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Phylogenetic investigations in the genus *Pseudoperonospora* reveal overlooked species and cryptic diversity in the *P. cubensis* species cluster

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Abstract *Pseudoperonospora cubensis* is one of the most devastating diseases of cucurbitaceous crops. The pathogen has a worldwide distribution and occurs in all major cucurbit growing areas. It had been noticed for the first time at the end of the 19th century, but it became a globally severe disease as recently as 1984 in Europe and 2004 in North America. Despite its economic importance, species concepts in *Pseudoperonospora* are debated. Here, we report that the genus *Pseudoperonospora* contains cryptic species distinct from the currently accepted ones. *Pseudoperonospora* on *Celtis* is split into two phylogenetic lineages and *Pseudoperonospora humuli* is confirmed as a species distinct from the Cucurbitaceae-infecting lineages. A cryptic species occupying a basal position within the *Pseudoperonospora cubensis* complex is revealed to be present on *Humulus japonicus*, thus providing evidence that the host jump that gave rise to *Pseudoperonospora cubensis* likely occurred from hops. Notably, Cucurbitaceae infecting pathogens are present in two cryptic sister species or subspecies. Clade 1 contains primarily specimens from North America and likely represents *Pseudoperonospora cubensis* s.str.. Pre-epidemic isolates in clade 2 originate from Japan and Korea, suggesting this cryptic species or subspecies is indigenous to East Asia. Recent samples of this lineage from epidemics in Europe and the United States cluster together with clade 2. It thus seems possible that this lineage is associated with the recent severe epidemics of cucurbit downy mildew and is now naturalised in North America and Europe.

Keywords *cox2* · Cucurbit downy mildew · Cryptic species · nrITS · Peronosporaceae · *Pseudoperonospora humuli* · Species delimitation · *ypt1*

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Introduction

The genus *Pseudoperonospora* includes six widely recognised species, of which *P. humuli* and *P. cubensis*

are economically most important. *Pseudoperonospora humuli* occurs in all of the hop-growing countries of the northern hemisphere and in Argentina (Royle and Kremheller 1981). The pathogen was first reported in Japan in 1905 (Miyabe and Takahashi 1906), and has caused severe losses in the major hop growing areas in the 20th century. Johnson et al. (1994) reported that Washington State had the highest disease incidences in 1988, 1990, and 1991. In 2002 and 2003 the disease re-emerged in Argentina and the reduction in hop cone yield of infested fields varied between 20% and 34% (Pérez et al. 2009). But due to advances in forecasting and disease management, hop downy mildew control seems feasible (Mozny et al. 1993; Dolinar and Žolnir 1994; Gent et al. 2009).

Downy mildew of cucurbits caused by *P. cubensis* occurs on wild and cultivated Cucurbitaceae in all major cucurbit growing countries (Cohen 1981; Holmes et al. 2004), and more than 60 host species have been reported (Lebeda 1990, 1999), including all cucurbits of economic interest. It is most widespread on *Cucumis sativus* (about 80 countries) and *Cucumis melo* (more than 50 countries) (Lebeda 1990). In 2006 the European countries Spain, the Netherlands, and Germany together produced about 2 million tons of cucumbers. In East Asia, in Japan and in the Republic of Korea, altogether more than 1 million tons of cucumbers were grown and Canada, Mexico, and the United States produced more than 1 million tons of cucumbers (www.ers.usda.gov) in total. The US cucumber production had a value of more than 180 million US$ in 2009 (Battaglia 2010), highlighting the value of this crop.

In the 1930s and 1940s, in the USA most of the efforts against cucurbit downy mildew were centred on chemical control and breeding for resistance (Palti and Cohen 1980). Until the 1940s, downy mildew was the most important disease of cucumber (Selby 1899; Holmes et al. 2006). This changed with the release of cultivar Palmetto in 1948, which had moderate resistance to downy mildew. In 1966, cultivar Poinsett was released with high resistance to downy mildew. This resistance has been bred into most of the popular cucumber cultivars (Holmes et al. 2006). Also in *Cucumis melo* some effective sources of resistance are available (Pitrat et al. 1998; Lebeda 1999) and the cantaloupe cultivar Smith Perfect is in the pedigree of most cantaloupe material in use in the USA today (Palti and Cohen 1980). In addition Lebeda and Widrlechner (2004) demonstrated broad genetic variation in resistance to *P. cubensis* among wild and weedy *Cucurbita* species. Because of the efficient breeding for resistance, US growers have hardly used fungicides to control downy mildew since the late 1960s (Holmes et al. 2006).

This situation changed in the mid 1980s in Europe and around 2004 in the USA. From 1984 onwards, downy mildew has become the economically most important pathogen of cucurbit vegetables in Central Europe (Lebeda 1990; Lebeda and Urban 2004), and disease control through growing of previously resistant cultivars has not been effective (Lebeda and Schwinn 1994; Lebeda and Widrlechner 2003; Lebeda and Urban 2004). Subsequently, in the Czech Republic the yield of field cucumbers decreased by ~80% in 1985 and 83% in 1989 (Lebeda 1991) and a considerable shift in pathogenicity in the Czech pathogen population was evident during 1986–2000 (Lebeda and Gadasová 2002). Cappelli et al. (2003) reported that in 2002 the disease spread rapidly in Italy and because the control measures used were not effective, growers suffered from severe yield losses. Since 2004 *P. cubensis* has re-emerged as a destructive pathogen in the USA. Outbreaks were found on cucumber about 2 months earlier than previously and it had been decades since growers had faced a similar level of destruction from downy mildew. In 2004 North Carolina, Delaware, Maryland, and Virginia lost 40% of their crop with a value of approximately 20 million US$ (Holmes et al. 2006). Downy mildew occured in several regions in Michigan in 2005, including major cucumber production regions, where the disease was so severe that many of the state's largest growers considered no longer growing the crop (Hausbeck 2007).

As the commercially available resistant cucumber cultivars were no longer effective in controlling downy mildew, fungicides were necessary to manage the disease (Urban and Lebeda 2006, 2007; Colucci et al. 2008a,b). In 1996 the global fungicide market was estimated at approximately 6.25 billion US$, of which 16.7% were chemicals to control downy mildews (Gisi 2002). It can be expected that the recent epidemics will lead to a significant increase in these figures. Unfortunately, not only the deployment of resistant cultivars, but also the effectiveness of systemic fungicides has been compromised by the

high adaptability of *P. cubensis* (Ishii et al. 2001; Shetty et al. 2002; Urban and Lebeda 2004, 2006, 2007; Keinath et al. 2007; Colucci et al. 2008a, b; Gugino et al. 2009).

Overcoming host defence or developing fungicide resistance may be due to high intraspecific genetic variation in *P. cubensis*, and strong evidence supports the presence of races of *P. cubensis* differing in virulence (Bains and Jhooty 1976; Palti and Cohen 1980). Thomas et al. (1987) distinguished five different pathotypes. Horejsi et al. (2000) and Shetty et al. (2002) revealed the presence of different races in Asia and the USA. On the basis of field evaluations of cucumbers Horejsi et al. (2000) hypothesised that Europe and the United States may harbour different races of *P. cubensis*. But no evidence for race differences in the pathogen was observed between North America and Europe. However, European locations tended to have higher downy mildew scores compared to the North American locations and resistance of cucumber to *P. cubensis* held up against isolates from the US, but not against European isolates (Lebeda 1992). Lebeda and Urban (2004) determined 34 different pathotypes of *P. cubensis* and concluded that there were no substantial geographical patterns in the occurrence of various pathotypes among 106 locations in the Czech Republic. Holmes et al. (2006) mentioned that the post 2004 downy mildew incidences might indicate the presence of a more virulent type of pathogen than in previous years, corresponding to a new race or pathotype (Holmes et al. 2006). But the paucity of characters suitable for species delineation in *Pseudoperonospora* (Shin and Choi 2003) renders it possible that the new epidemics were caused by a cryptic species of *Pseudoperonospora*.

Of the six widely accepted species, *P. humuli* and *P. cubensis* are the economically most important and are known since the end of the 19th and the beginning of the 20th century from the northern hemisphere. However, all species of *Pseudoperonospora* are morphologically similar, leading to a species classification that is primarily based on the host matrix. Earlier investigations have revealed that *P. cubensis* is closely related to *P. humuli*, despite parasitising the only distantly related Cucurbitaceae (Fraymouth 1956; Constantinescu 2000; Choi et al. 2005). Based on morphological characteristics, Shin and Choi (2003, 2006) and Choi et al. (2005) indicated that *P. cubensis* and *P. humuli* might be conspecific. Molecular phylogenetic analysis of Choi et al. (2005) revealed that evolutionary recent host shifts between Cannabinaceae and Cucurbitaceae have taken place in the genus *Pseudoperonospora*. The directionality of the host jumps could not be inferred, as based on nrITS, the sequences of *P. cubensis* from cucurbit specimens are poorly differentiated from *P. humuli* from hops. But a high degree of sequence similarity does not rule out the existence of closely related, yet distinct species, and Voglmayr (2008) comments that for detailed insights into the evolutionary processes in closely related downy mildew species, the ITS-based phylogenies offer too little resolution, necessitating multilocus analyses, including additional molecular markers with high phylogenetic resolution.

It was the aim of this study using a combined analysis of *cox*2, *ypt*1, and nrITS sequence data for obtaining a higher resolution of the *P. cubensis* species complex for inferring, whether *P. cubensis* and *P. humuli* are distinct species, whether additional cryptic species might be present in this group, and whether the new *Pseudoperonospora* epidemics in Europe and North America might be caused by a genetically distinct lineage which should be hindered from further spread by appropriate quarantine regulations.

Materials and methods

Oomycete material

The oomycete material used in this study is listed in Table 1. Herbarium samples originated from the herbarium of the New York Botanical Garden (NY), the Herbarium of the Plant Diseases Division of New Zealand (PDD), the Farlow Herbarium of the Harvard University (FH), the Mycological Herbarium of the Korea University (KUS-F), and the Herbarium of the University of Hohenheim (HOH). The laboratory strains originated from North America, the Czech Republic, and Germany.

DNA-extraction, PCR and sequencing

DNA was extracted using the method best suited for herbarium samples as described in Telle and Thines (2008). For amplification of the mitochondrial *cox*2 gene, primers designed by Hudspeth et al. (2000)

Table 1 Oomycete material used in this study

Pathogen	Host	DNA accession number	Year	Country	Source or herbarium number	GenBank accession number *cox2*	GenBank accession number *ypt1*	GenBank accession number nrITS
P. cannabina	*Cannabis sativa*	NY409	1958	Poland	NY, *ex* Kochman, Mycotheca Polonica, Fasc. I, No. 8	HM635956	HM636004	HM636052
P. cannabina	*Cannabis sativa*	U181	1936	Latvia	FH, *ex* Herbario J. Smarods, No. 316	HM635955	HM636003	HM636051
P. celtidis	*Celtis occidentalis*	NY411	1981	USA, PA	NY, *ex* Carnegie Museum (CM) Herbarium, No. 14551	HM635949	HM635997	HM636045
P. cubensis	*Citrullus vulgaris*	NY419	1887	USA, LA	NY, *ex* Flora Ludiviciana, No. 1/22	HM635918	HM635966	HM636014
P. cubensis	*Citrullus vulgaris*	NY435	1935	USA, FL	NY, *ex* University of Florida Herbarium, No. 10591	HM635917	HM635965	HM636013
P. cubensis	*Cucumis anguria*	NY416	1923	USA, St. Croix	NY, No. 881	HM635924	HM635972	HM636020
P. cubensis	*Cucumis melo*	D320	2006	Korea	KUS-F, No. 22001	HM635912	HM635960	HM636008
P. cubensis	*Cucumis melo*	NY420	1903	USA, SC	NY, *ex* United States Department of Agriculture, No. 1038	HM635910	HM635958	HM636006
P. cubensis	*Cucumis melo*	NY421	1905	USA, SC	NY, *ex* United States Department of Agriculture, No. 1040	HM635919	HM635967	HM636015
P. cubensis	*Cucumis melo*	NY442	1902	USA, CT	NY, *ex* Reliquiae Seymourianae	HM635915	HM635963	HM636011
P. cubensis	*Cucumis melo*	U187	1899	USA, ME	FH, *ex* Herbarium of W.G. Farlow	HM635914	HM635962	HM636010
P. cubensis	*Cucumis melo*	U189	1910	USA, MA	FH, *ex* United States Department of Agriculture, Mycological Collections	HM635909	HM635957	HM636005
P. cubensis	*Cucumis sativus*	NY423	1892	USA, MA	NY, *ex* Herbarium of the Massachusetts Agricultural College, Dept. Bot., No. 949	HM635922	HM635970	HM636018
P. cubensis	*Cucumis sativus*	NY429	1889	USA, NJ	NY, *ex* Economic Fungi. A.B. Seymour and F.S. Earle, No. 41	HM635930	HM635978	HM636026
P. cubensis	*Cucumis sativus*	NY446	1909	Germany	NY, *ex* Sydow, Phycomyces et Protomycten, No. 266	HM635911	HM635959	HM636007
P. cubensis	*Cucumis sativus*	U190	1889	USA, NJ	FH, *ex* Herbarium of W.G. Farlow	HM635921	HM635969	HM636017
P. cubensis	*Cucumis sativus*	U191	1921	USA, WI	FH, *ex* Herbarium of the University of Wisconsin	HM635923	HM635971	HM636019
P. cubensis	*Cucumis sativus*	U195	1912	Czech Republic	FH, *ex* F. Petrak, Flora Bohemiae et Moraviae exsiccata, Lfg. 9 No. 446	HM635913	HM635961	HM636009
P. cubensis	*Cucumis sativus*	U197	1918	Philippines	FH, *ex* Flora of the Philippines Herbarium, No. 30627	HM635942	HM635990	HM636038
P. cubensis	*Cucurbita maxima*	NY430	1905	USA, NJ	NY, *ex* Herbarium of George Massee	HM635926	HM635974	HM636022
P. cubensis	*Cucurbita maxima*	NY452	1907	USA, AL	NY, *ex* Wellesley College Herbarium (WELC), No. 67485	HM635929	HM635977	HM636025
P. cubensis	*Cucurbita pepo*	NY427	1943	USA, MA	NY, *ex* Univ. Massachusetts Fungus Herbarium (MASS), No. 3431	HM635928	HM635976	HM636024

P. cubensis	*Cucurbita pepo*	NY434	1935	USA, FL	NY, *ex* University of Florida Herbarium, No. 10172	HM635933	HM635981	HM636029
P. cubensis	*Cucurbita pepo*	NY447	1935	USA, FL	NY, *ex* Herbarium of Erdman West	HM635931	HM635979	HM636027
P. cubensis	*Lagenaria leucantha*	NY448	1925	USA, FL	NY, *ex* University of Florida Herbarium	HM635920	HM635968	HM636016
P. cubensis	*Melothria crassifolia*	NY431	1935	USA, FL	NY, *ex* University of Florida Herbarium, No. 11222	HM635927	HM635975	HM636023
P. cubensis	*Sicyos angulatus*	U192	1920	USA, WI	FH, *ex* Herbarium of the University of Wisconsin	HM635916	HM635964	HM636012
P. humuli	*Humulus lupulus*	10124	2005	Germany	HOH, No. HUH1134	HM635946	HM635994	HM636042
P. humuli	*Humulus lupulus*	NY454	1940	Czech Republic	NY, *ex* Cryptogamae exsiccatae a Museo Hist. Natur. Vindobonensi, No. 3404	HM635944	HM635992	HM636040
P. humuli	*Humulus lupulus*	NY455	1946	Germany	NY, *ex* Herbarium K. Starcs Flora Bavarica, No. 2073	HM635945	HM635993	HM636041
P. humuli	*Humulus lupulus*	NY458	1957	Poland	NY, *ex* Kochman, Mycotheca Polonica, Fasc. I, No. 7	HM635943	HM635991	HM636039
P. humuli	*Humulus lupulus*	NY459	1980	Argentina	NY, *ex* Universidad Nacional de la Plata, No. 42094	HM635948	HM635996	HM636044
P. humuli	*Humulus lupulus*	NY460	1925	Germany	NY, *ex* F. Petrak, Mycotheca Carpatica, No. 421	HM635947	HM635995	HM636043
P. urticae	*Urtica dioica*	HUH1111	2009	Italy	HOH, No. HUH1111	HM635952	HM636000	HM636048
P. urticae	*Urtica dioica*	PDD1	1975	Austria	PDD, No. 64564	HM635953	HM636001	HM636049
Pseudoperonospora sp.	*Celtis sinensis*	D232	2004	Korea	KUS-F, No. 20975	HM635954	HM636002	HM636050
Pseudoperonospora sp.	*Cucumis sativus*	21226	2007	Czech Republic	HOH, No. HUH1130	HM635937	HM635985	HM636033
Pseudoperonospora sp.	*Cucumis sativus*	22238	2007	Czech Republic	HOH, No. HUH1131	HM635939	HM635987	HM636035
Pseudoperonospora sp.	*Cucumis sativus*	23231	2007	USA, MI	HOH, No. HUH1132	HM635940	HM635988	HM636036
Pseudoperonospora sp.	*Cucumis sativus*	24230	2007	Germany	HOH, No. HUH1133	HM635938	HM635986	HM636034
Pseudoperonospora sp.	*Cucumis sativus*	NY425	1924	Japan	NY, *ex* Herb. Agr. Dept. Imp. Univ. Kyoto, No. 98	HM635934	HM635982	HM636030
Pseudoperonospora sp.	*Cucumis sativus*	U196	1904	Japan	FH, *ex* Herbarium of Marioka Imperial College of Agriculture and Forestry	HM635932	HM635980	HM636028
Pseudoperonospora sp.	*Cucumis sativus*	U201	1934	Japan	FH, *ex* Herbarium of Morioka Imperial College of Agriculture and Forestry, No. 14	HM635941	HM635989	HM636037
Pseudoperonospora sp.	*Cucurbita moschata*	D190	2002	Korea	KUS-F, No. 19205	HM635925	HM635973	HM636021
Pseudoperonospora sp.	*Cucurbita pepo*	NY440	1890	Japan	NY, *ex* Herbarium of Kingo Miyabe, No. 107	HM635935	HM635983	HM636031
Pseudoperonospora sp.	*Humulus japonicus*	D149	2003	Korea	KUS-F, No. 19582	HM635936	HM635984	HM636032
Pseudoperonospora sp.	*Humulus lupulus*	NY461	1909	USA, WI	NY, *ex* Herbarium of the University of Wisconsin, No. 8	HM635950	HM635998	HM636046
Pseudoperonospora sp.	*Lagenaria siceraria*	D265	2005	Korea	KUS-F, No. 21327	HM635951	HM635999	HM636047

were employed as described previously (Choi et al. 2007), but using the MangoTaq Polymerase (Bioline, Luckenwalde, Germany), adding BSA to a final concentration of 8 μg/ml in the PCR reaction mix. For amplification of the Ras-related protein (*ypt1*) gene the primers Ypt1F described in Chen and Roxby (1996) and Ypt4R described in Moorman et al. (2002) were used. Amplification was carried out with MangoTaq Polymerase and the addition of BSA to a final concentration of 8 μg/ml. Amplification of the nrITS region was carried out as described by Thines (2007) using the primers LR-0 (reverse complement to LR-0R, Moncalvo et al. 1995) and DC-6 (Cooke et al. 2000). Sequencing was carried out by a commercial sequencing provider (GATC Biotech, Konstanz, Germany) using the primers employed for PCR.

Phylogenetic inference

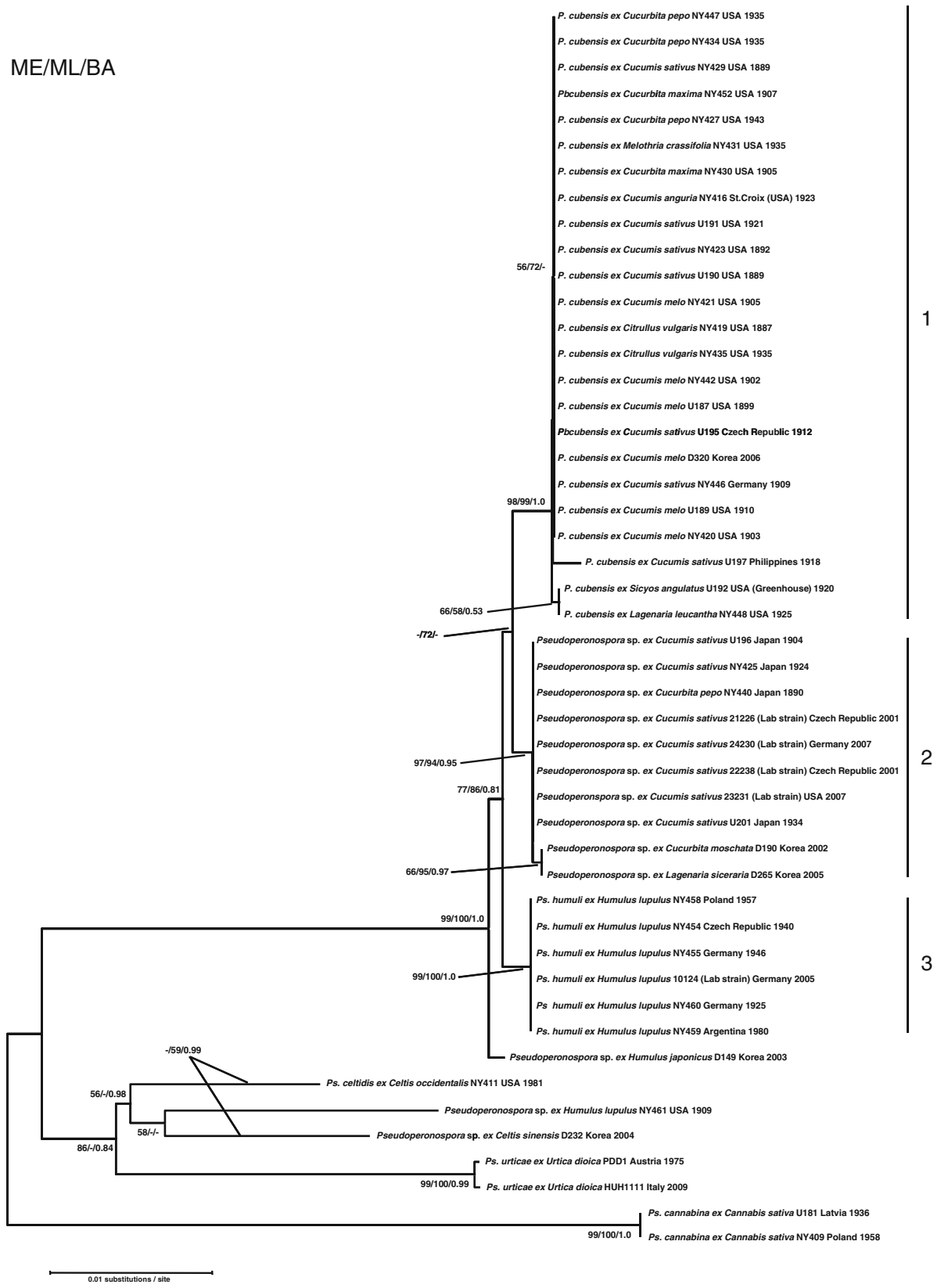
Minimum Evolution analysis was done with MEGA 4.0 (Tamura et al. 2007), under the Tamura-Nei substitution model. All other parameters were set to default values. For testing tree robustness, 1000 bootstrap replicates (Felsenstein 1985) were carried out. For Maximum Likelihood analysis RAxML (Stamatakis 2006) on the webserver at CIPRES was used (http://8ball.sdsc.edu:8889/cipres-web/Home.do), with likelihood search and estimation of invariable sites. All other parameters were set to default values. The analysis was repeated five times with 100 bootstrap replicates each. The bootstrap values obtained were averaged, because the rapid bootstrapping algorithm (Stamatakis et al. 2008) can lead to deviation in the range of several percent. For Bayesian analysis MrBAYES (Huelsenbeck and Ronquist 2001), version 3.1.2 was used. Four incrementally heated chains were run for 10,000,000 generations, with a sampling frequency of 1000, under the general time reversible (GTR) model and assuming gamma-distributed substitution rates. The first 2500 trees sampled this way were discarded, and the remaining 7500 trees were used to compute a majority based consensus tree and for inferring posterior probabilities. According to Choi et al. (2005) *P. cannabina* is considered to be the most basal species of the genus *Pseudoperonospora* and therefore the phylogenetic trees were rooted with sequences from this species.

Results

All phylogenetic analyses, including Minimum Evolution (ME), Maximum Likelihood (ML), and Bayesian Analysis (BA), yielded highly similar topologies, and no strongly supported inconsistencies were observed. The best scoring tree from the ME analysis, with the support values from all phylogenetic analyses is given in Fig. 1. Specimens of *Pseudoperonospora cannabina* formed a monophyletic group with high to maximum support in all analyses. *Pseudoperonospora urticae* clustered with *P. celtidis* with moderate support. *Pseudoperonospora celtidis* was split into two distinct lineages; one representing *P. celtidis* originating from *Celtis occidentalis* in the USA and the other representing an undescribed species of *Pseudoperonospora* on *Celtis sinensis* in Korea. The third lineage clustering with the two lineages from *Celtis* originated from *Humulus lupulus*, representing a rare, undescribed species.

The closely related species *P. humuli* and *P. cubensis* formed a monophyletic group together with two cryptic lineages with high to maximum support in all analyses. *Pseudoperonospora* sp. from *Humulus japonicus* in Korea is placed basal to the other lineages in the *P. humuli-P. cubensis* cluster. Clade 3 contains all *P. humuli* strains from *Humulus lupulus* and was supported with high to maximum support in all analyses. This lineage was placed sister to the two *Pseudoperonospora* clades 1 and 2 pathogenic to the Cucurbitaceae with moderate support. The sister group relationship of the clades 1 and 2 received low bootstrap support in ML analysis (72%) but was unsupported in the other analyses. Clade 2, which monophyly was highly supported in all analyses, contains only *Pseudoperonospora* strains from East Asia, i.e. Japan and Korea, except for the post-epidemic lineages represented by recently sampled laboratory strains originating from the USA, the Czech Republic, and Germany. The two Korean strains in clade 2 were separated from the other

Fig. 1 Phylogenetic tree for the downy mildew genus *Pseudoperonospora* based on Minimum Evolution analysis. Numbers on branches denote statistical support in Minimum Evolution, Maximum Likelihood, and Bayesian Analysis, in the respective order. Numbers on vertical lines are clade numbers referred to in the text. DNA accession numbers are given behind host taxon names, followed by country of origin and year of collection


ME/ML/BA
P. cubensis ex Cucurbita pepo NY447 USA 1935
P. cubensis ex Cucurbita pepo NY434 USA 1935
P. cubensis ex Cucumis sativus NY429 USA 1889
Pbcubensis ex Cucurbita maxima NY452 USA 1907
P. cubensis ex Cucurbita pepo NY427 USA 1943
P. cubensis ex Melothria crassifolia NY431 USA 1935
P. cubensis ex Cucurbita maxima NY430 USA 1905
P. cubensis ex Cucumis anguria NY416 St.Croix (USA) 1923
P. cubensis ex Cucumis sativus U191 USA 1921
P. cubensis ex Cucumis sativus NY423 USA 1892
56/72/-
P. cubensis ex Cucumis sativus U190 USA 1889
P. cubensis ex Cucumis melo NY421 USA 1905
P. cubensis ex Citrullus vulgaris NY419 USA 1887
P. cubensis ex Citrullus vulgaris NY435 USA 1935
P. cubensis ex Cucumis melo NY442 USA 1902
P. cubensis ex Cucumis melo U187 USA 1899
Pbcubensis ex Cucumis sativus U195 Czech Republic 1912
P. cubensis ex Cucumis melo D320 Korea 2006
P. cubensis ex Cucumis sativus NY446 Germany 1909
98/99/1.0
P. cubensis ex Cucumis melo U189 USA 1910
P. cubensis ex Cucumis melo NY420 USA 1903
P. cubensis ex Cucumis sativus U197 Philippines 1918
P. cubensis ex Sicyos angulatus U192 USA (Greenhouse) 1920
66/58/0.53
P. cubensis ex Lagenaria leucantha NY448 USA 1925
1
-/72/-
Pseudoperonospora sp. ex Cucumis sativus U196 Japan 1904
Pseudoperonospora sp. ex Cucumis sativus NY425 Japan 1924
Pseudoperonospora sp. ex Cucurbita pepo NY440 Japan 1890
Pseudoperonospora sp. ex Cucumis sativus 21226 (Lab strain) Czech Republic 2001
Pseudoperonospora sp. ex Cucumis sativus 24230 (Lab strain) Germany 2007
97/94/0.95
Pseudoperonospora sp. ex Cucumis sativus 22238 (Lab strain) Czech Republic 2001
77/86/0.81
Pseudoperonspora sp. ex Cucumis sativus 23231 (Lab strain) USA 2007
Pseudoperonospora sp. ex Cucumis sativus U201 Japan 1934
Pseudoperonospora sp. ex Cucurbita moschata D190 Korea 2002
66/95/0.97
Pseudoperonospora sp. ex Lagenaria siceraria D265 Korea 2005
2
Ps. humuli ex Humulus lupulus NY458 Poland 1957
99/100/1.0
Ps. humuli ex Humulus lupulus NY454 Czech Republic 1940
Ps. humuli ex Humulus lupulus NY455 Germany 1946
99/100/1.0
Ps. humuli ex Humulus lupulus 10124 (Lab strain) Germany 2005
Ps humuli ex Humulus lupulus NY460 Germany 1925
Ps. humuli ex Humulus lupulus NY459 Argentina 1980
3
-/59/0.99
Pseudoperonospora sp. ex Humulus japonicus D149 Korea 2003
Ps. celtidis ex Celtis occidentalis NY411 USA 1981
56/-/0.98
Pseudoperonospora sp. ex Humulus lupulus NY461 USA 1909
58/-/-
Pseudoperonospora sp. ex Celtis sinensis D232 Korea 2004
86/-/0.84
Ps. urticae ex Urtica dioica PDD1 Austria 1975
99/100/0.99
Ps. urticae ex Urtica dioica HUH1111 Italy 2009
Ps. cannabina ex Cannabis sativa U181 Latvia 1936
99/100/1.0
Ps. cannabina ex Cannabis sativa NY409 Poland 1958
0.01 substitutions / site

specimens and clustered together with low support in ME and high support in ML and BA. The monophyly of *P. cubensis* s.str. (clade 1) received high to maximum support in all analyses and contains pre-epidemic strains, in particular from the USA, but also a few specimens from Europe and Asia. Within clade 1 most specimens formed a uniform group. Specimens from *Sicyos angulatus* and *Lagenaria leucantha* clustered together with low support in all analyses. No clear-cut host preferences for clade 1 and clade 2 could be observed, both clades include hosts in the genera *Cucumis*, *Cucurbita*, and *Lagenaria*.

Discussion

Judging from the results of Choi et al. (2005) *P. cannabina* is the most basal species of the genus *Pseudoperonospora* and therefore the phylogeny was rooted with this species. In the present study, the species *P. cannabina* and *P. urticae* were monophyletic, while *P. celtidis* s.l. has to be divided into two different species. One of the phylogenetic lineages occurring on *Celtis* represents *P. celtidis* parasitic to the North American species *Celtis occidentalis*, the type host of *P. celtidis* (Waite 1892; Wilson 1907b). The second species is currently undescribed and is parasitic to the East Asian species *Celtis sinensis*, but more specimens should be investigated for clarifying the possibility of morphological delineation between the two species and also for clarifying their geographic range. We thus refrain from introducing the new species in this manuscript. Interestingly, a third phylogenetic lineage is clustering with the two isolates from *Celtis* species. The host for this lineage is *Humulus lupulus* and possibly represents *Pseudoperonospora celtidis* var. *humuli* described by Davis (1910) based on morphological similarities to *Pseudoperonospora celtidis*. Based on the assumption that only one downy mildew species occurs on a specific host plant (e.g. Constantinescu 1989), this variety has been treated as conspecific with *P. humuli*. Whether hop is the native host of the pathogen or an accidental host of a pathogen of another host (e.g. another species of *Celtis*) needs to be determined by a broader sampling of North American specimens of downy mildew from *Humulus lupulus* and species of *Celtis*.

Because of the unsatisfactory resolution of ITS sequences alone for the delineation of closely related species (Voglmayr 2008), we used a multilocus approach in this study, using two additional markers with high phylogenetic resolution. Previous studies (Choi et al. 2007, 2008, 2009; Thines et al. 2009) have proven that in addition to nrITS the mitochondrial *cox2* region offers good phylogenetic resolution in closely related species. For obtaining an even higher resolution, we also included the highly variable Ras-related protein (*ypt1*) gene for additional phylogenetic resolution.

Our results provide evidence that the degradation of *P. humuli* to a synonym of *P. cubensis* as suggested by Choi et al. (2005) should not be upheld. Due to the higher resolution of the present phylogenetic reconstruction, *P. humuli* was found to be distinct from *P. cubensis* with high support. Interestingly, a distinct phylogenetic lineage of *Pseudoperonospora* was observed from *Humulus japonicus* from South Korea, this clade was represented by two specimens from this host. For one of these only the *ypt1* and the *cox2* locus could be obtained and is thus not included in Fig. 1. This cryptic species is basal to all other lineages in the *P. cubensis* complex, suggesting a host jump from hop to Cucurbitaceae gave rise to *P. cubensis*. All other specimens of *Pseudoperonospora* parasitising *Humulus lupulus* were placed in a monophyletic clade, which received high to maximum support in all analyses. This clade contained specimens from several regions of the world, probably reflecting anthropogenic spread. A sample from Japan, the type locality of *P. humuli*, was also placed within this clade based on *ypt1* sequence, but had to be excluded from the multigene phylogeny as other loci did not amplify from this historic specimen. Further investigations encompassing a larger set of samples and the type specimen of *P. humuli* will be necessary to ascertain that this group (clade 3) represents *P. humuli* and to determine the origin of this species.

After the re-emergence of *P. cubensis* as the most destructive pathogen in cucurbit production since the 1980s in Europe (Lebeda 1990; Lebeda and Urban 2004) and since 2004 in the USA (Holmes et al. 2006), uncertainty remained about the reason for the breach of the resistance of the cultivars previously used. Although the occurrence of new pathotypes in *P. cubensis* is known (Lebeda et al. 2006), no molecular phylogenetic basis to distinguish among them is available to date (Sarris et al. 2009). Choi et

al. (2005) have shown a generally low diversity in nrITS of the *P. cubensis* species cluster. However, the present investigations using multi-locus sequences reveal a significant degree of genetic divergence within this species cluster, offering an unexpected, yet plausible explanation for the recently occurring epidemics of cucurbit downy mildew. It is noteworthy that two distinct phylogenetic lineages are parasitic to Cucurbitaceae, which are now widely distributed, but previously might have been geographically restricted.

Clade 1 contains specimens from all around the world but exhibits some genetic variation in Northern America and is considered to represent *P. cubensis*. The type species of *Pseudoperonospora* is *Peronospora cubensis* Berk. & Curt., and since Rostovzev (1903) based the description of *P. cubensis* on the original specimen from Cuba and ranked the Russian specimen only as a variety of this species (Wilson 1907a), no nomenclatorial complications with the previous description of Berkeley & Curtis (Berkeley 1869) arise. The higher degree of genetic variation within the North American specimens compared to samples from other parts of the world renders it likely that *Pseudoperonospora cubensis* originated from this region and was later distributed by international trade.

Clade 2 contains primarily East Asian specimens. As these mostly date from about half a century before the recent epidemics in Europe and the USA, it is probably indigenous to Asia. But it also contains the recent Czech and German isolates, as well as the US American laboratory strain of a genotype commonly found on cucumbers in the USA (Lina María Quesada, Hausbeck lab, personal communication). In spring of 1889 *Pseudoperonospora cubensis* was only known in Japan and the Americas (Halsted 1891). Given the geographic isolation, this suggests that the pathogen in Japan was not *P. cubensis* s.str., but a member of clade 2. This is in line with one specimen from 1890 and others from the turn of the century from Japan being placed in clade 2 in this study. In the light of the present results it thus seems likely that the resistance-overcoming lineage might have originated from East Asia. As specimens from Korea showed some genetic divergence, while Japanese samples were identical in sequence compared to the laboratory strains representing isolates from recent outbreaks, it is possible that the causal agent was introduced from Japan. The cryptic species or subspecies became invasive most likely through anthropogenic introduction into Europe and later into Northern America. The paths and invasion routes by which the pathogen entered into Europe and the United States should be clarified by population genetic studies to improve future risk assessment.

In earlier studies a variety of pathotypes of *Pseudoperonospora* on cucurbits was found (Thomas et al. 1987; Cohen et al. 2003; Lebeda and Urban 2004) and the question arose, if the recent epidemics are caused by a pathogen with a spontaneous mutation giving rise to higher virulence of the prevalent population or whether it represents a newly introduced pathogen with a high degree of virulence plasticity (Holmes et al. 2006). Although the differentiation in pathotypes is well known, hitherto no molecular data to distinguish these pathotypes has been available (Sarris et al. 2009). A set of twenty different pathotypes currently present in Europe was tested on differences in their nrITS and *cox2* sequences (data not shown). All pathotypes were identical in these loci and are also identical to recent strains from North America, providing further evidence that the current epidemics are not caused by a single race that has evolved to higher virulence, but by a new phylogenetic lineage with a high degree of adaptability and virulence plasticity, which likely represents a new subspecies or possibly even a new species of *Pseudoperonospora*. In Italy observations of downy mildew from the last millennium suggested that squash was not susceptible (Ciccarese et al. 1990). A more recent study of Cappelli et al. (2003) however obtained isolates on squash and linked it due to its high pathogenicity to pathotype 5 (the most virulent pathotype designated in Thomas et al. 1987). It seems possible that the observations were not merely a result of pathotype differentiation, but that the observations of Ciccarese et al. (1990) were referring to the 'old' pathogen *P. cubensis* s.str., and that the pathogen observed by Cappelli et al. (2003) was representing the 'new' and more virulent pathogen of the cryptic lineage uncovered here. Whether these previously geographically separated lineages are still able to exchange genetic material will be an important question for future research.

Both *P. cubensis* s.str. and the cryptic lineage have a very broad host range with several hosts in common. Thus these remain the downy mildew pathogens with the broadest known host ranges. We refrain from the formal introduction of the cryptic

lineage as a new taxon in this manuscript, as morphology of the two species or subspecies is highly similar, and thus the morphological variation caused by ecological conditions (Iwata 1942; Cohen 1981; Dudka et al. 2007) made it impossible to distinguish between them. Measurements of sporangiophores from the same host species harvested under the same controlled conditions might reveal morphological differences suitable for delineation (Runge and Thines 2010) and should be done on a broad set of isolates in future studies. In addition a multitude of isolates should be phylogenetically investigated using several loci, in order to determine if the phylogenetic lineage should be considered a distinct subspecies or species.

The recommendation of Palti (1974) that precautions must be taken not to transfer cucurbits infected with downy mildew from one region to another and his suggestion that varieties bred for resistance in one region have to be retested in any region into which they may be introduced was a remarkable foresight in the light of the present study. Although both cucurbit-affecting lineages are now widespread in cucurbit growing areas, precautions should to be taken for not distributing unknown cryptic pathogen species that might still reside in the *P. cubensis* species cluster, like the cryptic species uncovered on hop. Recent reports suggest an ongoing spread of *P. cubensis* in Korea (Choi and Shin 2008) and Taiwan (Ko et al. 2008) and recently, Voglmayr et al. (2009) reported a member of the *P. cubensis* species complex colonizing the Balsaminaceae. This highlights both the high adaptability and current radiation in this species complex and underscores the necessity for multilocus genotyping of these morphologically cryptic lineages. In addition it should be clarified by extensive population genetic studies, if the cryptic subspecies or species related to *P. cubensis* are still capable of hybridising with each other, for assessing the risk of the emergence of hyper-virulent hybrids.

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