsuitable for a genetic diversity study of this species.

Because of the lack of genetic variation among the isolates, it was not possible to study the phylogeography of the species. In particular, the lack variation between American and European isolates was surprisingly. In the case of *E. necator*, genetic diversity was greater in

Eastern U.S. populations than European and Western U.S. population (Brewer and Milgroom, 2010). This means that introduced populations have lower genetic diversity than the Eastern U.S. native population from which the pathogen originated. In the case of *P. fusca*, no differences were observed within American isolates even when this continent is the area of origin of the genus *Cucurbita*. Considering the hypothesis that the clonal population observed could be an introduced population that lost genetic diversity, it could be interesting to analyze a larger number of the isolates from South America and also from Africa and Asia where the *Cucumis* and *Citrullus* genera originated (Bisognin, 2002). Maybe wild populations of the pathogen from these continents could be more diverse populations.

A similar, but a bit more informative result was obtained by Amplified Fragment Length Polymorphism (AFLP) analysis. In this case, genetic similarity of isolates was distributed between similarity coefficients of 0.91 and 1.00. This result indicated that diversity observed by this technique was also very low, a result that was congruent with that obtained by the MLST method. In particular, it is interesting to note that in both dendrogram and neighbor-joining tree, isolates seem to be randomly grouped, with no clustering in function of geographical origin, host plant or mating types. The results from this molecular analysis also suggested that indeed *P. fusca* populations showed a clonal structure.

The results of genetic diversity analysis in *P. fusca* are in contrast with the occurrence of chasmothecia in North of Italy and the possibility that, at least in this area, recombination may occur. So, if there is no genetic variation at all and recombination does not take place, which is the role of chasmothecia for this species? Is it perhaps the main role to serve as a overwintering structure? The dendrogram obtained by AFLP analysis showed clearly that isolates from North of Italy are grouped isolates from the South of the peninsula and Spain. This reinforces the hypothesis that the populations of *P. fusca* that infect cucurbits in the middle spring and summer could come from Southern countries and that those areas are continuous sources of inoculum for the populations of the North of Italy. By these

observations, importance of the sexual stage in the life cycle of the pathogen seems to be very low. However, because of the lack of host species during the winter and to overcome the unfavorable climatic conditions, the species goes under sexual stage to overwinter and, therefore, an equilibrium between the two mating type is maintained.

To better investigate the relevance of sexual (ascospores) and asexual (conidia) infections may play during the growing season in the North of Italy, a systematic analysis of the genetic diversity of *P. fusca* populations during the entire growing season should be conducted. A similar approach has been used to study the contribution of sexual recombination to population structure of *Mycosphaerella graminicola* (Cowger *et al.*, 2008). If genetic differences are found at the beginning of the season when the first powdery mildew symptoms start to appear, it could be possible to affirm that the primary inoculum is from ascospores and thus from chasmothecia. This could justify the delay in the appearance of the species, because ascospore infections are presumably slower than infections from mycelia (Glawe, 2008). As indicated above, no ascospore infections have been obtained using chasmothecia produced under laboratory conditions (McGrath, 1994). It could be of great interest to demonstrate the viability of ascospores from chasmothecia collected in the field. To address this, one method could be the use of fluorescein diacetate (Widholm, 1972). By this method, ascospore viability was recently determined in chasmothecia of *E. necator* (Portillo *et al.*, 2012).

From the results obtained in this study, asexual reproduction producing a lot of conidia and fungal biomass appears to be the most important type of reproduction in *P. fusca* and represents an important source of inoculum for the dispersion of the pathogen that can be efficiently spread by the wind (Bardin *et al.*, 1997; Pérez-García *et al.*, 2009; Miazzi *et al.*, 2011). However, how can one explain the high biological diversity exhibited by *P. fusca* in terms of, for example, variety of races and pathotypes or resistance to fungicides? Cloning and sequencing of some of the polymorphic bands from the AFLP analysis may add some

clues on that. From the 12 AFLP polymorphisms that were analysed any of them was associated with housekeeping genes, which is in agreement with the results of the MLST analysis. Genetic variation was found to be linked to transposable elements (TEs) that are presumably very abundant in the *P. fusca* genome, to genes involved in activities related to environmental survival such as cell detoxification and defense, or involve in metabolic functions such as the respiratory chain. These little differences that characterize particular individuals within a given population could probably be the result of a selection pressure exerted by different factors. As already mentioned, *P. fusca* is considered a difficult pathogen to control (McGrath, 2001; Pérez-García *et al*., 2009) and, for this reason, it is submitted to a continuous selection pressure of different origins such applications of fungicide or the use of resistant cultivars (Brunelli and Gengotti, 2007; Nuñez-Palenius *et al.*, 2009).

The scarce genetic variation observed in this study could be result of the selection of new genotypes as a consequence of changes in agricultural practices. As pointed out by McDonald and Linde, (2002), factors affecting the evolution of a population as mutations and selection induce changes in populations and create strains capable of overcoming resistance genes or develop resistance to fungicides. Indeed, changes in genotypes caused by mutations have been observed in the pathogen in response to fungicides (Collina *et al.*, 2006; Miyamoto *et al.*, 2010; Ishii *et al.*, 2011). Similarly, races and pathotypes have been developed by the pathogen in response to the use of resistance cultivars, affecting the virulence of the species (del Pino *et al.*, 2002; Lebeda *et al.* 2011). In *B. graminis* a family of virulence effectors seems to have coevolved with a particular family of retransposons (Sacristán *et al.*, 2009). As suggested by the authors, the coevolution of these two entities may reflect a mutual benefit to the association, which could ultimately contribute to parasite adaptation and success. Genomes of powdery mildews are full of transposable elements, which seem to be key elements for the evolutionary success of these pathogens (Spanu *et al.*,

2010). Although the *P. fusca* genome has not been sequenced yet, several TEs have been already identified. Our hypothesis is that these elements could be also essential for the rapid adaptation of *P. fusca* to overcome the deployment of new resistant cultivars.

According to McDonald and Linde (2002), rusts and powdery mildews stand among the plant pathogens with the highest potential risk of evolution because these species are particularly subjected to the 5 evolutionary forces. In their theoretical approach, a mixed reproduction system is a key element to provide genetic variation through recombination. In *P. fusca*, however, the apparent absence of recombination does not seem to be an obstacle for evolutionary success. The main forces driving *P. fusca* evolution could be mutation and selection that are the forces responsible for the creation and selection of new genotypes that could allow the pathogen to rapidly adapt to changes in agricultural practices. In addition, the massive production of conidia through asexual reproduction and the easy dispersion of these propagules by the wind would ensure the spread of the more adapted genotypes through gene flow. The fifth evolutionary force, genetic drift, maybe has occurred in *P. fusca* for example when the pathogen became pathogenic to cucurbits as a consequence of a founder effect. This process that causes genetic loss may have been relatively recent in time and thus being responsible for the lack of genetic diversity observed in housekeeping genes. In conclusion, as shown in Figure 27, from an epidemiological perspective, asexual reproduction is the most important system of reproduction and spread of the pathogen and probably the means of creating of new genetic variants that will be selected or not depending on agricultural practices.

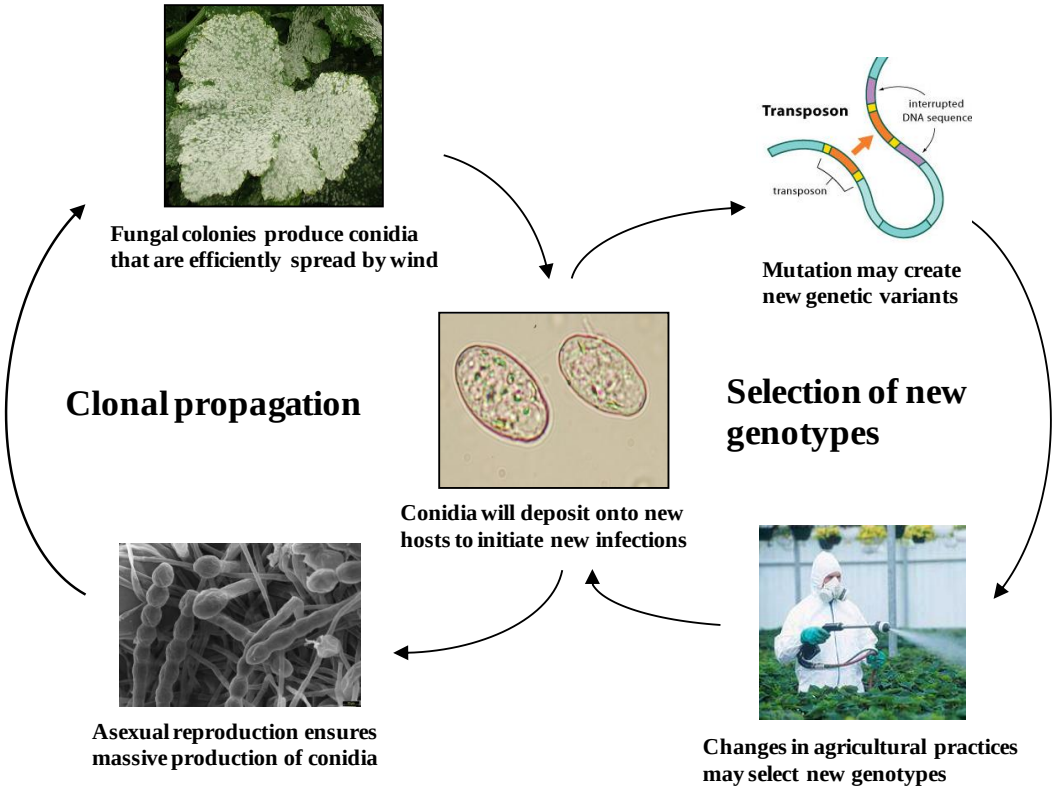

Figure 27 Epidemiological importance of the asexual life cycle of *P. fusca*. See text for details.

The genetic structure of *P. fusca* illustrated by this study and the high risk of the pathogen to evolve rapidly must be taken into consideration to improve the control of the disease when planning control strategies. The second cucurbit powdery mildew agent, *G. orontii*, has a relative minor distribution and lower economic importance as a species causing of cucurbit powdery mildew disease in South of Europe. This study showed its occurrence for a short period during the growing season in the North of Italy where no chasmothecia were collected. These factors affected the collection of an appropriate number of isolates for a population genetics study and, in consequence, the genetic structure of this species was not analyzed. This powdery mildew species has a wide host range than *P. fusca* and in literature some isolates from non cucurbitaceous plants were able to infect cucurbits (Cohen and Eyal, 1988). It could be interesting to address the genetic structure of this species to compare with that of *P. fusca*, especially considering that the two species are in competition for a common host habitat.

Conclusions

The main conclusions arising from this work are the following:

1. In Northern Italy there is a replacement of species of fungi causing cucurbit powdery mildew, *P. fusca* being the predominant species during most of the growing season.

2. The temporal variations observed in the occurrence of cucurbit powdery mildew fungi in Northern Italy should lead to reconsider the current management strategies of cucurbit powdery mildew disease in the examined areas.

3. The occurrence of *P. fusca* chasmothecia and a mating type ratio of 1:1 suggest that populations of *P. fusca* could be actively mating in Northern Italy.

4. The low genetic diversity evidenced by MLST and AFLP analyses suggests the existence of populations of *P. fusca* with a clonal structure.

5. Although chasmothecia can be easily collected in the North of Italy and other cucurbit production areas, sexual reproduction seems to be a minor source of genetic variation in *P. fusca* populations, suggesting that the sexual cycle is of minor importance for the epidemiology of the disease.

6. The AFLP polymorphisms indentified in *P. fusca* suggest that the pathogen could evolve rapidly in response to selection pressure and adapt to changes in agricultural practices.

7. The high risk of epidemics evidenced for *P. fusca* is not linked to a mixed reproduction system. In this case, other evolutionary forces should be responsible for the high evolutionary potential and biological success exhibited by the pathogen.

References

Ahmed, N., Manjulata Devi, S., de los Á Valverde, M., Vijayachari, P., Machang'u, R.S., Ellis, W.A. and Hartskeerl, R.A. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. *Ann Clin Microbiol Antimicrob*. 5: 28.

Ali, N. (1988). Untersuchungen zur Fruchtkörperentwicklung der Echten Mehltaupilze (Erysiphales). Dissertation, Martin-Luther-Universität Halle-Wittenberg.

Álvarez, B. and Torés, J.A. (1997). Cultivo in vitro de *Sphaerotheca fuliginea* (Schlecht. ex Fr.), efecto de diferentes fuentes de carbono sobre su desarrollo. *Bol. San. Veg. Plagas*, 23, 283–288.

Álvarez, B. and Torés, J.A. (1995). Ausencia de huéspedes alternativos del oídio de las cucurbitáceas en la costa oriental de Málaga. *Bol. San. Veg. Plagas*, 21:185-193, 1995.

Avise, J.C. (1994). Molecular Markers, Natural History, and Evolution. Chapman & Hall, New York 511 pp.

Bardin, M., Nicot, P. C., Normand, P. and Lemaire, J. M. (1997). Virulence variation and DNA polymorphism in *Sphaerotheca fuliginea*, causal agent of powdery mildew of cucurbits. *European Journal of Plant Pathology*, 103, 545–554.

Bardin, M., Dogimont, C., Nicot, P. and Pitrat, M. (1999) Genetic analysis of resistance of melon line PI 124112 to *Sphaerotheca fuliginea* and *Erysiphe cichoracearum* studied in recombinant inbred lines. *Acta Hortic* 492:163–168.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., and Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Manage. Sci.* 58:649-662.

Bertrand, F. (1991). Les oidiums des Cucurbitacées: Maintien en culture pure, Etude de leur variabilite´ et de la sensibilite´ chez le melon. PhD thesis, Université Paris-Sud-Orsay, Paris

Bertrand, F., M. Pitrat, A. Glandard, and J.M. Lemaire. (1992). Diversité et variabilité des champignons responsables de l'oïdium des cucurbitacées. *Phytoma* 438, 46-49.

Bertrand., F. (2002) AR Hale's Best Jumbo, a new differential melon variety for *Sphaerotheca fuliginea* races in leaf disk test. In: Proc. Cucurbitaceae 2002. Naples, Florida, pp 234–237

Bisognin, D.A. (2002). Origin and evolution of the cultivated cucurbits. *Ciência Rural*, Santa Maria, v.32, n.5, 715-723

Boeswinkel, H. J. (1977). Morphology of the imperfect states of powdery mildews (Erysiphasceae). The New York Botanical Garden Archive.

Bojórquez-Ramos, C., León-Félix, J., Allende-Molar, R., Muy-Rangel, M.D., Carrillo-Fasio, J.A., Valdez-Torres, J.B., López-Soto, F.S.M. and García-Estrada, R.S. (2012). Characterization of powdery mildew in cucumber plants under greenhouse conditions in the Culiacan Valley, Sinaloa, Mexico. *African Journal of Agricultural Research* Vol. 7(21), pp. 3237-3248.

Branzanti, B. and Brunelli, A. (1987). Distribuzione e frequenza degli attacchi di *Erysiphe cichoracearum* e *Sphaerotheca fuliginea* su zucchino in Emilia-Romagna. *Informatore fitopatologico*., 9, p. 39-45

Branzanti, B. and Brunelli, A. (1992).Etiology study on powdery mildew of Cucurbitaceae family in Emilia-Romagna. *Informatore fitopatologico*. v. 42(11) p. 37-44.

Braun, U. (1987). A monograph of the Erysiphales (powdery mildews). *Beih. Nova Hedwigia* 89, 1–700.

Braun, U. and Takamatsu, S. (2000). Phylogeny of *Erysiphe, Microsphaera, Uncinula (Erysipheae) and Cystotheca, Podosphaera, Sphaerotheca* (Cystotheceae) inferred from rDNA ITS sequences—some taxonomic consequences. *Schlechtendali*a, 4, 1–33.

Braun, U., Shishkoff, N., and Takamatsu, S. (2001). Phylogeny of *Podosphaera* sect. *Sphaerotheca* subsect. Magnicellulatae (*Sphaerotheca fuliginea* auct. s. lat.) inferred from rDNA ITS sequences-a taxonomic interpretation. *Schlechtendalia*, 7, 45–52.

Braun, U., Cook, R.T.A., Inman, A.J. and Shin, H.-D. (2002). The taxonomy of the powdery mildew fungi in Bélanger, R., W.R. Bushnell, A.J. Dik, T.L.W. Carver, ed, The Powdery Mildews. A Comprehensive Treatise. The American Phytopathological Society, St. Paul, Minnesota, pp 13-55

Brewer, M.T. and Milgroom, M.G. (2010). Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse Vitis species. *BMC Evolutionary Biology*, 10, 268.

Brewer, M. T., Cadle-Davidson, L., Cortesi, P., Spanu, P. D. and Milgroom, M. G. (2011). Identification and structure of the mating-type locus and development of PCR-based markers for mating type in powdery mildew fungi. *Fungal Genetics and Biology*. 48:704-713.

Brookfield, J. F. Y. and Parkin, D. T. (1993). Use of single-locus DNA probes in the establishment of relatedness in wild populations. *Heredity* 70, 660–663

Brown, J.K.M. and Hovmøller, M.S. (2002). Aerial dispersal of fungi on the global and continental scales and its consequences for plant disease. *Science* 297: 537-541.

Brunelli, A. and Gengotti, S. (2007). Cucurbitacee: come difenderle dall'oidio. *Agricoltura*, September, 2007, 121-123.

Brunelli, A., Sedda, G., Portillo, I., Pirondi, A. and Collina, M. (2010). Ulteriori esperienze sperimentali sull'attività di recenti fungicidi contro l'oidio delle cucurbitacee. *Atti Giornate Fitopatologiche*, 2, 419-426.

Butt, D.J. (1978). Epidemiology of Powdery mildews in: The Powdery Mildews. D.M. Spencer, ed. Academic press, London

Chat-Locussol, I. and Lavoy, M. (1990). La détermination des espèces de oïdium du concombre. *P.H.M - Revieu Horticole* 304, 35-37

Chen, R.S., Chu, C., Cheng, C.W., Chen, W.Y. and Tsay, J.G. (2008). Differentiation of two powdery mildews of sunflower (*Helianthus annuus*) by a PCR-mediated method based on ITS sequences. *Eur J Plant Pathol* 121:1–8

Choi, G.H., Chen, B. and Nuss, D.L. (1995). Virus-mediated or transgenic suppression of a G-protein alpha subunit and attenuation of fungal virulence. *Proc Natl Acad Sci USA* 92(1):305-309.

Corbaz, R. (1992). Les oïdiums des cucurbitacées en Suisse romande et au Tessin. *Vitic. Arboricult. Hort.* 24, 83-84.

Cohen, Y. And Eyal, H. (1988). Pathogenicity of *Erysiphe cichoracearum* to cucurbits. CGC 11:90

Collina, M., Alberoni, G. and Brunelli, A. (2006). Sensibilità di *Podosphaera xanthii* (agente dell'oidio delle cucurbitacee) agli analoghi delle strobilurine. *Atti Giornate Fitopatologiche*, 2, 371-372.

Collina, M., Pirondi, A., Portillo, I., Vignini, M., Vigna, F., Nanni, I. and Brunelli, A. (2012). Studio di campo e in ambiente controllato dell'attività di fungicidi verso *Podosphaera fusca*, agente dell'oidio delle cucurbitacee. *Atti Giornate Fitopatologiche*, 2, 539-546.

Cortesi, P., Gadoury, D.M., Seem, R.C. and Pearson, R.C. (1995). Distribution and retention of cleistothecia of *Uncinula necator* on the bark of grapevines. *Plant Disease*, 79: 15–19.

Cowger, C., Brunner, P. and Mundt, C.C. (2008). Frequency of Sexual Recombination by Mycosphaerella graminicola in Mild and Severe Epidemics. *Phytopathology*. 98:752-759.

Cunnington, J. H., Lawrie, C. and Pascoe, I. G. (2010). Genetic characterization of the *Golovinomyces cichoracearum* complex in Australia. *Plant Pathology*. Volume 59, Issue 1, pp. 158–164.

de Melo Aguiar, B., Batista Vida, J., Tessmann, D.J., Ribeiro de Oliveira, R., Lana Aguiar, R. and Albuquerque Alves, T.C. (2012). Fungal species that cause powdery mildew in greenhouse-grown cucumber and melon in Paraná State, Brazil. *Acta Scientarum. Agronomy*. Vol. 34 n°3.

del Pino, D., Olalla, L., Perez-Garcia, A**.,** Rivera, M.E., García, S., Moreno, R., de Vicente, A. and J.A. Torés (2002) Occurrence of races and pathotypes of cucurbit powdery mildew in Southeastern Spain. *Phytoparasitica* 30:459–466

del Sorbo, G., Schoonbeek, H.J. and de Waard, M.A. (2000). Fungal transporters involved in efflux of natural toxic compounds and fungicides. *Fungal Genet. Biol.* 30, 1-15.

Dörfet, H., Ali, N. and Schröder, M.-B. (1989). Studien zur Fruchtkörperentwicklung un Ultrastruktur von *Sphaerotheca fusca* (Ascomycetes/Erysiphales). *Flora* 183: 39-55.

Dörfet, H., and Ali, N. (1996). Studien zur sexuellen Fortplanzung und Fruchtkörperentwicklung Echter Mehltaupilze (Erysiphales). *Flora* 191:201-220.

Doyle , J. J. and Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19 :11 – 15 .

Dlugosch, K.M. and Parker, I.M. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Mol. Ecol.*, 17:431-449.

El-Ammari S.S. and Wajid Khan, M.(1983) Leveillula taurica Powdery Mildew on Greenhouse Cucumbers in Libia. *Plant Disease*. 553-555.

El-Kazzaz, M. K. (1981). *Sphaerotheca fuliginea* (Schlecht. ex Fr.) Poll., the causal agent of powdery mildew on many cucurbits in Egypt. *Egyptian Journal of Phytopathology*, 13, 65-66.

Endo. T., El Guilli, M., Farih, A. and Tantaoui, A. (2012). Identification of powdery mildew fungus on Moroccan cucurbitaceous plants. *Al Awamia* 125-126 December 2011/June 2012

Erschadi, S., Haberer, G., Schoniger, M. and Torres-Ruiz, R.A. (2000): Estimating genetic diversity of Arabidopsis thaliana ecotypes with amplified fragment length polymorphism (AFLP). *Theoretical and Applied Genetics*, 100: 633–640.

Feau, N., Lauron-Moreau, A., Piou, D., Marçais, B., Dutech, C. and Desprez-Lostau, M.-L. (2012). Niche partitioning of the genetic lineages of the oak powdery mildew complex. *Fungal Ecology*. Volume 5, Issue 2 pp. 154–162

Félix-Gastélum, R., Apodaca-Sánchez, M.A., Martínez-Valenzuela, M.C., Espinoza-Matías, S. (2005). *Podosphaera* (Sect. *Sphaerotheca*) *xanthii* (Castagne) U. Braun and N. Shishkoff on cucurbits in the northern of Sinaloa, Mexico. *Mex. Rev Fitopathol*., 23: 162-168.

Fernández-Ortuño, D., Pérez-García, A., López-Ruiz, F., Romero, D., de Vicente, A. and Torés, J.A. (2006) Occurrence and distribution of resistance to QoI fungicides in populations of *Podosphaera fusca* in south central Spain. *Eur. J. Plant Pathol.* 115, 215–222.

Fernández-Ortuño, D., Torés, J.A., de Vicente, A. and Pérez-García, A. (2007). Multiple Displacement amplification, a powerful tool for molecular genetic analysis of powdery mildew fungi. *Curr. Genet.* 51, 209–219.

Fernández-Ortuño, D., Torés, J.A., de Vicente, A. and Pérez-García, A. (2008). Field resistance to QoI fungicides in *Podosphaera fusca* is not supported by typical mutations in the mitochondrial cytochrome b gene. *Pest Manag. Sci.* 64, 694–702.

Flachowsky, H., Schumann, E., Weber, W.E. and Peil, A. (2001) Application of AFLP for the detection of sex-specific markers in hemp. *Plant Breeding* 120: 305- 309.

Gadoury, D.M., Cadle-Davidson, L., Wilcox, W.F., Dry, I.B., Seem, R.C. and Milgroom, M.G. (2012). Grapevine powdery mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Mol Plant Pathol*,.13(1):1-16.

Gause, G. F. (1934). The struggle for existence. New York, NY: Hafner Publishing Company.

Glawe, D.A. (2008). The Powdery Mildews: A Review of the World's Most Familiar (Yet Poorly Known) Plant Pathogens. *Annual Review of Phytopathology*, Vol. 46: 27 -51

Godt, M. J. W. and J. L. Hamrick. 1999. Genetic divergence among infraspecific taxa of *Sarracenia purpurea*. *Syst. Bot.* 23:427-438.

Goodwin, S.B., Cohen, B.A. and Fry, W.E. (1994). Panglobal distributions of a single clonal lineage of the Irish potato famine fungus. *Proc Natl Acad Sci*, 91:11591-11595.

Gupta, M.K. and Sharma, G.K. (2012). Studies on the dynamics of powdery mildew on cucurbits in Haryana, India. *Indian J. Sci. Res.* 3(1): 101-106.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. and Hebert, P.D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences, USA. 103:968–971.

Hebert PD, Stoeckle MY, Zemlak TS and Francis CM. Identification of birds through DNA barcodes.

Halldén, C., Hansen, M., Nilsson, N-O. and Hjerdin, A. (1996). Competition as a source of errors in RAPD analysis. *Theoretical and Applied Genetics* 93:1185–1192.

Hansen, M., Halldén, C., and Säll, T. (1998). Error rates and polymorphism frequencies for three RAPD protocols. *Plant Mol Biol Rep.* 16:139-146.

Hansen, M. (2009). Powdery Mildew of Cucurbits. Produced by Communications and Marketing. College of Agriculture and Life Sciences. Virginia Polytechnic Institute and State University. 1-2.

Heluta, V.P. (1988). Phylogenetic connections among genera of powdery mildew fungi and some questions of systematics of Erysiphales. *Biological Journal of Armenia* 41(5): 351-358.

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., and Francis, C. M. (2004). Identification of birds through DNA barcodes. *PloS Biology* 2, 1657–1663.

Hong, H., Maccaroni, M.,Figuli, P.J., Pryor, B. M., and Bellisario, A. (2006). Polyphasic classification of Alternaria isolated from hazelnut and walnut fruit in Europe. *Mycological Research* 110(11):1290-41.

Hosoya, K., Narisawa, K., Pitrat and M., Ezura, H. (2000). Race identification in powdery mildew (*Sphaerotheca fuliginea*) on melon (*Cucumis melo*) in Japan. *Plant Breed* 118:259–262

Huys, G., Coopman, R., Janssen, P., and Kersters, K. (1996). High-resolution genotypic analysis of the genus Aeromonas by AFLP fingerprinting. *International Journal of Systematic. Bacteriology* 46, 572–580.

Ibrahim, I. F., Kadhim, L. J. and Mahmood, A. H. (1985). A study of some biological characters for *Sphaerotheca fuliginea* as a new powdery mildew causative agent of cucumber in Iraq. *Journal of Biological Sciences Research*, 16, 1–12.

Inuma T., Khodaparast S.A. and Takamatsu S. (2007). Multilocus phylogenetic analyses within *Blumeria graminis*, a powdery mildew fungus of cereals. *Molecular phylogenetics and evolution*. 44(2), 741-751.

Ishii, H., Miyamoto, T., Ushio, S. and Kakishima, M. (2011). Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. Pest management science. Vol. 67, N° 4, pp. 474-482(9).

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K., and Dijkshoorn, L. (1997). Discrimination of Acinetobacter genomic species by AFLP fingerprinting, *International Journal of Systematic. Bacteriology* 47:1179–1187

Jeffrey, D. (1990) Appendix: An outline classification of the Cucurbitaceae. In: Bates, D.M., Robinson, R.W., Jeffrey, C. Biology and utilization of the Cucurbitaceae. Ithaca and London : Cornell University, 449-463.

Jimenez-Gasco, M.M., Milgroom, M.G. and Jimenez-Diaz, R.M. (2002) Gene genealogies support Fusarium oxysporum f.sp. ciceris as a monophyletic group. *Plant Pathology*, 51: 72-77.

Kable, F.P. and J.B. Ballantyne. (1963). Observation on the cucurbit powdery mildew in the Ithaca district. *Plant Dis. Rptr.* 47:482.

Karp, A. and Edwards, J. (1997) DNA markers: a global overview. In: Caetano-Anolles, G. and Gresshoff, P.M. (eds) DNA Markers – Protocols, Applications and Overviews. Wiley-Liss, Inc., New York, pp. 1-3

Kirkbride, J.H. Jr. (1993) Biosystematic monograph of the genus Cucumis (Cucurbitaceae). North Carolina : Parkway, Boone, 159p.

Kiss, K. (2010). A more than 100-year old enigma solved: fibrosin bodies, crystalline cell inclusions in conidia of some species of the Erysiphales, consist of lipids. IMC9, The biology of fungi. Edinburgh, 1-6 August 2010.

Kliebenstein, D.J., Lambrix, V.M., Reichelt, M., Gershenzon, J. and Mitchell-Olds, T. (2001). Gene duplication in the diversification of secondary metabolism: Tandem 2-oxoglutarate dependent dioxygenases control glucosinolate biosinthesys in Arabidopsis. *Plant Cell* 13: 681-693

Křístková, E., Lebeda, A. and Sedláková, B. (2009). Species spectra, distribution and host range of cucurbit powdery mildews in the Czech Republic, and in some other European and Middle Eastern countries. *Phytoparasitica*, 37: 337-350.

Kroon, L.P., Bakker, F.T., van den Bosch, G.B., Bonants, P.J. and Flier, W.G. (2004). Phylogenetic analysis of Phytophthora species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology*. 41:766–782.

Lebeda A. (1983). The genera and species spectrum of cucumber powdery mildew in Czechoslovakia. *Phytopath Z* 108, 71-77.

Lebeda, A., Sedláková, B. and Krístková, E. (2004) Distribution, harmfulness and pathogenic variability of cucurbit powdery mildew in the Czech Republic. *Acta Fytotech Zootech*. 7:174–176.

Lebeda, A. and Sedláková, B. (2006). Identification and survey of cucurbit powdery mildew races in Czech populations. In: Proceedings of Cucurbitaceae 2006, G.J. Holmes (Ed.), pp. 444-452, Asheville, NC, USA, September, 17-21, 2006, Universal Press, Raleigh, NC, USA.

Lebeda, A., Sedláková, B. and Krístková, E. (2007). Temporal Changes in Pathogenicity Structure of Cucurbit Powdery Mildew Populations Proc. IIIrd IS on Cucurbits Eds. R. McConchie and G. Rogers. *Acta Hort.* 731, ISHS.

Lebeda, A., Krístková, E., Sedláková, B., McCreight, J.D. and Coffey, M.D. (2008) New concept for determination and denomination of pathotypes and races of cucurbit powdery mildew. Cucurbitaceae. In: Proc 9th EUCARPIA Meet Genetics Breeding Cucurbitaceae. Avignon, pp. 125–134.

Lebeda, A. and Sedláková, B. (2010). Screening for resistance to cucurbit powdery mildew (Golovinomyces cichoracearum, *Podosphaera xanthii*). In: Mass Screening Technigues for Selecting Crops Resistant to Diseases, chapter 19, 295-307, IAEA, ISBN 978-92-0-105110-3, Vienna, Austria.

Lebeda, A., Krístková, E., Sedláková, B., Coffey, M.D. and McCreight, J.D. (2011). Gaps and perspectives of pathotype and race determination in *Golovinomyces cichoracearum* and *Podosphaera xanthii*. *Mycoscience* (2011) 52:159–164.

Lerceteau, E. and Szmidt, A.E. (1999). Properties of AFLP markers in inheritance and genetic diversity studies of *Pinus sylvestris* L. *Heredity* (Edinb). 82 (Pt 3):252-60.

Letham, D. B. and Priest, M. J. (1989). Occurrence of cleistothecia of *Sphaerotheca fuliginea* on cucurbits in South Australia and New South Wales. *Australian Plant Pathology*, 18, 35–37.

Leus, L., Van Huylenbroeck, J., Van Bockstaele, E. and Höfte, M. (2002). Powdery mildew on roses: pathotype screening. Proc. XX EUCARPIA Symp. on New Ornamentals II Eds. J. Van Huylenbroeck et al. *Acta Hort*. 572, ISHS 2002.

Liu, S., Wang, L., Jiang, W., Li, Y. and Liu, Y. (2011). Morphological and molecular characterizations of powdery mildew, *Podosphaera xanthii* occurring on cucurbits in Changchun Agri-Expo Garden, China. *Mycosystema*. 30(5): 702-712.

López-Ruiz, F.J., Pérez-García, A., Fernández-Ortuño, D., Romero, D., García, E., de Vincente, A., Brown, J.KM. and Torés, J.A. (2010). Sensitivities to DMI fungicides in populations of *Podosphaera fusca* in south central Spain. *Pest Manag. Sci*., 66, 801-808, ISSN 1526-98X.

Majer, D., Mithen, R., Lewis, B.G., Vos, P. and Oliver, R.P. (1996). The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Mycological Research* 100:1107-1111.

Majer, D., Lewis, B.G., and Mithen, R. (1998). Genetic variation among field isolates of *Pyrenopeziza brassicae*. *Plant Pathology* 47:22-28.

Mallick, M.F.R. and Masui, M. (1986). Origin, distribution and taxonomy of melons. *Scientia Horticulturae*, v.28, 252- 261.

Martin, et al. (2010) Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. *Nature* 464:1033–1038.

Martin, F.N. and Tooley, P.W. (2003). Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia*. 95:269–284.

Martinez-Palacios, A., Eguiarte, L. E. and Furnier, G. R. (1999). Genetic diversity of the endangered endemic *Agave victoriae-reginae* (Agavaceae) in the Chihuahuan Desert. *Am. J. Bot.*, 861, 1093-1094.

McCreight, J.D. (2006) Melon-powdery mildew interactions reveal variation in melon cultigens and *Podosphaera xanthii* races 1 and 2. *J Am Soc Hortic Sci* 131:59–65.

McDonald, B.A. (1997) The population genetics of fungi: tools and techniques. *Phytopathology* 87, 448–453

McDonald, B.A. and Linde, C. (2002). Pathogen population genetics, evolutionary potential and durable resistance. *Annual Review of Phytopathology* 40: 349-379.

McDonald, B.A. and Linde, C. (2002). The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica* 124 (2): 163-180.

McGrath, M.T. (1994). Heterothallism in *Sphaerotheca fuliginea*. *Mycologia*, 86, 517–523.

McGrath, M. T, Staniszewska, H., Shishkoff, N., and Casella, G. (1996). Distribution of mating types of *Sphaerotheca fuliginea* in the United States. *Plant Disease* 80:1098-1102.

McGrath, M.T. (2001). Fungicide resistance in cucurbit powdery mildew: experiences and challenges. *Plant disease*. Vol. 85 (3), 236-245.

McGrath, M. (2011). Powdery mildew of cucurbits. Fact Sheet Page: 732.30. http://vegetablemdonline.ppath.cornell.edu/factsheets/Cucurbits_PM.htm. Cooperative Extension- New York State – Cornell University

Meng, X. and Chen, W. 2001. Applications of AFLP and ISSR techniques in detecting genetic diversity in the soybean brown stem rot pathogen *Phialophora gregata*. *Mycological Research* 105(8):936-940.

Miazzi, M., Laguardia, C. and Faretra, F. (2011). Variation in *Podosphaera xanthii* on cucurbits in Southern Italy. *Journal of Phytopathology*. 04/2011; 159(7-8):538 - 545.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annu. Rev. Phytopathol.* 34:457-477

Miyamoto T., Ishii H. and Tomita Y. (2010). Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron–sulfur protein of succinate dehydrogenase of the causal fungus. *Journal of General Plant Pathology*, 76, 261-267.

Mouyna, I. and Brygoo, Y. (1993) Les champignons et la PCR. In: Larzul D (ed) La PCR, un procede de replication in vitro Collection Genie Genetique, Tech. et Doc., Lavoisier, Paris. (pp 335–341)

Myrta, A., Medico, E. and Boscolo, S. (2012). Valutazione di un nuovo fungicida a base di cyflufenamid per il controllo dell'oidio delle cucurbitacee e delle solanacee. *Atti Giornate Fitopatologiche*, 2, 521-526.

Mohamed, Y. F., Bardin, K., Nicot, P. C. and Pitrat, M. (1995). Causal agents of powdery mildew of cucurbits in Sudan. *Plant Disease*, 79, 634–636.

Moncalvo, J.-M. (2005) Molecular systematics: major fungal phylogenetic groups and fungal species concepts. In: Evolutionary Genetics of Fungi (Xu, J., ed.), Wymondham, Norfolk: *Horizon Bioscience.* 1–33.

Mori, Y., Sato, Y. and Takamatsu, S. (2000). Evolutionary analysis of the powdery mildew fungi using nucleotide sequences of the nuclear ribosomal DNA. *Mycologia* 92: 74 – 93 .

Mori, Y., Sato, Y. and Takamatsu, S. (2000). Molecular phylogeny and radiation time of Erysiphales inferred from the nuclear ribosomal DNA sequences. *Mycoscience* 41: 437 – 447 .

Nagy, G. S. (1976) Studies on powdery mildews of cucurbits I Life cycle and epidemiology of Erysiphe cichoracearum and Sphaerotheca fuliginea. *Acta Phytopathologica Academiae Scientiarum Hungaricae* 1977; 11(3/4): 205-210.

Nagy, G.SZ. and Kiss, L. (2006). A Check-list of Powdery Mildew Fungi of Hungary. *Acta Phytopathologica hungarica* 41(1-2), 79-91.

Naruzawa, E. S., Vale, R. K. D., Silva, C. M. and Camargo, L. E. A. (2011) Estudo da diversidade Genética de *Podosphaera xanthii* através de marcadores AFLP e seqüências ITS. Botucatu. *Summa Phytopathologica*, v. 37 n. 2, p. 94-100.

Nei M, Maruyama T and Chakraborty R (1975). The bottleneck effect and genetic variability in populations. *Evolution*, 29:1-10.

Nuñez-Palenius, H.G., Hopkins, D. and Cantliffe, D.J. (2009). Powery mildew of cucurbits in Florida. HS1067 Horticultural Sciences Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Pane, C., Rekab, D., Firrao, G., Ruocco, M. and Scala, F. (2008). A novel gene coding for an abc transporter in *Botrytis cinerea* (*Botryotinia fuckeliana*) is involved in resistance to H_2O_2. *Journal of Plant Pathology*, 90(3), 453-46

Park, M.S., Romanoski, C.E., and Pryor, B.M. (2008). A re-examination of the phylogenetic relationship between the causal agents of carrot black rot, *Alternaria radicina* and *A. carotiincultae*. *Mycologia* 100(3):2008:511–527.

Pearson, R.C. and Gadoury, D.M. (1987): Cleistothecia, the source of primary inoculum for grape powdery mildew in New York. *Phytopatology*, 77: 1509–1514.

Pérez-García, A., Mingorance, E., Rivera, M.E., del Pino, D., Romero, D., Torés, J.A. and de Vicente, A. (2006). Long-term preservation of *Podosphaera fusca* using silica gel. *Journal of Phytopathology*. 154, 190–192.

Pérez-Garcia, A., Romero, D., Fernández-Ortuño, D., López- Ruiz, F., De Vicente, A. and Torés, J.A. (2009). The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*), a constant threat to cucurbits. *Molecular Plant Pathology*, 10, 153–160.

Pitrat, M,, Dogimont, C. and Bardin, M. (1998) Resistance to fungal diseases of foliage in melon. In: Proc. Cucurbitaceae '98: Evaluation Enhancement of Cucurbit Crops. Pacific Grove, pp. 167–173.

Portillo, I. (2010). Studio genetico ed epidemiologico di *Erysiphe necator*, agente eziologico dell'oidio della vite. PhD thesis.

Portillo, I., Pirondi, A. and Brunelli, A. (2012). Studio in ambiente controllato dell'attività di Polithiol nei confronti dei cleistoteci di *Erysiphe necator*. *Atti Giornate Fitopatologiche*, 2, 3781-384.

Pryor, B.M., and Gilbertson, R.L. (2000). Molecular phylogenetic relationships among Alternaria species and related fungi based upon analysis of nuclear ITS and mtSSU rDNA sequences. *Mycological Research* 104(11):1312-1321.

Pryor, B.M., and Michaelides, T.J. (2002). Morphological, pathogenic and molecular characterization of Alternaria isolate associated with Alternaria late blight of Pistachio. *Phytopathology* 92(4):406-415.

Rabouam, C., Comes, A.M., Bretagnolle, V., Humbert, J-F., Periquet, G., and Bigot, Y. (1999). Features of DNA fragments obtained by random amplified polymorphic DNA (RAPD) assays. *Molecular Ecology* 8:493–503.

Rehner, S. A. (2001) Primers for Elongation Factor 1-alpha (EF1-alpha). Http://ocid.nacse.org/research/deephyphae/EF1primer.pdf.

Reifschneider, J.B., Boiteux, L.S. and Occhiena, E.M. (1985). Powdery mildew of melon (*Cucumis melo*) caused by *Sphaeroteca fuliginea* in Brazil. *Plant disease* 69:1069-1070.

Reimann, S. and Deising H.B. (2005). Inhibition of efflux transporter-mediated fungicide resistance in *Pyrenophora tritici-repentis* by a derivative of 4_-hydroxyflavone and enhancement of fungicide activity. *Appl Environ Microbiol* 71:3269–3275.

Roa, A.C., Maya, M.M., Duque, M.C., Tohme, J., Allem, A.C. and Bonierbale, M.W. (1997). AFLP analysis of relationships among cassava and other Manihot species. *Theor. Appl. Genet* 95:741–750.

Roberts, R. G., Reymond, S. T., and Andersen, B. (2000). RAPD fragment pattern analysis and morphological segregation of small-spored Alternaria species and species groups. *Mycological Research* 104:151-160.

Robideau, G.P., de Cock, A.W.A.M., Coffey, M.D. Voglmayr, H., Brouwer, H., Bala, K., Chitty, D.W., Désaulniers, N., Eggertson, Q.A., Gachon, C.M.M., Hu, C.-H., Küpper, F.C., Rintoul, T.L., Sarhan, E., Verstappen, E.C.P., Zhang, Y., Bonants, P.J.M., Ristaino, J.B. and Lévesque, C.A. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Mol Ecol Resour.* 11(6): 1002–1011.

Robinson, R.W. and Decker-Walters, D.S. (1997) Cucurbits. New York Cab International. *Crop Production Science in Horticulture* 6-226 pp.

Robles, J.C., Koreen, L., Park, S. and Perlin, D.S. (2004). Multilocus Sequence Typing Is a Reliable Alternative Method to DNA Fingerprinting for Discriminating among Strains of *Candida albicans*. *J. Clin. Microbiol.* 42-6 2480-2488

Rosendahl, S., and Taylor, J.W. (1997). Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. *Molecular Ecology* 6:821–829.

Rotondo, F. (2011) Study of Italian isolates of *Alternaria* spp.: molecular and morphological characterization and pathogenesis on apple tree. PhD thesis.

Sacristán, S., Vigouroux, M., Pedersen, C., Skamnioti, P., Thordal-Christensen, H., *et al.* (2009) Coevolution between a Family of Parasite Virulence Effectors and a Class of LINE-1 Retrotransposons. PLoS ONE 4(10): e7463. doi:10.1371/journal.pone.0007463

Saenz, G.S. and Taylor, J.W. (1999). Phylogeny of the Erysiphales (powdery mildews) inferred from internal transcribed spacer ribosomal DNA sequences. *Can. J. Bot.* 77:150-168.

Saunders, G.W. (2005). Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philosophical Transactions of the Royal Society of London B: *Biological Sciences.* 360:1879–1888.

Schoonbeek, H., Del Sorbo, G. and De Waard, M. A. (2001) The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. *Molecular Plant-Microbe Interaction*, 14:562-571.

Sedláková, B. and Lebeda, A. (2008) Fungicide resistance in Czech populations of cucurbit powdery mildews. *Phytoparasitica* 36:272–289.

Seifert, K.A., Samson, R.A. and Dewaard, J.R. (2007). Prospects for fungus identification using CO1 DNA barcodes, with Penicillium as a test case. Proceedings *of the National Academy of Sciences*, 104:3901–3906.

Sharma, P.D. (1989). Microbiology and Plant Pathology. p. 112

Shin, H.-D. (2000). Erysiphaceae of Korea. Suwon, Korea: *National Institute of Agricultural Science and Technology*.

Solè, M. (2003). Factors affecting the genotypic and genetic diversity of the dioecious clonal plant *Cirsium arvense* at the metapopulation level. Dissertation zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) vorgelegt an der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg.

Spanu, P.A. *et al.* (2010). Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* 10;330(6010):1543-6

Stansfield, W.D. (1986). Theory and problems of genetics. McGraw-Hill Book Company, New York, pp.140-156.

Stergiopoulos, I, Suviers, L.H. and De Waard, M.A. (2002). Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitor superfamily. *European Journal of Plant Pathology* 108: 719-734

Takamatsu, S. (2004). Phylogeny and evolution of the powdery mildew fungi (Erysiphales, Ascomycota) inferred from nuclear ribosomal DNA sequences. *Mycoscience* 45: 147 – 157.

Takamatsu, S. and Matsuda, S. 2004. Estimation of molecular clocks for ITS and 28S rDNA in Erysiphales. *Mycoscience* 45: 340 – 344 .

Taylor, J. W. and Fisher, M. C. (2003). Fungal multilocus sequence typing-it's not just for bacteria. *Curr Opin Microbiol*, 6(4):351-356.

Tomason, Y. and Gibson, P.T. (2006). Fungal characteristics and varietal reactions of powdery mildew species on cucurbits in the steppes of Ukraine. *Agronomy Research*. 4(2), 549-562

Ulbrich, A. and Smolka, E. (1994). First report of cleistothecia of *Sphaerotheca fuliginea* and *Erysiphe cichoracearum* on powdery mildew-infected greenhouse cucumbers (*Cucumis sativus*) in Germany. *Nachtrichtenble. Deut. Pflanzenschutzd*., 46 (7) 154-159.

Vakalounakis, D. J., E. Klironomou and A. Papadakis. (1994) Species spectrum, host range and distribution of powdery mildew on Cucurbitaceae in Crete. *Plant Pathology*. 43: 813-818.

Vakalounakis, D. J. and Klironomou, E. (1995). Race and mating type identification of powdery mildew on cucurbits in Greece. *Plant Pathology* 44, 1033-1038.

Valmori, I. (2012). Agrofarmaci, Ed. Informatore Agrario pp. 456

Velkov, N. and Masheva, S. (2002). Species and races composition of powdery mildew on cucurbits in Bulgaria. *Cucurbit Genetics Cooperative Report*, 25: 7-10.

Vermeulen, T., Schoonbeek, H. and De Waard, M. (2001). The ABC transporter BcatrB from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil. *Pesticide Management Science* 57: 393-402

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, and T., Hornes, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23:4407-4414.

Vos, P. and Kupier, M. (1997). AFLP analysis. DNA markers: Protocols, applications and overviews. Editor: Caetano-Anoll6s. G. & Cresshoff, P.F., Wiley-VCH Verlag, New York:115-131.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., and Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. Philosophical Transac-tions of the Royal Society of London. Series B, *Biological Sciences* 360 ,1847–1857. doi:10.1098/RSTB.2005.1716

Weir, T.L., Huff, D.R., Christ, B.J. and Romaine, C.P. (1998). RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *Alternaria alternata* from potato and tomato. *Mycologia* 90:813-821.

Whitaker, T.W. and Davis, G.N. (1962). Cucurbits: botany, cultivation and utilization. New York: *Interscience*, 250 pp.

Widholm, J.M. (1972). The use fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology*, 47-4, pp. 89-94.

Wyand, R.A., Brown, J.K.M. (2003). Genetic and formae speciales diversity in *Blumeria graminis* of cereals and its implications for host–pathogen co-evolution. *Mol. Plant Pathol.* 4, 187–198.

Zaracovitis, C. (1965). Attempts to identify powdery mildew fungi by conidial characters. *Transactions of the British Mycological Society* 48: 553–558.

Zietkiewicz, E., Rafalski, A., and Labuda, D. (1994). Genomic fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20:176-183.

Zitter, T.A., Hopkins, D.L. and Thomas, C.E. (1996). Compendium of Cucurbits Diseases. Saint Paul, MN: APS Press.

Annexes

GEL 1 M48-E24

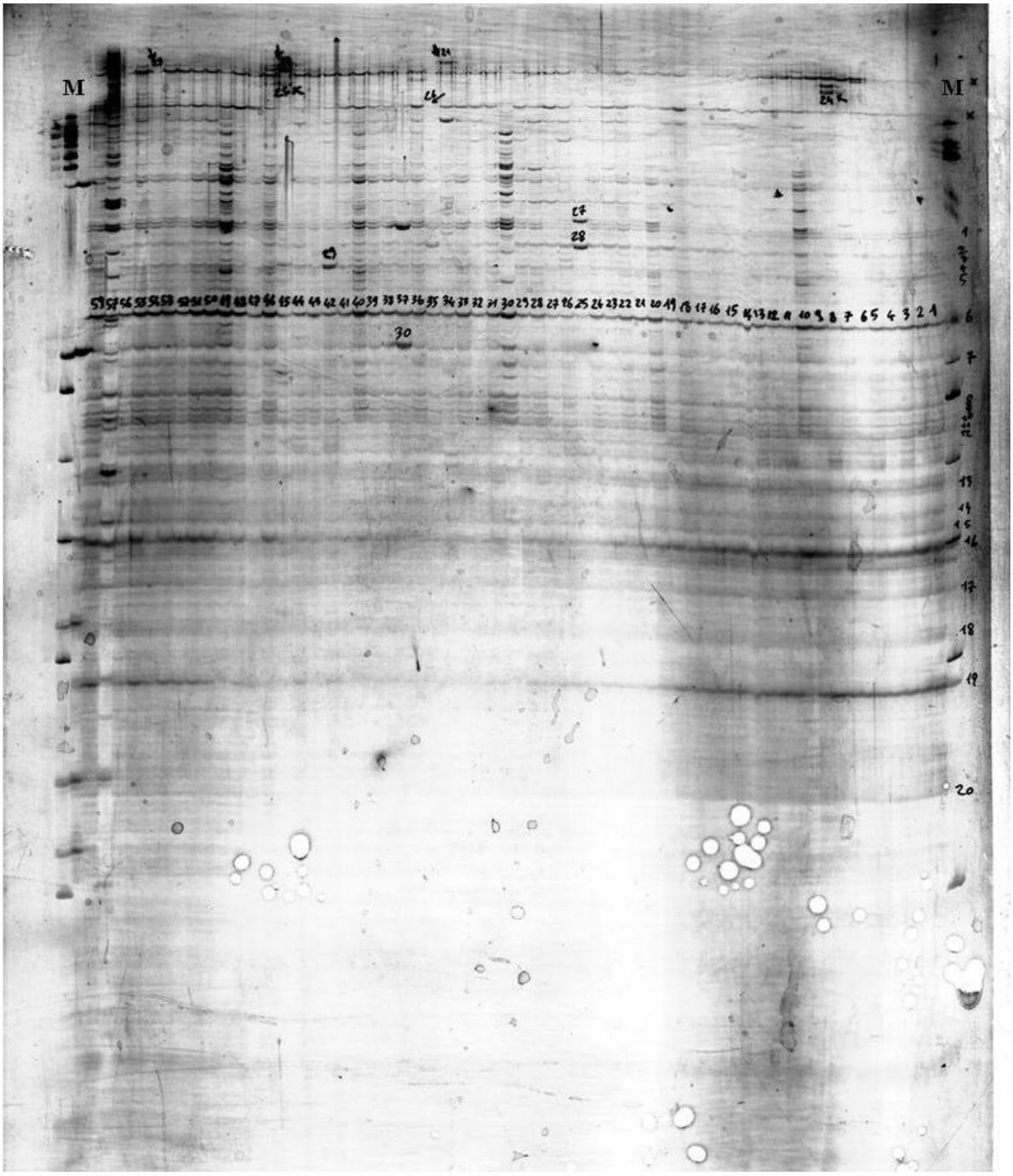

Figure 28 AFLP Gel 1 picture. Progressive numbers from to 59 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). As isolate 30 was not well digested, it was not included in the analysis.

GEL 2 M47-E24

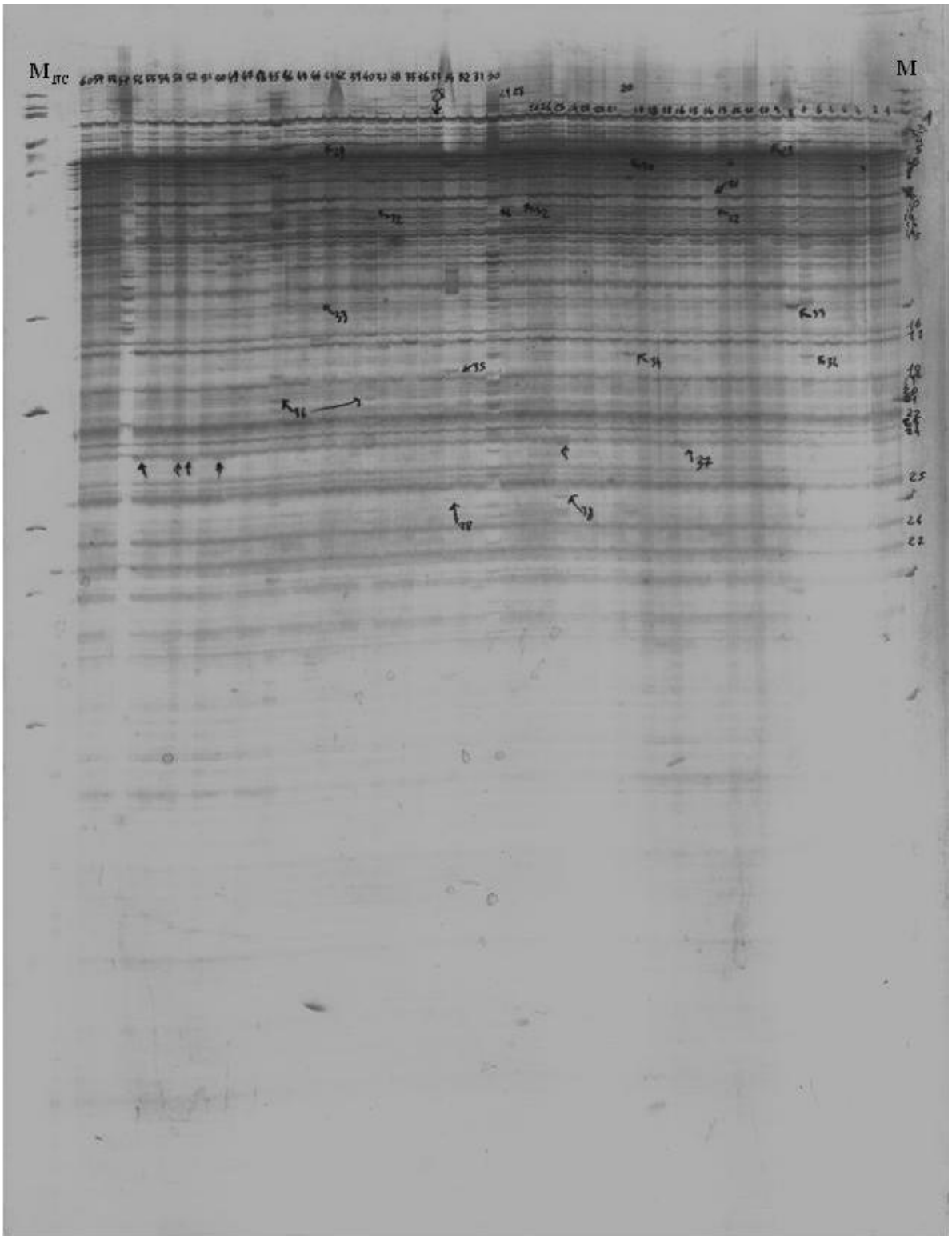

Figure 29 AFLP Gel 2 picture. Progressive numbers from 1 to 60 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). NC is the negative control (no DNA was added) As isolate 30 was not well digested, it was not included in the analysis.

GEL 3 M50-E20

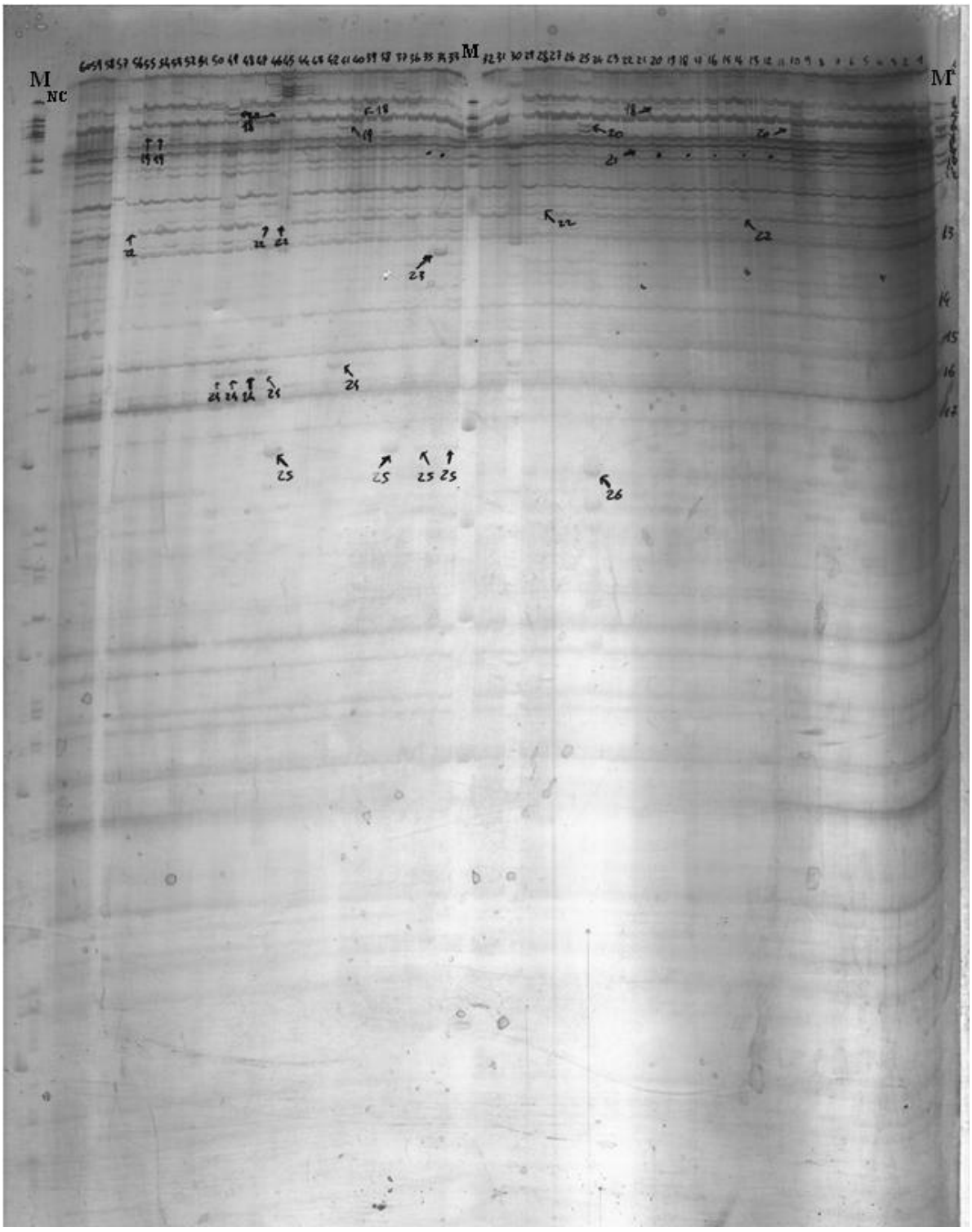

Figure 30 AFLP Gel 3 picture. Progressive numbers from 1 to 60 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). NC is the negative control (no DNA was added) As isolate 30 was not well digested, it was not included in the analysis.

GEL 4 M50-E24

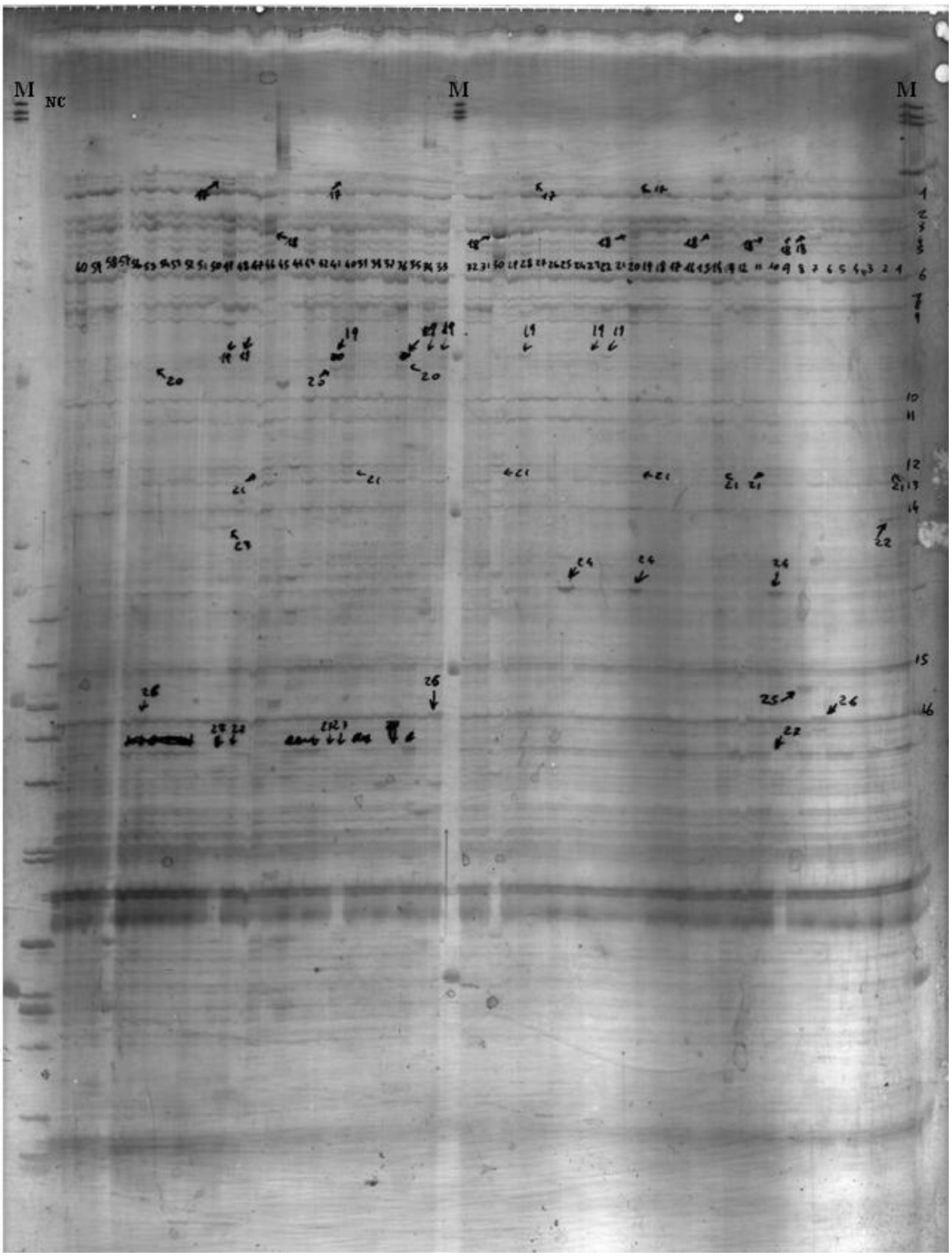

Figure 31 AFLP Gel 4 picture. Progressive numbers from 1 to 60 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). NC is the negative control (no DNA was added) As isolate 30 was not well digested, it was not included in the analysis.

GEL 5 M48-E31

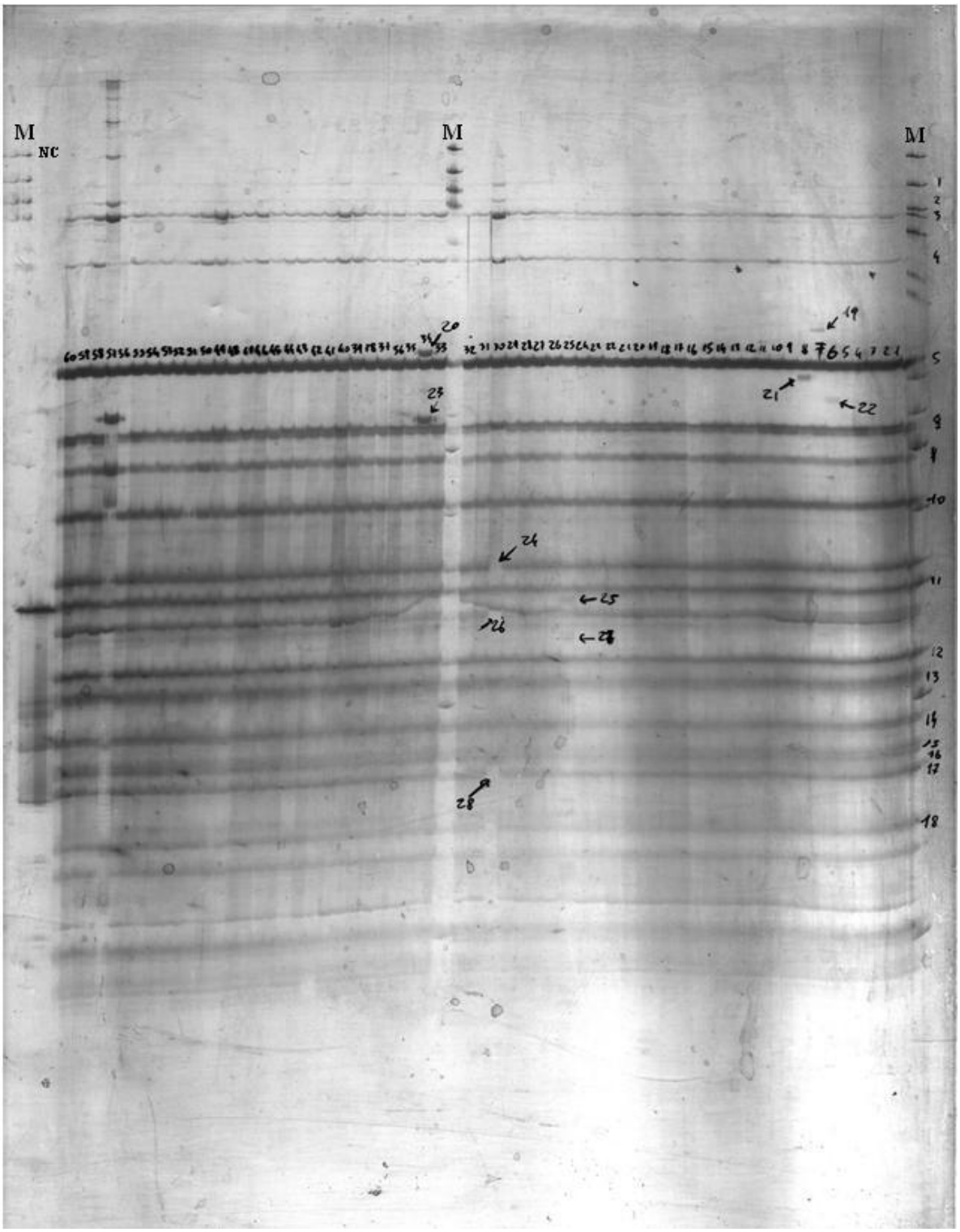

Figure 32 AFLP Gel 5 picture. Progressive numbers from 1 to 60 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). NC is the negative control (no DNA was added) As isolate 30 was not well digested, it was not included in the analysis.

GEL 6 M48-E32

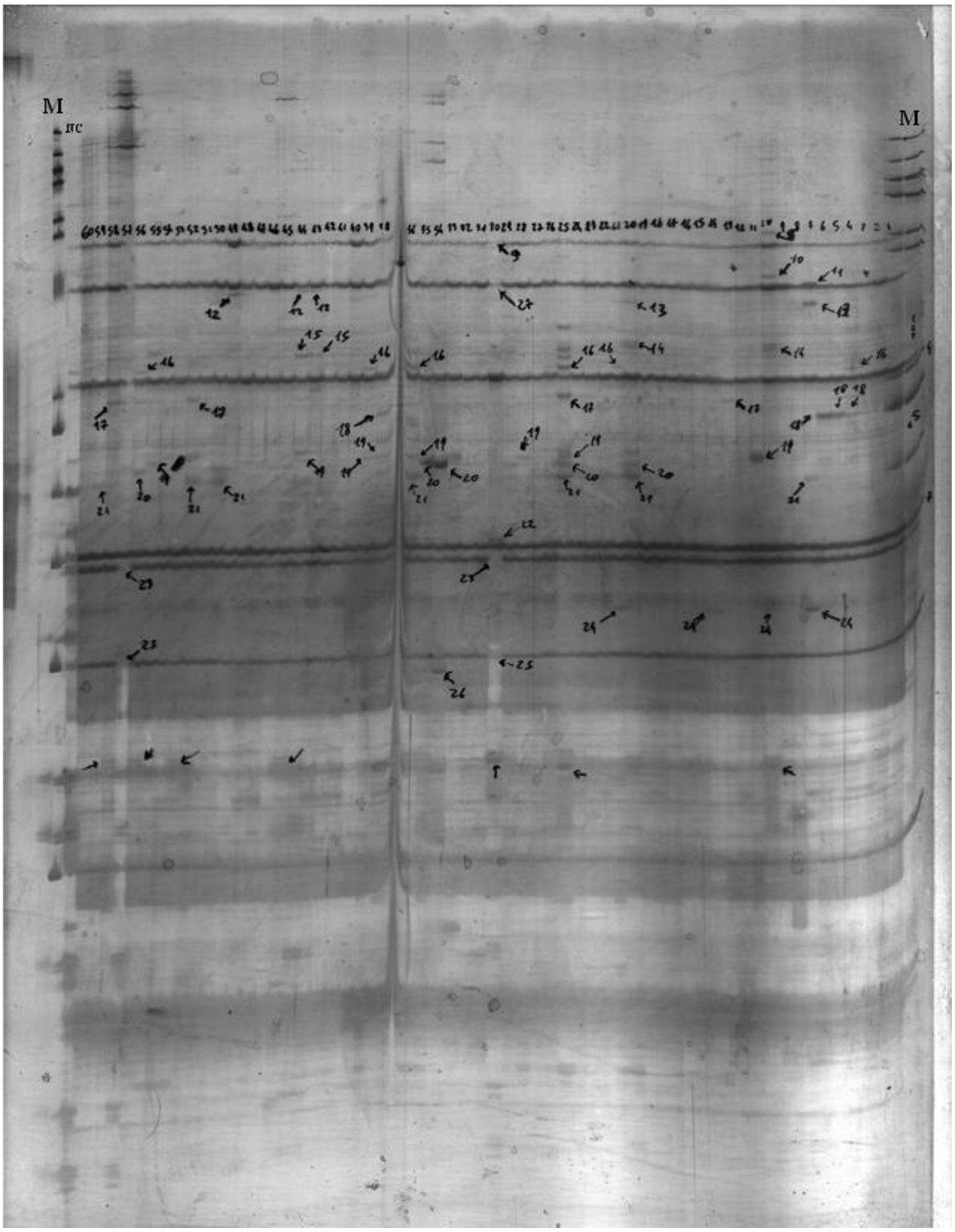

Figure 33 AFLP Gel 6 picture. Progressive numbers from 1 to 60 indicate the isolates, numbers in column on the right the common main bands considered and numbers in the middle of gel with arrows the polymorphic bands considered. M is the molecular DNA marker 100 bp Gene Ruler supplied by Fermentas® (Thermo Fisher Scientific, Vilnius, Lithuania). NC is the negative control (no DNA was added) As isolate 30 was not well digested, it was not included in the analysis.

Ackowledgments

I would like to thank:

- my family for all the help that gave me in these years, thank you a lot;

- my grandmothers, Beatrice and Nerina tha passed away last year. Thank you for all you teached me about life;

- my girlfriend Irene. Thank you for your patience in the last months: this is the last thesis…

- prof. Agostino Brunelli that inspired this work and Dr. Marina Collina for the collaboration;

- prof. Alejandro Pérez-García for gave me the possibility to spend 6 months in his laboratory in Malaga, for all his experience about cucurbit powdery mildew and for all the help, support and ideas that contributed to this work. Thank you also for your time and patience.
Thank you also to prof. Antonio de Vicente, Juan Antonio Torés, Francisco Cazorla and Juan Josè Borrego for host me in the department;

- prof. Ales Lebeda (Department of Botany, Olomouc, Czech Republic), prof. Marc Bardin (INRA, Avignon, France), prof. Meg McGrath (Cornell University, Riverhead, U.S.A.), Dr. Monica Miazzi (Faculty of Agriculture, Bari, Italy), Dr. Nikolay Velkov (Institute of Horticulture and Canned Foods, Plovdiv, Bulgaria) and Dr. Cosme-Bojorquez Ramos (Centro de Investigación en Alimentación y Desarrollo, Sinaloa, Mexico) that gently supplied powdery mildew strains;

- prof. Michael Milgroom from Cornell University (Ithaca, New York, USA) for all the precious ideas and suggestions about the population genetics of *P. fusca*;

- Dr. Luca Dondini (Dip.S.A.) for the kind help and support to plan and perform AFLP analysis and also for interpretation of data. Thank you a lot;

- my collegues and friends Ivan and Marco for give me support and suggestions about the work, for collecting strains and also for all the happy years and moments spent togheter. Thank you Ivan for all your ideas and for you experience about powdery mildews; Thank you also to Ceren Turan, Irene Maja Nanni and Alessandro Ciriani;

- the wonderful Spanish collegues and friends: the “powdery mildew” guys David (thank you for you patience and collaboration) Jesus and Davinia, Houda, Laura, Raul, Guti, Irene and Victor. Six months were to short…..

- Greta for all the help about counting germinated conidia, chasmothecia extraction and identification and performing of Multiplex-PCR.

- Federico “The Beast” Vigna for enjoy our group with his home-made beer and humor;

- Dr. Mirco Iotti (Dip.S.A.) for all the suggestions and ideas.

- Giulia Alberoni for all the patience and for teaching and helping me in the lab during the first year of Ph.D;

- Giulia Toschi and Tonino Romagnoli for all the help and for the pleasant company;

- the farms that allowed me to collect samples during these years: Tagliavini, Zanarini, Zamboni and Landuzzi (Bologna), Nadalini, Aguzzi (Mantua) and Lorenzini (Mantua and Ragusa);