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Phytophthora ×*serendipita* sp. nov. and *P.* ×*pelgrandis*, two destructive pathogens generated by natural hybridization

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Abstract: The first natural hybrids in the genus *Phytophthora* were described in 1998, and they were the result of hybridization between *P. nicotianae* and *P. cactorum*. They were described formally as *Phytophthora* ×*pelgrandis* in 2009. In 2007 a second type of *P. cactorum* hybrid species was described, generated by hybridization between *P. hedraiandra* and *P. cactorum*; it is described formally here as *P.* ×*serendipita* sp. nov. The morphological description of *P.* ×*pelgrandis* was incomplete and here we also add several important diagnostic characters of *P.* ×*pelgrandis* that were not in its original description. In addition, ITS-SSCP profiles are presented confirming the hybrid identity of both *P.* ×*pelgrandis* and *P.* ×*serendipita*.

Key words: hosts, hybrids, ITS-SSCP, morphology

Introduction

Natural hybrid individuals derive from crosses between individuals from two populations or groups of populations that are distinguishable on the basis of one or more heritable characters (Arnold 1997). Hybrids harbor new features because their allopolyploid genome results in buffering of disadvantageous alleles; they may contain novel combinations of virulence factors (Paun et al. 2007), novel combinations of enzymes (e.g. pectinases) facilitating the penetration of the bark and/or the cuticula of the host at the infection court and hence high hybrid fitness. The question of hybridization in *Phytophthora* is important because hybrids may exhibit a broader ecological amplitude than each of their parents, resulting in exploring new niches in terms of new hosts, new host specialization and aggressiveness (Brasier 1995).

Identification of *Phytophthora* species traditionally has been based on morphology. However, *Phytophthora* species are difficult to identify morphologically due to variation of characteristics within and among species. It has been suggested that naturally occurring interspecific hybrids of *Phytophthora*, if they exist, would be difficult to detect due their presumed atypical morphology (Brasier 1991). Molecular approaches may be more suitable for revealing the possible hybrid nature of atypical strains and identifying their progenitors because the fusion of the two parental genomes inevitably must result in heterozygous loci.

In 1995 several isolates were detected that were heterozygous at the malic enzyme (*Mdhp*) locus (Man in 't Veld et al. 2007), identical to the *P. nicotianae* ×*cactorum* hybrids (Man in 't Veld et al. 1998), but unlike these hybrids the malate dehydrogenase (*Mdh-2*) pattern only contained a single band comigrating with the single *P. cactorum* band. The morphology superficially resembled that of *P. cactorum*, albeit that large numbers of abortive oogonia also were present. At that time we hypothesized that these strains might have been the result of backcrossing of *P. nicotianae* ×*cactorum* with *P. cactorum* or, on the other hand, these strains were the result of chromosome loss. As more strains of the same type were isolated from diseased plants they were stored in our collection and we paid no attention to them for many years.

Since the first molecular phylogeny of the genus *Phytophthora*, based on ITS sequences, was published (Cooke et al. 2000a) sequencing has become a routine tool in the identification process. Applying ITS sequencing to our anomalous hybrid isolates revealed double bases at four positions, and at the same time sequencing revealed *P. hedraiandra* as a new species (de Cock and Man in 't Veld 2004) closely related to *P. cactorum*. The double bases were present at positions where the sequences of *P. cactorum* and *P. hedraiandra* differed (GenBank DQ836127) and, moreover, cloning experiments revealed that the bases occupying these positions were typical for both species. As a matter of serendipity, a new hybrid was discovered and its identity was confirmed by *CoxI* sequence analysis because both parental *CoxI* types were found in the population (Man in 't Veld et al. 2007).

Hybrids between *P. hedraiandra* and *P. cactorum* represent a distinct, well defined genetic entity and they are generated de novo whenever both parents are present. Moreover, there is also a need to describe their morphological and molecular characteristics to aid identification and hence their description as a

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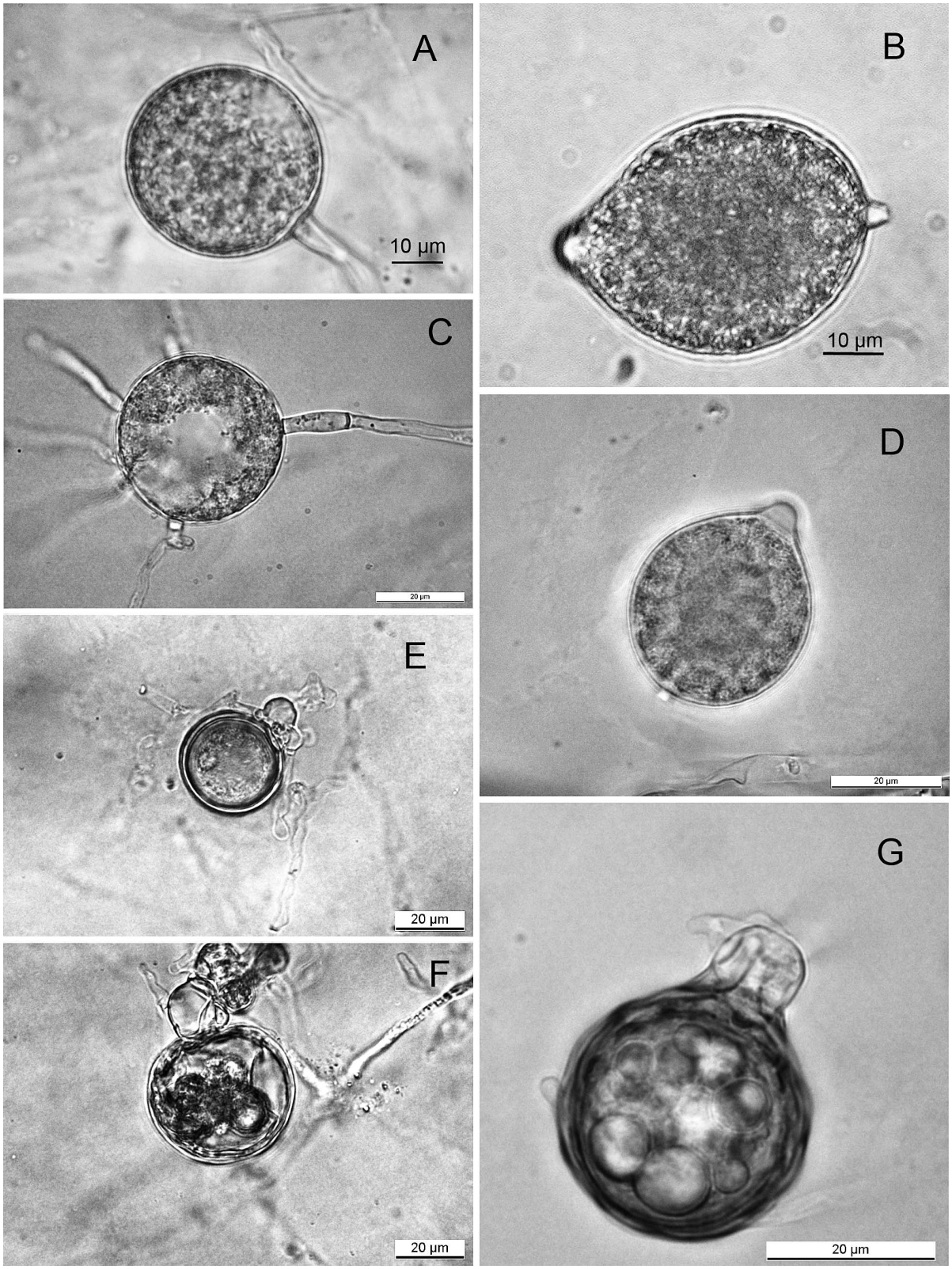

FIG. 1. A, B. Morphological structures of *Phytophthora* ×*pelgrandis* strain CBS123385. A. Chlamydospore. B. Caducous, ovoid, spherical, papillate sporangium with short pedicel. C–G. CBS100421. C. Chlamydospore with radiating hyphae. D. Caducous, ovoid, spherical, papillate sporangium with short pedicel. E. Oogonium with plerotic oospore and paragynous antheridium. F. Oogonium with abortive oospore and distorted antheridium. G. Oogonium with abortive oospore and amphigynous antheridium. (A, B courtesy of József Bakonyi).

new taxon is justified. Therefore, the hybrids of *P. hedraiandra* and *P. cactorum* are described formally here as *P.* ×*serendipita* sp. nov.

Since the initial discovery of hybrids of *Phytophthora nicotianae* ×*cactorum* in the Netherlands (Man in 't Veld et al. 1998), they have been found in Germany (Nirenberg et al. 2009), Hungary (József Bakonyi pers comm), Peru and Taiwan (Hurtado-Gonzales et al. 2009) and the United States (Leonberger 2010). Pathogenicity tests showed that *Eriobotrya japonica* (Chern et al. 1998) and *Spathiphyllum* (Man in 't Veld et al. 1998) developed disease symptoms after inoculation with a *P. nicotianae* ×*cactorum* hybrid. The pathogen was re-isolated successfully from symptomatic plants and its identity was confirmed, thus fulfilling Koch's postulates. Apparently these hybrids are well established and successful. In 2009 the *P. nicotianae* ×*cactorum* hybrids were described formally as *P.* ×*pelgrandis* (Nirenberg et al. 2009). Additional morphological characteristics we have observed for *P.* ×*pelgrandis* are presented here.

MATERIALS AND METHODS

Morphology.—Cultures were maintained on V8 agar slants (Crous et al. 2009). Isolates were cultivated on V8 agar at 22 C in the dark to study the dimensions of oogonia, oospores and chlamydospores and colony growth characteristics. Sporangia formation and morphology were studied on colonized pepper seeds in sterile pond water. About five seeds were placed at the margins of actively growing colonies; when the seeds were covered by mycelium they were transferred to water agar and filtered sterile pond water was added; sporangia usually formed after ~5 h. For all characteristics studied, at least 25 measurements were made for each isolate and the average value was calculated. The attachment of the antheridia was scored only when it could be unequivocally determined.

Temperature-growth profiles were determined on potato dextrose agar (PDA) by incubating the colonies (at 10, 22, 26, 28, 30, 31, 32 C). Growth of the mycelium was measured after 7 d in the dark. The highest temperature at which growth occurred was considered to be the maximum growing temperature.

ITS-SSCP analysis.—The single-strand conformation polymorphism (SSCP) technique is capable of distinguishing two well defined DNA sequences with only one nucleotide difference (Orita et al. 1989). It also has proven capable of differentiating *Phytophthora* species based on ITS-SSCP (Kong et al. 2003). Therefore this technique is suitable for visualizing at a glance the presence of the both parental ITS sequences in hybrids. Purified DNA was amplified with forward primer ITS6 and reverse primer ITS7, and PCR products were electrophoresed in acrylamide gel, followed by silver staining (Kong et al. 2003). The ITS-SSCP profiles of both types of hybrids are presented here.

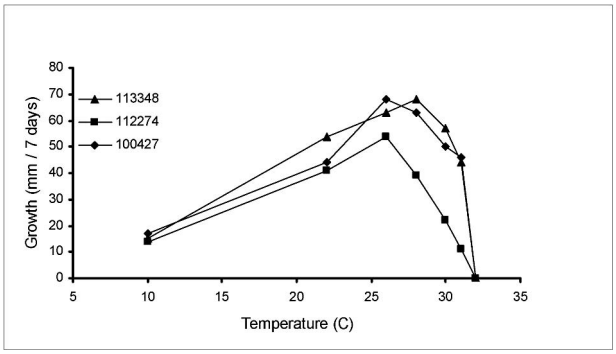

FIG. 2. Growth-temperature curves of *P.* ×*serendipita* isolates CBS100427, CBS112274 and CBS113348 on potato dextrose agar (PDA).

RESULTS

Examination of the morphological structures of the type strain of *P.* ×*pelgrandis* CBS123385 (FIG. 1A, B) and CBS100421 (= PD 94/1166, FIG. 1C–G) ex *Spathiphyllum* (Man in 't Veld et al. 1998) resulted in the discovery of chlamydospores (30.0–41.3 μm, av 35.9), caducous sporangia (pedicel 2–3 μm) plerotic oospores and abortive oogonia. The maximum growing temperature of the type strain of *P.* ×*pelgrandis* CBS 123385 and CBS100421 was ~36 C.

Temperature-growth relationships on PDA of three strains of anomalous *P. cactorum*-like hybrids, described as *P.* ×*serendipita* below, were determined. Strain CBS100427 grew slightly slower than CBS112274 and CBS113348. The average optimum temperature for growth was ~27 C and no growth was observed at 32 C in all three strains (FIG. 2).

ITS-SSCP.—Single-strand conformation polymorphism (SSCP) profiles of anomalous *P. cactorum*-like hybrids, described as *P.* ×*serendipita* below, are presented (FIG. 3 lanes 5–7 and parent lanes 4, 8). *P.* ×*serendipita* generated four bands, two of which comigrated with those typical for *P. cactorum* and two with those typical for *P. hedraiandra*. Single-strand conformation polymorphism (SSCP) profiles of *P.* ×*pelgrandis* are presented (FIG. 3 lane 2 and the parent lanes 1 and 3). *P.* ×*pelgrandis* generated four bands, two of which comigrated with those typical for *P. cactorum* and two with those typical for *P. nicotianae*.

TAXONOMY

Phytophthora ×*serendipita* Man in 't Veld, K. Rosendahl, nothosp. nov. FIGS. 4, 5

MycoBank MB560686

≡ *Phytophthora hedraiandra* ×*cactorum* Man in 't Veld, de Cock, Summerbell Eur. J. Plant Pathol. 117:32 (2007). Holotype not indicated. Nom. inval., Arts 36.1, 37.1, 37.2, 37.3.

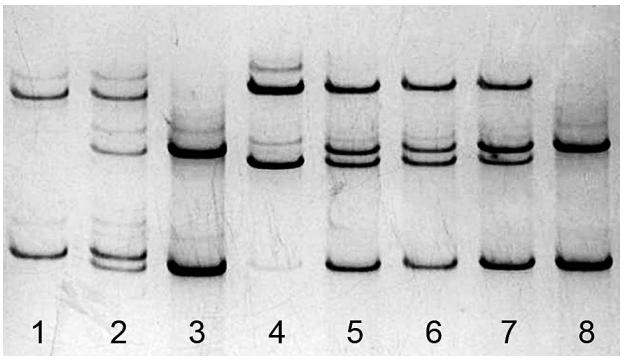

FIG. 3. Single-strand conformation polymorphism profiles of *Phytophthora ×serendipita* and *Phytophthora ×pelgrandis* and their parents. Lanes: 1. *P. nicotianae* 22G1, MYA4036. 2. *P. ×pelgrandis* CBS100420. 3. *P. cactorum* 22E8, MYA3653. 4. *P.hedraiandra* 33F3. 5. *P. ×serendipita* CBS100425. 6. *P. ×serendipita* CBS100427. 7. *P. ×serendipita* CBS113348. 8. *P. cactorum* 22E8, MYA3653.

A natural hybrid of *Phytophthora hedraiandra* de Cock & Man in 't Veld and *Phytophthora cactorum* (Lebert & Cohn) J, Schröter.

Colony morphology in V8 agar slightly stellate. In water sporangia abundant, papillate, single or sympodially arranged, ovoid and spherical, circa 32–49 × 24–37 µm, average 40.5 × 30.6 µm, length : width ratio 1 : 33, non-caducous and caducous, with a short pedicel 2–5 µm, sometimes sporangia formed by external proliferation; T_{opt} ~ 27 C, T_{max} ~ 31 C; chlamydospores were not observed. It is a homothallic species, oogonia, circa 26–33 µm, av 29.9 µm, oospores plerotic and aplerotic, circa 21–29 µm, av 24.8 µm, often abortive, antheridia paragynous, sometimes distorted; a unique combination of isozyme alleles at the *Mdhp* locus, and a unique combination of ITS sequences of *P. cactorum* and *P. hedraiandra* resulting in a unique SSCP profile.

Holotype. CBS100427, dried culture of isolate from stembase rot of *Idesia polycarpa*, isolated by Wil Veenbaas, the Netherlands, Boskoop 1995. Culture ex holotype CBS100427 (= PD95/5111). Holotype in CBS-KNAW, Fungal Biodiversity Centre, Utrecht, the Netherlands. Isotypes CBS112274 ex *Penstemon*, CBS113348 ex *Rhododendron* (Man in 't Veld et al. 2007).

Etymology. Serendipity, making an unexpected discovery by coincidence and sagacity.

DISCUSSION

The morphological features of *P. ×serendipita* do not differ significantly from those of the one parent, *P. cactorum*, except for the absence of chlamydospores or those of the other parent, *P. hedraiandra*, except for its higher optimum growth temperature. *P.*

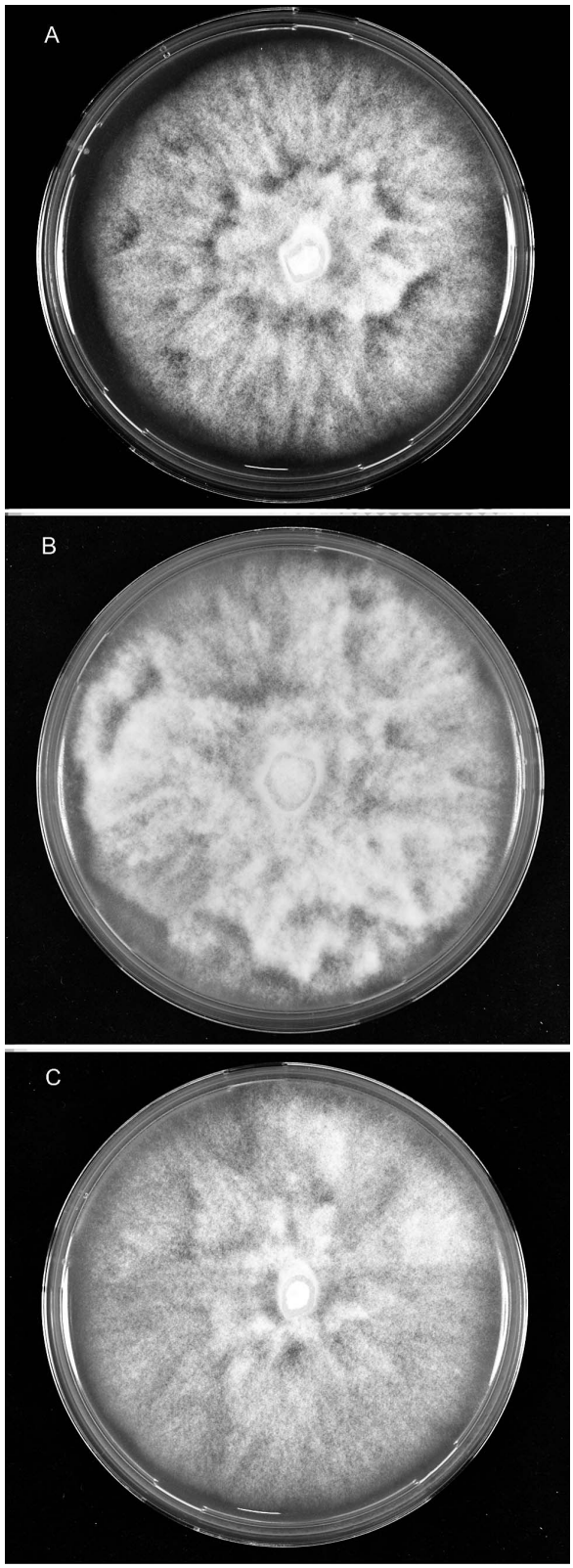

FIG. 4A–C. Colony morphology on V8 of *P. ×serendipita* of isolates CBS100427 (A), CBS112274 (B) and CBS113348 (C).

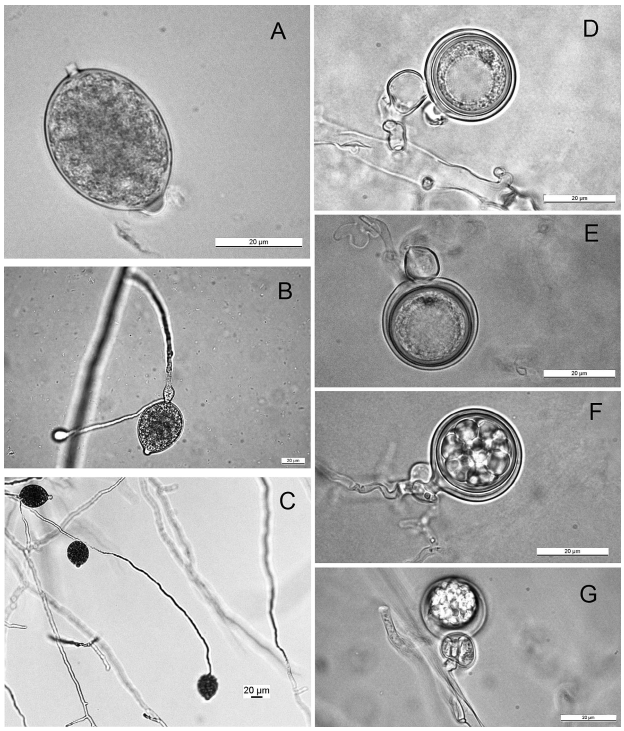

FIG. 5A–G. Morphological structures of *Phytophthora* ×*serendipita*. A. Caducous ovoid, spherical, papillate sporangium with short pedicel. B. Sporangiophore with terminal papillate sporangium and external proliferation. C. Sympodially arranged papillate sporangia. D. Oogonium with plerotic oospore and paragynous antheridium. E. Oogonium with aplerotic oospore and paragynous antheridium. F. Oogonium with abortive oospore and distorted antheridium. G. Oogonium with abortive oospore and distorted antheridium.

×*serendipita* differs from both parents by producing abortive oospores and distorted antheridia and its greater sporangia length : width ratio. *P.* ×*serendipita* has *Mdhp* patterns intermediate between the two parents and contains ITS sequences of both parents as well, resulting in additive SSCP profiles. Two de novo hybridization events could be deduced from the presence of strains with the *P. hedraiandra* type *CoxI* gene and one strain with the *P. cactorum* type *CoxI* gene (Man in 't Veld et al. 2007).

Phytophthora. ×*serendipita* was isolated from basal stem rot of *Idesia* and *Penstemon* from leaf spots (*Allium cepa*, *Allium porrum*) and from wilting shoots (*Rhododendron*), usually resulting in the death of the hosts. Since its initial discovery, seven more *P.* ×*serendipita* strains have been discovered on *Rhododendron* in the Netherlands (Man in 't Veld unpubl) and 38 strains have been reported on *Rhododendron* in Belgium (Kris van Poucke pers comm) on *Kalmia latifolia* in Slovenia (Alenka Munda pers comm) and on *Dicentra* and *Rhododendron* in the United States (Leonberger 2010). These isolations indicated that *P.* ×*serendipita* is well established and successful and even is proliferating on new hosts. Pathogenicity tests showed that *Dicentra* developed disease symptoms; the pathogen was re-isolated successfully and hence in this case Koch's postulates were fulfilled (Leonberger 2010). The presence of *P. hedraiandra* had been reported (Schwingle et al. 2006) and hybridization de novo could have happened because both parents are present in the United States.

Hybrid *P.* ×*serendipita* strains appear to be genetically stable and do not readily revert to parent types either in artificial culture or in nature. Nine *P.* ×*serendipita* strains contained ITS sequences of both parents. Strains CBS100425, isolated in 1992, and CBS 100427, isolated in 1995 (Man in 't Veld et al. 2007), both retained both parental ITS sequences when they were sequenced in 2006. Since their initial isolation these strains passed numerous replications of the genome during mitosis, and the heterozygous ITS sequence apparently still has not changed in 14 and 11 y respectively. Two other strains, CBS116572 and CBS111731, isolated in 1997 and 1998 respectively, also still possessed both parental ITS sequences when they were sequenced in 2006. In addition, both malate dehydrogenase (*Mdh-2*) and malic enzyme (*Mdhp*) patterns of *P.* ×*pelgrandis* strain PD93/1339, isolated in 1993, were still heterozygous in 2003. Furthermore, two *P.* ×*pelgrandis* strains, isolated in 1995 in Taiwan, had both parental ITS and *Pheca* genes 14 y after isolation (Hurtado-Gonzales et al. 2009). Moreover, sequencing of the *β-tub* gene of strain 95023 from Taiwan and PD94/1166 (GenBank JQ681269), isolated in 1994, demonstrated that both parental genes were still present in 2011 (Man in 't Veld unpubl). Together these examples indicated that hybrid strains in general are able to survive as such for a decade or more and prolonged periods of time seem to be feasible.

Hybrids have several routes to evolve: (i) by intercrossing, (ii) by backcrossing to either one of the parents (introgression), (iii) by mitotic chromosomal rearrangements, (iv) by chromosome loss, (v) by epigenetic alterations leading to changes in local patterns of gene expression and gene silencing (Adams et al. 2003). A typical example of hybrid evolution in *P.* ×*serendipita* is CBS114342, which is homozygous for the *P. cactorum Mdhp*^{A} allele, which has the *P. cactorum CoxI* type but contains the homozygous ITS characteristic for *P. hedraiandra*. In this case it is impossible to reconstruct by which route this strain has evolved. Also strain BBA 5/94 seems to have evolved from *P.* ×*serendipita* by the loss of the *P. hedraiandra* ITS type (Man in 't Veld et al. 2007).

The natural hybrids described to date in *Phytophthora*, *P. alni* subsp. *alni* (Brasier et al. 2004),

P. ×pelgrandis and *P. ×serendipita* all seem to have these characteristics in common: considerable numbers of abortive oospores, heterozygous isozyme patterns (*Mdh-2*, *Mdhp* or *Gpi*) and ITS sequences of both parents. When encountering strains with anomalous features, the aforementioned combination of characteristics should be kept in mind as indicative of hybrids. In addition, sequences of single-copy genes are equally suitable to detect hybridization by additive bases at those positions where the sequences of the parents differ. It is advisable to establish monosporic strains to exclude physical mixtures of strains of different species.

Hybrids are a complicating factor in DNA barcoding based on *CoxI*, the standard DNA barcode supported by GenBank (Robideau et al. 2011), or other mitochondrial genes or sequences, because the analysis of mitochondrial genes will reveal only one of the parents. The ITS sequences of hybrids, however, may reveal crucial data about the parental strains involved because, if the parental sequences are equal in length, double bases will be present at well defined positions where the parental sequences differ. This is the case for *P. ×serendipita* and *P. alni* subsp. *alni*. For *P. ×pelgrandis*, on the other hand, the ITS sequence is unreadable due to indels but restriction enzyme analysis (Cooke et al. 2000b) or cloning will reveal the parental strains.

Although hybrids have a nonfunctional sexual reproduction system, as indicated by numerous abortive oospores in both types, *Phytophthora* hybrids have ''the eternal life'' because Muller's ratchet, which states that a small population will decline due to accumulation of mutations (Muller 1964), is bypassed; deleterious mutations are counterbalanced by multiple alleles in the allopolyploid genome and hence the hybrid fitness will decrease slowly. The hybrids could be created de novo wherever the two parents are present (Man in 't Veld et al. 2007, Hurtado-Gonzales et al. 2009), but they are also able to spread clonally in the environment due to their caducous sporangia and, when present, due to chlamydospores in infected plant material. Even long range transmission is feasible through the intestines of birds (Keast et al. 1979). Both types of hybrids are successful; they represent a genetically distinct entity, and due to their hybrid fitness they are able to explore new hosts and new niches. The hybrid character of both types was consistently supported by isozymes, ITS sequence analysis (Man in 't Veld et al. 1998, 2007; Hurtado-Gonzales et al. 2009) and ITS-SSCP profiles (this paper).

The major source of hybridization is probably the introduction of formerly allopatric species, resulting from divergence through geographical isolation, that meet with local species. Allopatric species can diverge without premating barriers toward local species. Hence hybrids have a bright future because the global horticultural trade encompasses the introduction of new invasive *Phytophthora* species (Brasier 2008). Although this is considered to be collateral damage, it is unintentionally increasing the chance of new hybridizations between resident and introduced species. Hybrids have the potential to occupy new niches and new hosts and might pose new threats to horticultural crops and ecosystems in the future.

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