Eur J Plant Pathol (2014) 139:641–653
DOI 10.1007/s10658-014-0420-y

ORIGINAL RESEARCH

Characterization of *Pseudoperonospora cubensis* isolates from Europe and Asia using ISSR and SRAP molecular markers

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Accepted: 12 March 2014 / Published online: 28 March 2014
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Abstract Downy mildew caused by *Pseudoperonospora cubensis* is a major disease of cucurbits worldwide. New genotypes of the pathogen have recently appeared in the USA, EU and Israel causing breakdown of genetic resistance, expansion of host range, and the appearance of a new A2 mating type. Seventy-eight *P. cubensis* isolates were collected during 1996–2011 from cucurbits fields in different regions of Turkey, Israel and the Czech Republic and genetic diversity was analysed using highly polymorphic ISSR and SRAP molecular markers. The data acquired showed remarkable genetic diversity within and among the isolates. While isolates from Turkey and Czech Republic exhibited uniform genetic background, the isolates from Israel were clearly distinguished from the others. The results may indicate on migration and/or frequent sexual reproduction of the pathogen in Israel. Moreover the selected markers can be suggested for monitoring genetic diversity within *P. cubensis* isolates in further studies.

Keywords *Cucumis sativus* · Cucurbits · Cucurbit downy mildew · Genetic diversity · Pathotypes · Population structure · Mating type

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Introduction

The obligate oomycete pathogen *Pseudoperonospora cubensis* (Berk.et Curt.) Rost. is the causal agent of downy mildew in Cucurbitaceae, a family of vegetable crops that includes cucumber, melon, pumpkin, squash, watermelon, butternut gourd, bottle gourd and sponge gourd (Lebeda and Cohen 2011). The market value of cucurbits is estimated at nearly $1.6 billion in the USA (Savory et al. 2011) and about $200 million in Israel (The Plant Council, Israel).

More than 60 cucurbit species, in at least 70 countries, have been reported as hosts of this pathogen (Lebeda and Cohen 2011). Because of the devastating nature of this disease, cucumber cultivars in the U.S. had been bred for resistance to *P. cubensis*. However, since

2004, widespread crop failures have occurred, resulting in significant economic losses, in the U.S. (Holmes et al. 2004), and elsewhere. While losses associated with this disease are substantial, the genetic basis for host resistance, pathogen virulence, global pathogen migration and population composition remains poorly studied (Lebeda 1999; Savory et al. 2011) thus, making the work presented herein of important value.

Six pathotypes of *P. cubensis* have been identified in the USA, Israel and Japan (Cohen et al. 2003; Lebeda and Gadasova 2002; Sarris et al. 2009), and many more exist in Europe (Lebeda et al. 2013). Using *Citrullus, Cucumis and Cucurbita* spp., Thomas et al. (1987) identified five distinct pathotypes of *P. cubensis*: 1 and 2 from Japan, 3 from Israel, and 4 and 5 from the USA. In 2003, Cohen et al. (2003) identified a sixth pathotype in Israel based on its virulence to a wider range of susceptible cucurbits compared with pathotype 3. All six pathotypes that have been described are virulent on cucumber and muskmelon (*C. melo* var. *reticulatus*), but show differences in virulence on watermelon, squash or pumpkin. Subsequently, Lebeda and Widrlechner (2003) developed a set of differential taxa that included 12 representatives from six genera (*Benincasa*, *Citrullus*, *Cucumis*, *Cucurbita*, *Lagenaria* and *Luffa*), which represent natural hosts of *P. cubensis*. Using this set, they evaluated the virulence of 22 isolates from the Czech Republic, Spain, France and the Netherlands and classified them into as 13 pathotypes (Lebeda and Gadasova 2002; Lebeda and Widrlechner 2003). Most recent studies showed enormous virulence variation and spatio-temporal shift in the Czech *P. cubensis* populations, with 67 different pathotypes identified during 2001–2010 (Lebeda et al. 2012, 2013). Unfortunately, the genetic basis for the differences among pathotypes is not known.

Amplified fragment length polymorphisms (AFLP) and sequencing of nuclear and mitochondrial loci have been used for studying the genetic diversity, for taxonomy and phylogeny of downy mildew pathogens including *P. cubensis* (Mitchell et al. 2011; Quesada-Ocampo et al. 2012; Runge et al. 2011; Sarris et al. 2009; Voglmayr 2008).

The Sequence Related Amplified Polymorphism (SRAP) molecular markers system enables random amplification of coding regions in the genome in a more reproducible way than RAPD. It has also been applied extensively in genetic diversity analyses (Ferriol et al. 2003) and comparative genetics of different species (Lin et al. 2004) including fungi (Baysal et al. 2009) and root knot nematodes (Devran and Baysal 2012). SRAP is a PCR marker system combining simplicity, reliability and a moderate throughput ratio, which was used in genetic diversity analysis and map construction (Lin et al. 2005). It has also been suggested as a simple, middle-yield, high-dominant total, repetitive way on genetic diversity of *Gibberella zeae* isolates (Fernando et al. 2006).

The Inter-Simple Sequence Repeat (ISSR) is a single primer amplifying DNA fragments between two identical microsatellite repeat (SSR) regions oriented in opposite directions. ISSR molecular markers allow for cost-effective detection and quantification of the pathogen compared to AFLP (Dubey and Singh 2008). The works with ISSR demonstrated the hypervariable nature of ISSR markers and its potential for population studies, which was proved in plants (Martins-Lopes et al. 2007) and insects (Soliani et al. 2010). These markers were also suggested to implications related to resistance management on *Ceratitis capitata* (Beroiz et al. 2012).

To the best of our knowledge, *P. cubensis* has never been genetically characterized using ISSR and SRAP markers. We employed these markers to a larger number of isolates of *P. cubensis* that have originated from distinct areas and are well characterized in their phytopathological attributes. This information may provide the basis for investigating the sources and shifts in genetic diversity within and between *P. cubensis* populations worldwide.

Material and methods

Pathogen collection

Leaves infected with *Pseudoperonospora cubensis* were collected from the field or greenhouses. A total of 78 isolates (Table 1) were genotyped in the present study: 28 from Israel, 19 from Turkey and 31 from the Czech Republic. Israeli isolates were collected from cucumber, melon, squash or pumpkin whereas all other isolates were collected from cucumber (Table 1). The isolates were subjected to pathotyping according to Cohen et al. (2003) and mefenoxam sensitivity testing. Czech and other European isolates were characterized in our previous papers (Lebeda and Gadasova 2002; Lebeda et al. 2013; Lebeda and Widrlechner 2003; Urban and Lebeda 2007).

Table 1 Samples list of *Pseudoperonospora cubensis* originating from Israel, Turkey and the Czech Republic, date of collecting, geographic origin, host of recovery, pathotype differentiation and mefenoxam sensitivity

No.	ID	Country	Date collected	Zone of sampling	Host	Pathotype[a]	Mefenoxam sensitivity[b]
1	15	Israel	2007	Benei darom	*Cucumis melo*	3	R
2	32	Israel	2006	Bar-ilan Univ. farm	*Cucumis sativus*	3	R
3	18	Israel	2009	Achituv shalman	*C. sativus*	3	R
4	35	Israel	2009	Hof carmel	*C. sativus*	3	R
5	36	Israel	2009	Netazim naan	*C. sativus*	3	R
6	20	Israel	2008	Achituv efrayim	*C. sativus*	3	R
7	None	Israel	2008	Achituv pardes	*C. sativus*	3	R
8	74	Israel	2007	Unknown	*C. melo*	3	nd
9	75	Israel	2011	Bet ezra	*Cucurbita pepo*	6	R
10	76	Israel	2011	Bet ezra	*C. pepo*	6	S
11	77	Israel	2011	Bet ezra	*C. pepo*	6	R
12	78	Israel	2011	Bet ezra	*C. pepo*	6	S
13	79	Israel	2011	Bar-ilan Univ. farm	*Cucumis sativus*	3	nd
14	80	Israel	2011	Bar-ilan Univ. farm	*C. sativus*	3	nd
15	85	Israel	2011	Bet ezra	*Cucurbita pepo*	5	S
16	86	Israel	2011	Bet ezra	*Cucurbita maxima*	6	I
17	88	Israel	2011	Bet ezra	*C. maxima*	6	I
18	89	Israel	2011	Bet ezra	*C. maxima*	6	S
19	90	Israel	2011	Kfar Haim	*Cucurbita pepo*	6	nd
20	91	Israel	2011	Zofit	*C. pepo*	6	nd
21	96	Israel	2011	Unknown	unknown	6	R
22	98	Israel	2011	Kalkilia	*Cucurbita maxima*	6	S
23	101	Israel	2011	Bar-ilan Univ. farm	*C. maxima*	6	R
24	105	Israel	2011	Bar-ilan Univ. farm	*Luffa cylindrica*	–	R
25	124	Israel	2011	Nahalal	*Cucurbita pepo*	6	nd
26	125	Israel	2011	Bar-ilan Univ. farm	*Cucurbita maxima*	6	I
27	127	Israel	2011	Bet ezra	*Cucurbita pepo*	6	S
28	132	Israel	2011	Bar-ilan Univ. farm 29	*Cucumis melo*	3	R
29	Batem2	Turkey	2010	Antalya	*Cucumis sativus*	3	I
30	Bartin4	Turkey	2010	Bartin	*C. sativus*	3	S
31	Bartin3	Turkey	2010	Bartin	*C. sativus*	3	S
32	Izmir7	Turkey	2010	İzmir	*Cucumis sativus*	3	S
33	Izmir8	Turkey	2010	İzmir	*C. sativus*	3	I
34	Batem1	Turkey	2010	Antalya	*C. sativus*	3	I
35	Adana17	Turkey	2010	Adana	*C. sativus*	3	I
36	Adana18	Turkey	2010	Adana	*C. sativus*	3	I
37	Bartin5	Turkey	2010	Bartin	*C. sativus*	3	S
38	Bartin6	Turkey	2010	Bartin	*C. sativus*	3	S
39	Izmir9	Turkey	2010	İzmir	*C. sativus*	3	I
40	Izmir10	Turkey	2010	İzmir	*C. sativus*	3	I
41	Mugla1	Turkey	2010	Muğla	*C. sativus*	3	I
42	Mugla2	Turkey	2010	Muğla	*C. sativus*	3	I
43	Mugla3	Turkey	2010	Muğla	*C. sativus*	3	I
44	Adana19	Turkey	2010	Adana	*C. sativus*	3	I

Table 1 (continued)

No.	ID	Country	Date collected	Zone of sampling	Host	Pathotype[a]	Mefenoxam sensitivity[b]
45	Antalya3	Turkey	2010	Antalya	*C. sativus*	3	I
46	Antalya4	Turkey	2010	Antalya	*C. sativus*	3	I
47	Antalya5	Turkey	2010	Antalya	*C. sativus*	3	I
48	6/96	Czech	1996	Brno-Židenice	*C. sativus*	6	nd
49	6/97	Czech	1997	Hajany	*C. sativus*	6	nd
50	1/98	Czech	1998	Gene Bank, Olomouc	*C. sativus*	6	S
51	5/00	Czech	2000	Velká Bystřice	*C. sativus*	5	nd
52	7/00	Czech	2000	Olomouc-Holice, SRLS	*C. sativus*	–	nd
53	9/00	Czech	2000	Starý Jičín	*C. sativus*	–	nd
54	10/00	Czech	2000	Lutín	*C. sativus*	6	nd
55	11/00	Czech	2000	Lednice na Moravě	*C. sativus*	6	nd
56	14/00	Czech	2000	Dolní Moravice	*C. sativus*	6	nd
57	11/01	Czech	2001	Žehrov	*C. sativus*	7	R
58	24/01	Czech	2001	Dub nad Moravou	*C. sativus*	7	nd
59	26/01	Czech	2001	Kojetín	*Cucumis sativus*	7	R
60	39/01	Czech	2001	Vacenovice	*C. sativus*	7	nd
61	41/01	Czech	2001	Mistřín-Svatobořice	*C. sativus*	7	R
62	57/01	Czech	2001	Tasovice	*C. sativus*	7	R
63	64/01	Czech	2001	Valtice	*C. sativus*	7	nd
64	71/01	Czech	2001	Rokytnice	*C. sativus*	6	R
65	72/01	Czech	2001	Praha-Ruzyně	*C. sativus*	7	R
66	24/02	Czech	2002	Charváty	*C. sativus*	7	R
67	53/02	Czech	2002	Moravský Žižkov	*C. sativus*	6	R
68	84/02	Czech.	2002	Borušov	*C. sativus*	7	R
69	104/02	Czech	2002	Kojetín	*C. sativus*	5	R
70	11/1/03	Czech	2003	Rozstání	*C. sativus*	5	nd
71	13/03	Czech	2003	Kotvrdovice	*C. sativus*	6	nd
72	45/03	Czech	2003	Trávník	*C. sativus*	6	nd
73	54/03	Czech	2003	Ostrožská Nová Ves	*C. sativus*	7	R
74	61/1/03	Czech	2003	Moravský Žižkov	*C. sativus*	6	nd
75	83/03	Czech	2003	Benátky nad Jizerou	*C. sativus*	5	R
76	85/03	Czech	2003	Lysá nad Labem	*C. sativus*	5	R
77	4/04	Czech	2004	Věrovany-Nenakonice	*C. sativus*	5	R
78	9/04	Czech	2004	Oplocany	*C. sativus*	6	R

[a] *pathotype 3* sporulate on only *Cucumis* spp; *pathotype 5* differentiation according Lebeda et al. (2006); *pathotype 6* sporulates on *Cucumis* spp. *and Cucurbita* spp.; *pathotype 7* Lebeda et al. (2006); – missing data

[b] *S* sensitive; *I* tolerant; *R* resistant; *nd* missing data

DNA extraction

Total genomic DNA was extracted from sporangia of *P. cubensis* using a DNA isolation kit (Promega, Wizard Genomic DNA Purification Kit, Madison, US) according to the manufacturer's instructions. The extract was treated with DNase-free RNase (Roche Diagnostics, Germany) and quantified in agarose gels (1 %) using standard lambda DNA for comparison.

Molecular studies using ISSR and SRAP markers

Sixteen ISSR (Baysal et al. 2009; Levi et al. 2005) and 57 SRAP (Baysal et al. 2009; Yeboah et al. 2007) markers were used to determine the genetic diversity within the collection of 78 isolates. Twenty-four out of 73 markers were chosen (33 %) based on their polymorphic ratio and used to genotype the *P. cubensis* collection (Table 2).

ISSR analysis

The ISSR amplifications were carried out in reaction volumes of 25 μl containing 20 mM TriseHCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, 800 mM dNTP, 0.5 mM of each primer, 1 U Taq polymerase (Invitrogen, Life Technologies), and 20 ng of genomic DNA. PCR reactions were performed under the following cycle program: initial denaturation step for 4 min at 94 °C, followed by 36 cycles at 94 °C for 30 s (denaturation), 46–56 °C for 45 s (annealing) and 72 °C for 120 s (extension), followed by a final extension step at 72 °C for 7 min.

SRAP analysis

SRAP is a PCR-based marker system with two primers, a forward primer of 17 bases and a reverse primer of 18 bases. The PCR amplifications were carried out using a GeneAmp 9700 Thermal Cycler (Applied Biosystems) in reaction volumes of 15 μl containing 15 ng of genomic DNA and 0.2 μM each of forward and reverse primers. Reactions were performed under the following conditions: initial denaturation step for 90 s at 94 °C, the first five cycles were run at 94 °C for 1 min (denaturation), 35 °C for 1 min (annealing) and 72 °C for 1 min (extension), followed by 35 cycles where the annealing temperature was raised at 50 °C for 1 min. The final extension step was at 72 °C for 10 min.

PCR products obtained from different markers were separated on 2.5 % high resolution agarose gel in 1X TAE buffer at 100 V for 3.0 h. A 100 bp DNA ladder was used as molecular standard. To confirm the reproducibility of the banding patterns, all PCR experiments were repeated three times.

Table 2 List of molecular markers used for genetic characterization of *Pseudoperonospora cubensis* collection

Name	Type	T_a[a]	Reference
808	ISSR	54	Levi et al. (2005); Baysal et al. (2009)
809	ISSR	54	Levi et al. (2005); Baysal et al. (2009)
824	ISSR	52	Levi et al. (2005); Baysal et al. (2009)
827	ISSR	55	Levi et al. (2005); Baysal et al. (2009)
834	ISSR	54	Levi et al. (2005); Baysal et al. (2009)
889	ISSR	55	Levi et al. (2005); Baysal et al. (2009)
731	ISSR	45	Levi et al. (2005); Baysal et al. (2009)
825	ISSR	45	Levi et al. (2005); Baysal et al. (2009)
112	ISSR	45	Levi et al. (2005); Baysal et al. (2009)
me8em10	SRAP	[b]	Li and Quiros (2001); Baysal et al. (2009)
me8em14	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me8em16	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me1em16	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me2em4	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me2em9	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me4em9	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me4em14	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me9em14	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me6em4	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me6em7	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me12em5	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me12em7	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me12em10	SRAP		Li and Quiros (2001); Baysal et al. (2009)
me3em4	SRAP		Li and Quiros (2001); Baysal et al. (2009)

[a] Ta annealing temperature

[b] SRAP primers were at the first 5 cycle at 35 °C and later 35 cycle at 50 °C

Anneling temperatures were detected by optimizations

Phenotypic characterization

Pathotype determination assays of Israeli isolates were conducted according to Thomas et al. (1987) and the modification of Cohen et al. (2003). Briefly, leaf discs of nine cucurbit species were inoculated at 20 °C (14 h light/day) and sporulation of the pathogen, as evaluated at 7 dpi, was used for pathotype determination. Czech isolates were pathotyped according the scheme described by Lebeda and Widrlechner (2003). Interpretation of results was simplified on the level of previous system of pathotypes denomination (Cohen et al. 2003).

Mefenoxam sensitivity was determined as followed: 4 cm diameter leaf discs of cucumber were laid on wet filter paper in 14 cm Petri dishes, lower surface upword, and sprayed with mefenoxam of 0, 0.1, 1, 10, or 100 μg/ml (active ingredient). At 6 h after spray discs were each inoculated with 4×20 μl droplets of sporangial suspension (100 sporangia per droplet). Plates were incubated at 20 °C (14 h light/day). At 7 dpi the sporulation of the pathogen on leaf discs was visually examined. Isolates that sporulated on 100, 10 or 1 μg/ml mefenoxam were considered resistant, intermediately resistant or sensitive, respectively. Czech *P. cubensis* isolates were screened by a modified method described by Urban and Lebeda (2007).

Data analysis

Amplified bands from each primer were scored as present (1) or absent (0). Only those bands that were consistently amplified were considered; smeared and weak bands were excluded from the analysis.

The pairwise genetic distances for phylogenetic relationships among strains were estimated using Nei's (1973) coefficient. A dissimilarity matrix was computed and a weighted neighbour-joining (NJ) tree was generated with PowerMarker version 3.25 (Liu and Muse 2005) using the data sets obtained from ISSR and SRAP. A consensus tree was created in NEXUS format for viewing in Tree-View (Page 1996), the nodes being supported by bootstrap analysis (1,000 replicates).

Additional statistics were computed to estimate the grade of polymorphism among the studied isolates. The percentage of polymorphic loci, Shannon's Information index, and Nei's gene diversity within the collection analysed were calculated using POPGENE, version 1.31 (Yeh et al. 1999).

The number of genetic group in the collection analysed was estimated using the STRUCTURE software version 2.3.4 (Pritchard et al. 2000). This package employs a Bayesian clustering approach to identify different gene pools and to assign individuals to K populations based on the allele frequencies at each locus. The evaluation of the most probable number of genetic groups (K_s) was performed following Pritchard and Wen (2003) and the simulation analysis by Evanno et al. (2005), which proposed an ad hoc statistic, ΔK. Program settings used the admixture ancestry and correlated marker frequency models. Alpha was inferred from the data and lambda was set to 1 (Evanno et al. 2005; Pritchard and Wen 2003). For each K (ranging from 1 to 10), 20 independent runs (50,000 burn-in, 100,000 Marchov Chain Monte Carlo) were carried out. The 20 runs were averaged using the software CLUMPP (CLUster Matching and Permutation Program (Jakobsson and Rosenberg 2007)), and shown in histograms using the program DISTRUCT (Rosenberg 2004).

To determine the presence of significant genetic structuring among geographic origin, originating host genus and pathotypes, genotype profiles were analyzed by molecular variance analysis (AMOVA) using Arlequin 3.1 software (Excoffier et al. 2005). Variance was partitioned into components among and within isolate groupings based on the following parameters: (i) host origin (*Cucumis sativus*, *C. melo*, *Cucurbita maxima*, *C. pepo* and other hosts), (ii) geographical origin (Israel, Turkey and Czech) and (iii) pathotype (3, 5, 6 and 7). The variance components and fixation index (F_{ST}) were calculated for each grouping with 16,000 permutations.

Finally, to further understand the genetic distribution of *P. cubensis* isolates, we performed a principal coordinate analysis (PCoA) (Fig. 3) using GenAlEx 6 program (Peakall and Smouse 2012).

Results

Seventy-eight *P. cubensis* isolates collected from Israel (28), Turkey (19) and the Czech Republic (31) were analysed using two different PCR-based markers: ISSR and SRAP.

Nine out of the 16 ISSR primers tested were chosen for the analysis. ISSR primers generated a total of 17 well-resolved bands of which 88.24 and 11.76 % were

polymorphic in the isolates sampled from Israel and the Czech Republic, respectively. The amplified fragments ranged in size from 390 bp (primer 112) to 1.6 Kb (primer 809). A low level of genetic diversity among the Czech strains were obtained (h=0.0203, I=0.0367), whereas the results underlined a moderate variability in the Israeli genetic background (Table 3). Indeed, the ISSR markers used revealed a relatively high level of genetic distance among most isolates sampled in Israel. The genetic similarities among the isolates from three countries ranged from 0.6752 to 0.8876 (Table 4).

Fifty-seven random SRAP primer combinations were tested for SRAP analysis and 15 of which were successfully used for genotyping. A total of 34 bands were observed, 31 of these (95 %) were polymorphic within the Israeli collection and six (17.65 %) in the Czech panel. No polymorphism was observed within the Turkish group (data not shown). Individual data sets obtained from ISSR and SRAP markers were combined. In the pooled analysis, genetic similarities among all groups ranged from 0.6774 to 0.9608, with a mean similarity of 0.8346 (Table 4).

The weighted neighbour-joining (NJ) tree showed three main clusters (Fig. 1) and grouping of genotypes obtained agreed with the origin of sampling. Indeed, cluster (a) that included all samples collected from Israel, appeared to be distinct from other groups; cluster (b) grouped the isolates form the Czech Republic, shared into ten genotypes; while cluster (c) grouped all samples from Turkey, which appeared to be closely related genetically to the Czech isolates.

The assignment of gene pool of origin for each of the 78 isolates was accomplished as described in "Materials and methods" from $K=1$ to $K=10$, using the combined molecular markers dataset. The number of genetic pools (K) showed a clear peak at two, where two main groups (pool I and pool II) were distinguished (Fig. 2): the first pool included samples from Czech and Turkey group, whilst all samples from Israel belonged to the second pool, showing a genetic background that differed from other strains (Fig. 2). These results closely mirrored the pattern of diversity described in the NJ tree (Fig. 1).

The principal coordinate analysis (PCoA) for the *P. cubensis* collection is shown in Fig. 3. PCoA allowed the evaluation of population structure and geometric distances between all genotypes by underlining the clear subdivision in two major genepools, pool I and pool II, which are clearly distinguished as two separate clusters (Fig. 3, orange and

Table 4 Genetic distance and similarity revealed by ISSR, SRAP and combined markers among *Pseudoperonospora cubensis* collection analysed

Group	Israel	Turkey	Czech
ISSR			
Israel	****	0.6752	0.7480
Turkey	0.3928	****	0.8876
Czech	0.2904	0.1192	****
SRAP			
Israel	****	0.6785	0.6877
Turkey	0.3879	****	0.9979
Czech	0.3744	0.0024	****
Combined ISSR and SRAP			
Israel	****	0.6774	0.7077
Turkey	0.3895	****	0.9608
Czech	0.3457	0.0399	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Table 3 Diversity detected by ISSR and SRAP markers. No polymorphic loci were found in the strains sampled in different areas of Turkey (data not shown)

	Israel				Czech			
	Number of polymorphic loci	Polymorphic loci (%)	*h*	*I*	Number of polymorphic loci	Polymorphic loci (%)	*h*	*I*
ISSR	15	88.24	0.3801	0.5469	2	11.76	0.0203	0.0367
SRAP	31	91.18	0.3701	0.5392	6	17.65	0.0262	0.0463
Mean	23	89.71	0.3751	0.5430	4	14.70	0.0232	0.0415

h Nei's (1973) gene diversity, *I* Shannon's Information index (Lewontin 1972)

Fig. 1 Dendrogram of genetic relationships among the 78 isolates based on combined data sets of the two marker techniques (ISSR and SRAP), generated with Nei's coefficient (Nei 1973) and Neighbor-joining (NJ) cluster analysis. The letters on the right of the tree indicate phylogenetic groups: **a** Israel; **b** Czech and **c** Turkey

gray ellipses, respectively). Within each genepool, different subgroups were found, corresponding to the geographical origin of isolates (Fig. 3). The PCoA explained around the 72 % of the variability; a clear grouping for the two genepools was obtained and the first axis accounts for 60 % of variability while the second one for 12 %.

Of the 19 Turkish isolates tested, the majority (73.7 %) were classified as intermediately resistant to mefenoxam and the rest were sensitive (Table 1). Unlike the Czech and Israel strains, no isolate from Turkey was resistant. Most isolates from Czech were either resistant (46.43 %) or intermediate (10.71 %) to mefenoxam (Table 1). No obvious relationship was observed between mefenoxam response and geographical origin of the isolates. Finally, a significant genetic structure was determined with AMOVA among geographic origins with haplotypes obtained from multilocus genotypes analysis. When the 78 isolates of *P. cubensis* were sorted into three geographic origins (Israel, Turkey and Czech) AMOVA and the associated fixation index (F_{ST}) indicated that 49.36 % of the variance was attributable to origin of sampling ($P<0.001$, Table 5). Similarly, 18.43 % and 25.66 % of the variability was explained by the host of origin and pathotype, respectively (Table 5).

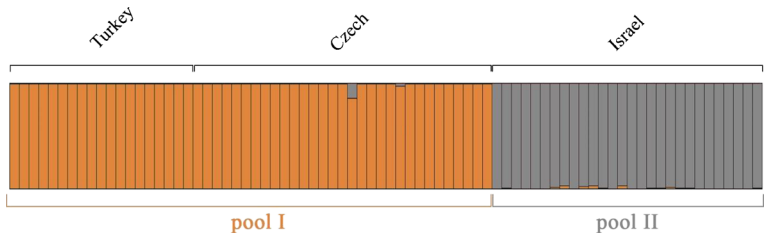

Fig. 2 Hierarchical organization of genetic relatedness of 78 strains based on ISSR and SRAP markers analysed by STRUCTURE software as described in "Materials and methods". Bar graphs were developed with the program DISTRUCT; each colour represents one genetic pool and the length of the coloured segment shows the estimated membership proportion of each sample to designed group

Discussion

In the present study we looked at the genetic diversity of 78 field isolates of *P. cubensis* from three countries, Turkey, Israel and the Czech Republic. We used 43 genetic markers (ISSR and SRAP) that were selected for polymorphism. These types of molecular markers combine simplicity, reliability and are extensively used in genetic diversity analysis. The results showed that the strains sampled in Israel are highly variable and strongly distinct from the other groups, suggesting on possible extensive sexual recombinations, migration, or both. On the other hand, the analysis of our data and suggested marker system have alleviated the difficulty of finding polymorphic markers in *P. cubensis* and differences found between Czech vs. Israel isolates.

As known epidemics of downy mildew caused by *P. cubensis* have recently devastated cucumber (*Cucumis sativus*) crops in Europe (Lebeda and Cohen 2011; Lebeda et al. 2011) and the USA (Holmes et al. 2004). *P. cubensis* attacks many species and genera of *Cucurbitaceae*, showing enormous variability in virulence. Pathotypes and physiological races, and possibly formae speciales, were reported (Lebeda and Cohen 2011; Lebeda et al. 2006, 2013). However, the genetic basis for this variability is poorly known. Our markers can also be suggested to understand of the genetic variability on the level of pathotype and physiological races differentiation of *P. cubensis* in further studies.

Advances in genome technologies have recently provided the genome sequence of the most economically important plant-pathogenic oomycetes, including *Phytophthora* species (Lamour et al. 2012), and *Pseudoperonospora cubensis* (Savory et al. 2012a). The genome sequence of *P. cubensis* isolates MSU-1 have been characterized on cucumber for virulence and susceptibility (Lebeda 1999; Savory et al. 2012a). The first extensive expression profiling of *P. cubensis* MSU-1 on susceptible cucumber elucidated major changes in gene expression during the interaction in both the host and the pathogen (Adhikari et al. 2012; Savory et al. 2012b).

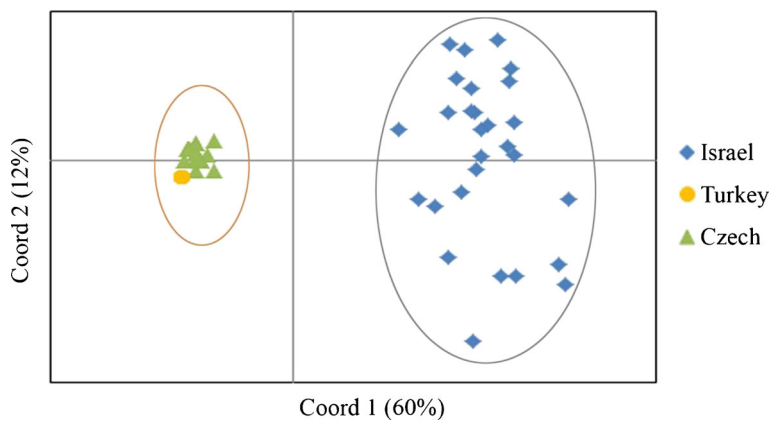

Fig. 3 Principal coordinate analysis of ISSR and SRAP diversity based on the presence/absence of alleles. The two ellipses indicate groups identified at $K=2$ in Fig. 2: orange ellipse=pool I; green ellipse=pool II. Colours and symbols correspond to the different sampling area of *P. cubensis* collection

Table 5 Analysis of molecular variance (AMOVA) of *Pseudoperonospora cubensis* collection based on multi-locus genotypes analysis

Source of variation[a]	Variance components	Variation (%)	Fixation index (Fst)	*P* value
Host				
Among all groups	0.0881	18.43	0.1843	0.0000
Within groups	0.3901	81.57	…	…
Geographical origin				
Among all groups	0.2513	49.36	0.4936	0.0000
Within groups	0.2578	50.64	…	…
Pathotype				
Among all groups	0.1199	25.66	0.2566	0.0000
Within groups	0.3473	74.34	…	…

[a] AMOVA considering originating host genus (Table 3), geographic origin of isolates (Israel, Turkey and Czech) and pathotypes (Table 3)

Quesada-Ocampo and co-workers (2012) investigated the genetic structure of 465 *P. cubensis* isolates from three continents, 13 countries, 19 states of the United States, and five host species using five nuclear and two mitochondrial loci. Bayesian clustering resolved six genetic clusters and suggested some population structure by geographic origin and host, because some clusters occurred more or less frequently in particular categories. All of the genetic clusters were present in the sampling from North America and Europe. Differences in cluster occurrence were observed by country and state. Isolates from cucumber had different cluster composition and lower genetic diversity than isolates from other cucurbit. Nevertheless, they identified relatively similar genetic profile of isolates from the Czech Republic and Turkey (brown colour on Fig 1 in Quesada-Ocampo et al. 2012) with slightly different cluster composition of Israelian isolates. A similar pattern is also evident from our data, where all 19 isolates from nine locations in Turkey sampled in 2010 exhibits genetic background close to the Czech isolates, contrary to the Israelian isolates. It implies on the joint origin of isolates in Turkey and the Czech Republic as well as joint putative dispersal route of genetic material from maternal Asian population to the crop environment in Central Europe.

Current molecular information suggested that population structure of this organism has been recently changed in Europe and subsequently in the USA, probably due to migration from the Far East (Runge et al. 2011). A major change in virulence has already been reported 10 year ago from Italy (Cappelli et al. 2003) and Israel (Cohen et al. 2003). In Italy, squash became a new host of *P. cubensis* and in Israel, *Cucurbita* species that have never been attacked, became susceptible. More recently, similar changes in virulence of *P. cubensis* populations were also described in isolates from the Czech Republic (Lebeda et al. 2010, 2012, 2013; Pavelkova et al. 2011). Our results obtained using SRAP and ISSR markers confirmed the differentiation and pathotype formation of *P. cubensis* in Israel compared to strain from Czech Republic and Turkey. Similarly, when compared genetic variability indices between larger sets of isolates sampled within single year in Israel and Turkey, we were able clearly find differences in the percentage of polymorphic loci (Israel= 88.2 %, Turkey=0 %) and gene diversity *h* (Israel= 0.319, Turkey=0). It implies higher possibility of frequent sexual reproduction of *P. cubensis* on the territory of Israel, or alternatively another explanation can be seen in possible influence of frequent mefonoxam spray which may trigger and/or promote the genetic differentiation.

Furthermore, in 2010, a new mating type, A2, appeared for the first time in Israel (Cohen and Rubin 2012), mainly on *Cucurbita* species (Cohen et al. 2013c) and in 2013 this mating type was reported for the first time in the USA (Thomas et al. 2013). A special survey revealed that the A2 mating type occurs in China (Cohen et al. 2013a) and Vietnam (Cohen and Rubin unpublished data) but not in Western Russia (Cohen and Rubin unpublished data). Data from China show that oospores of *P. cubensis* formed in the field in late season serve as initial inoculum in the spring (Zhang et al. 2012). The occurrence of both A1 and A2 mating types, which allow the formation of oospores, may explain not

only the over-seasoning of the pathogen but also the large diversity in its population structure.

Migration of *P. cubensis* may take place via air-borne sporangia which can travel to long distances (for example along the east coast of the USA (Palti and Cohen 1980)), via plants debris that contain oospores or via infected seeds (Cohen et al. 2013b). Continental migration was shown to influence spatio-temporal virulence variability in Europe, as well as genetic variation in the Czech populations of *P. cubensis* (Lebeda et al. 2013). As mentioned above, Czech and Turkey strains represent similar genetic pool (Fig. 2), and due to prevailing wind action, there is high probability that the inoculum is coming from identical maternal source in Asia. Little information is available on the genetic diversity in natural populations of *P. cubensis*. Amplified Fragment Length Polymorphisms (AFLP) and the nucleotide sequence of the ITS1-5.8S-ITS2 subunit of ribosomal DNA (rDNA ITS) have been used to study the genetic diversity in *Phytophthora infestans* (Cooke and Lees 2004) and taxonomy and phylogeny of downy mildew pathogens (e.g., Voglmayr 2008), including *P. cubensis* isolates from two geographically distant areas (Sarris et al. 2009). In all studies, isolates of *P. cubensis* originated from cucumber (*Cucumis sativus*). AFLP fingerprinting produced ample polymorphisms and isolates were grouped into two separate clusters; one included the Czech (Central Europe) and West European (the Netherlands, France) isolates, and the other included the isolates from Crete (Sarris et al. 2009). Significant differences were found between these two large geographical regions. Within each group some variations found were attributed to geographic origin, host cultivar, virulence and fungicide resistance. rDNA ITS analysis showed no variability among isolates in ITS1; however, all ITS2 rDNA sequences of Crete and Czech isolates clustered together with isolates from Austria, forming a large cluster together with *P. humuli*, indicating on their close taxonomic relationship (Sarris et al. 2009).

Recently, Runge et al. (2011) suggested that the 2004 severe epidemics of cucurbit downy mildew in Europe and USA resulted from the migration of Clade 2 isolates from East Asia to Europe and the USA, where clade 1 isolates have prevailed. Similarly, our suggested SRAP and ISSR markers were highly efficient and informative tool to understand possible pathotype formation, genetic structure on *P. cubensis* as studies carried out by AFLP and rDNA .

In this paper we show that also ISSR and SRAP markers can efficiently be used for discrimination of the genetic diversity between and among isolates of *P. cubensis*. This molecular genotyping is a powerful tool to follow the sexual recombinations between different isolates and tracking the changes in the population structure of the pathogen in different countries. Yet, highly specific molecular markers are required to better discriminate between pathotypes, mating types and fungicide-resistant isolates of *P. cubensis.*

Acknowledgments The research of IP and OB, FM, YC, FC was supported by Republic of Turkey Ministry of Food, Agriculture and Livestock Projects (TAGEM-BS-10/10-11/02-09). MK and AL were supported by Palacký University funds (IGA PrF-2014-001), by MSM 6198959215 (Ministry of Education, Youth and Sports of the Czech Republic) and QH 71229 (Czech Ministry of Agriculture).

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