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Purple coneflower with reddening and phyllody: a new host of clover phyllody phytoplasma

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Abstract *Echinacea purpurea* plants showing yellowing/reddening and phyllody symptoms were observed in southern Bohemia. Transmission electron microscopy observations showed phytoplasmas in sieve cells of symptomatic plants, but not in healthy ones. Direct and nested polymerase chain reaction with phytoplasma specific primers and subsequent restriction fragment length polymorphism analyses of 16S rDNA as well as sequence analyses of the 16S–23S ribosomal operon (1789 bp) enabled the classification of the detected phytoplasmas in the aster yellows group, ribosomal subgroup 16SrI-C. Identical analyses of the transcription factor *tuf* gene and the ribosomal protein *rpl22*, *rps3* genes were used for further classification, and revealed the affiliations of the phytoplasmas to the *tuf*IC and *rp*IC subgroups, respectively. The presence of clover phyllody phytoplasmas in purple coneflowers is a new record for this species.

Keywords *Echinacea purpurea* · Electron microscopy · PCR/RFLP · Phytoplasma characterization · Sequencing

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Purple coneflower (*Echinacea purpurea* syn. *Rudbeckia purpurea*), a member of the *Asteraceae* family, is an important medicinal plant used for the prevention and treatment of colds, flu, and respiratory ailments, to increase resistance to infections and to enhance the immune system. *Echinacea purpurea* is also widely planted as an ornamental plant in gardens and grown commercially for cut flowers. In September 2000, *E. purpurea* plants with flower abnormalities, purplish reddening of basal leaves, stunted growth and thickened and brittle stems were collected in a residential garden in Hluboká nad Vltavou. Severely infected plants neither produced seeds nor survived through to the next winter.

Plant cells were completely filled with spherical, ovoid or pleomorphic particles ranging in size from 50×50 to 460×680 nm diam (average 500×430 nm) and were observed in cross-sections of mature and immature phloem sieve tube elements of affected plants under Jeol 1010 transmission electron microscopy (Fig. 1). These particles were always found in diseased plants, but not in asymptomatic ones. No other microorganisms such as bacteria, viruses or virus-induced structures were noted.

Phloem tissue from stems, leaf midribs and flowers of the seven diseased *E. purpurea* plants and of reference phytoplasma strains [aster yellows (AY, ribosomal subgroup 16SrI-B), strawberry green petal (SGP, ribosomal subgroup 16SrI-C) and stolbur (STOL, ribosomal subgroup 16SrXII-A)] were sub-

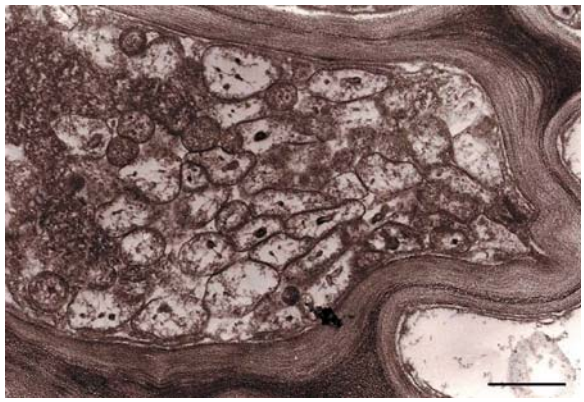

Fig. 1 Phytoplasma-like particles in a flower stalk phloem cell of diseased *E. purpurea*. Bar = 500 nm

jected to chloroform/phenol DNA extraction followed by isopropanol precipitation. The primer pair P1/P7 was used to prime the amplification of a 1.8 kbp product of the 16S ribosomal RNA (rRNA) gene, the spacer region between the 16S and 23S rRNA genes and the start of the 23S rRNA gene region of the phytoplasma genome. The PCR products were diluted 1:29 with sterile distilled water prior to nested amplification, using the general and group-specific primer pairs R16F2n/R2 and R16(I)F1/R1, respectively. When the primer pair P1/P7 was used in direct PCR assays, visible amplification products of about 1.8 kbp were obtained from two of the seven examined diseased purple coneflower plants. However, in nested PCR with R16F2n/R2 and R16F1(I)/R1(I) primers, products of the expected sizes of 1.2 and 1.1 kb, respectively, were obtained in all seven symptomatic plants.

Corresponding DNA fragments were also amplified from positive controls, but not from negative controls. About 100 ng of DNA from each R16F2n/R2 amplicon was separately digested with 2.5 U of *Alu*I, *Hae*III, *Hha*I, *Hpa*I, *Kpn*I, *Mse*I, and *Rsa*I restriction enzymes for ≥16 h according to the manufacturer's instructions (NE Biolabs, Beverly, MA, USA). Products obtained with R16(I)F1/R1 primers were digested with the *Tru*I restriction enzyme. All seven individually analysed RFLP samples from purple coneflowers showed the same restriction profiles with each enzyme, indicating the presence of phytoplasma identical to the SGP phytoplasma control (Fig. 2 and data not shown, for comparison see Lee et al. 1998; Bertaccini et al. 2005).

Purple coneflower samples and controls were screened using Tuf1f/r primers by direct PCR, followed by fTufu/rTufu and fTufAy/rTufAy in nested PCR to amplify the portion of the *tuf* gene encoding the elongation factor EF-Tu. RFLP analysis of the fTufu/rTufu and fTufAy/rTufAy amplicons was then performed with the *Tru*I, *Tas*I, *Alu*I, and *Mse*I; and *Hpa*II, Sau3AI and *Tru*I restriction enzymes, respectively (NE Biolabs, Beverly, MA, USA). In direct PCR assays with the Tuf1f/Tuf1r primer pair, expected length amplicons of 1080 bp were obtained from the same two of seven symptomatic plants as in the P1/P7 amplification. Two bands of unexpected

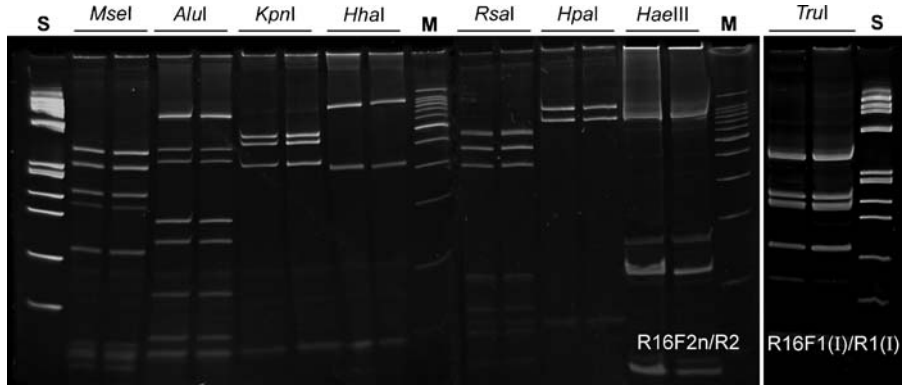

Fig. 2 RFLP analysis of 16S rDNA amplified by nested PCR primed with the oligonucleotide pairs R16F2n/R2 and R16F1(I)/R1(I) from phytoplasmas infecting two naturally diseased plants of *E. purpurea*. First round PCR was amplified by the P1/P7 primers. DNA products from the second, R16F2n/R2 nested PCR were digested with the restriction endonucleases *Mse*I, *Alu*I, *Kpn*I, *Hha*I, *Rsa*I, *Hpa*I and *Hae*III. The products of R16F1(I)/R1(I) nested PCR were digested with *Tru*I and separated on a 10% polyacrylamide gel. Marker S is a ϕX174/*Hae*III digest, with fragment sizes in base pairs from top to bottom of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72, and marker M is size standard with fragment sizes in base pairs from top to bottom of 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 and 50

size were amplified from the other five samples from symptomatic plants. Both nested PCRs with the TufAyf/TufAyr and fTufu/rTufu primer pairs were fairly sensitive, and resulted in fragments of the expected lengths of 946 bp and 841 bp, respectively, in all seven symptomatic plants but not in asymptomatic controls. The restriction patterns were indistinguishable from the SGP phytoplasma reference strain (Fig. 3).

A nested PCR using the primer pair rpF1/rpR1 followed by rp(I)F1A/rp(I)R1A was used to amplify a phytoplasma DNA segment of the ribosomal protein operon that encompasses the genes *rpl22* and *rps3*. The products of both amplification were digested with the *Mse*I, *Tas*I, and *Alu*I restriction enzymes (NE Biolabs, Beverly, MA, USA). Direct amplification of the *rp* genes resulted in the expected 1239 bp product in two purple coneflower plants, which probably had the highest concentrations of the pathogen as well as in the phytoplasma controls SGP and AY. Subsequent nested PCR revealed a specific amplification product of 1212 bp DNA in all symptomatic plants, SGP and AY. The restriction patterns of the products from purple coneflower phytoplasmas were in agreement with SGP (Fig. 4).

DNA amplified from one *E. purpurea* plant was used for sequencing. A set of overlapping PCR products from the infected plant was generated by amplification with the primers P1/U3 (position 6–1230), R16F2n/R16R2 (position 152–1397) and 16R758/P7 (position 758–1818). PCR amplicons of *rp* genes (primers rpF1/rpR1, position 12–1651) and the *tuf* gene (primers fTuf1/rTuf1, position 57–1094) were sequenced from both directions using a BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK). Sequencing was performed in an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequences were compared with sequences available in GenBank using the World Wide Web service BLASTN 2.2.16 (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The nucleotide sequences of the 16S rRNA were aligned for phylogenetic analysis. A phylogenetic tree was constructed using the neighbour-joining method in MEGA version 3.1.

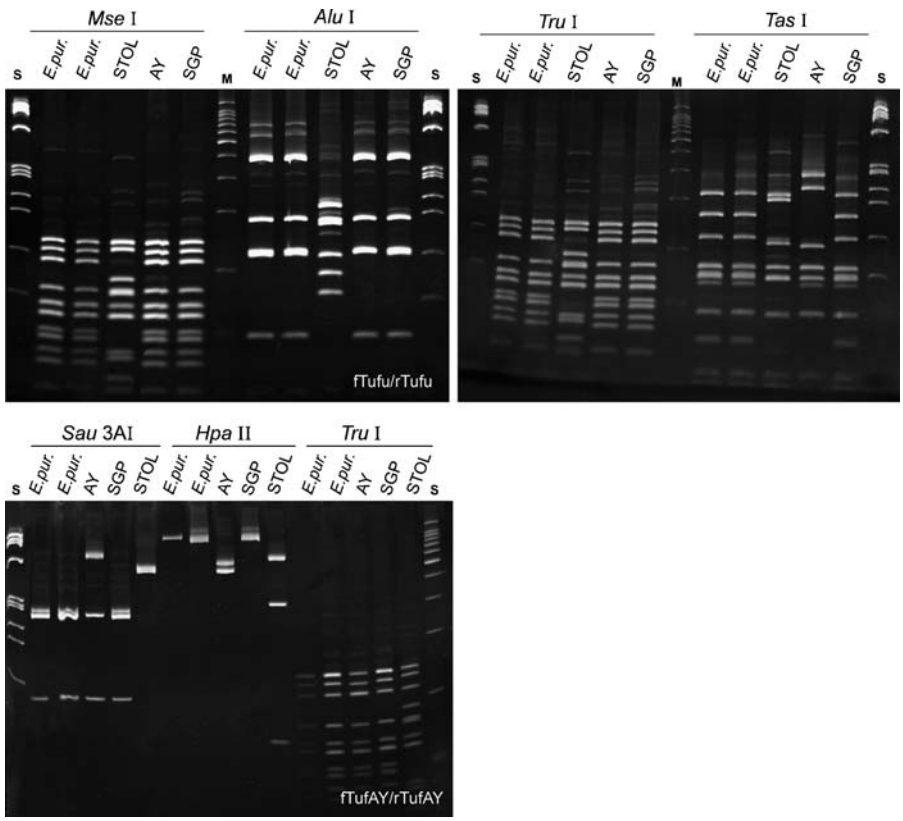

Fig. 3 Polyacrylamide gels showing the RFLP patterns of two *E. purpurea* samples and the control strains AY (aster yellows, 16SrI-B subgroup), SGP (strawberry green petals, 16SrI-C subgroup) and STOL (stolbur, 16SrXII-A subgroup), amplified with the primers fTufu/rTufu or fTufAY/rTufAy and digested with the enzyme listed. See Fig. 2 for markers

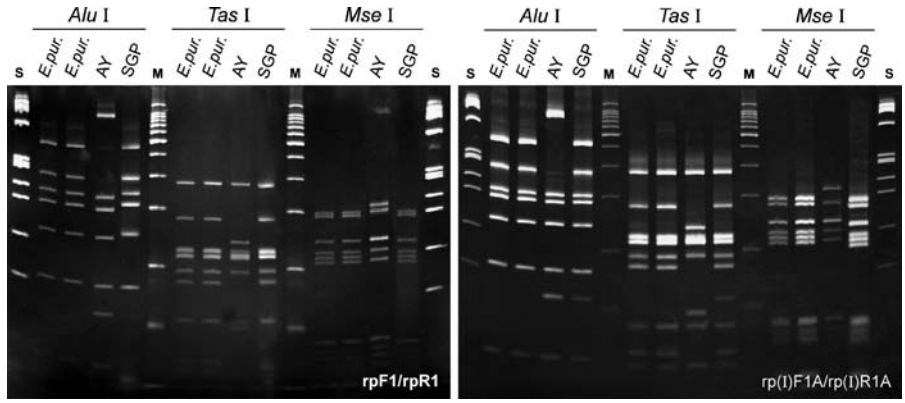

Fig. 4 RFLP profiles of ribosomal protein operon sequences amplified by direct PCR with the primer pair rpF1/rpR1, followed by nested PCR with the primer pair rp(I)F1A/rp(I)R1A from two samples of *E. purpurea* phytoplasma, and the control strain AY and SGP phytoplasmas. The PCR products were digested with the listed restriction enzymes. Lanes S and M contain size markers as described in the legend to Fig. 2. For phytoplasma reference strains, see the legend to Fig. 3

The 1789 bp region of the phytoplasma genome from the phytoplasma infecting purple coneflower in the Czech Republic containing a partial sequence of the 16S ribosomal RNA gene, the spacer region between the 16S and 23S rRNA genes, and the start of the 23S rRNA gene region, was deposited in the GenBank database under accession no. (AC) EF546778 (Czech *Echinacea purpurea* phyllody phytoplasma). A detailed comparison of the trimmed (1234 bp) 16S rRNA sequence revealed its identity as that of strawberry phylloid fruit (StrawbPhF) phytoplasma (AC: AY102275, 1246 bp available), originating in North America (Jomantiene et al. 2002), and *Ribes rubrum* phytoplasma (AC: AY669063, Czech Republic). One base difference was found between the *E. purpurea* phytoplasma and both the as yet unclassified *Cirsium* yellows phytoplasma (AC: AF200431) reported in *Cirsium arvense* in Lithuania and the clover phyllody phytoplasma, operon A (AC: AF222065). These sequences form a cluster of closely related phytoplasmas, but their separation from other branches of related 16SrI-C phytoplasmas is not statistically significant (Fig. 5). The sequence of the *rp* gene operon (1053 bp, encompassing the *rpl22* complete and *rps3* partial genes) was deposited in the GenBank database under AC: EF546779. Sequence comparison revealed a 100% identity of the *E. purpurea* phytoplasma with sequences of the ribosomal proteins of the CPh phytoplasma strain CPh (AC: AY264862) originating from *Trifolium sativum* in Canada, and strains KVG and KVE (AC: AY264860, AY264861), both of which were isolated from *T. repens* in Germany. The partial sequence (832 bp) of the *tuf* gene was deposited in the GenBank database under AC: EF551060. Sequence comparison revealed identity with the sequence of the non-culturable plant pathogenic bacterial species, clone KV (AC: L46369) and wheat blue dwarf phytoplasma (AC: DQ507200). One base difference was found out of 832 bp compared between the sequence from *E. purpurea* phytoplasma and aster yellows phytoplasma, strains KVM and KVF (AC: AJ271318, AJ271317) from white clovers growing in France.

A similar disease of *E. purpurea* was described about 10 years ago in Alberta (Canada) and Wisconsin (USA) (Hwang et al. 1997; Stanosz et al. 1997). The phytoplasma detected in the American plants was, however, classified on the basis of RFLP to the 16S rRNA group I, subgroup A (strain cluster I of AY) (Stanosz et al. 1997; Khadhair et al. 1997). Sequence comparison was not possible as the ribosomal and *tuf* genes from the American specimens have not yet been sequenced.

The phytoplasma detected in the Czech *E. purpurea* was identified as belonging to the aster yellows group, 16SrI-C ribosomal subgroup. This classification based on the 16S rRNA sequence was further supported by sequencing and RFLP analysis of the *tuf* and *rpl22*, *rps3* genes, which revealed the affiliation with the *tuf*IC and *rp*IC subgroups, respectively (Lee et al. 1998, 2004; Schneider et al. 1997).

Although our analysis of the 16S rRNA sequence demonstrated identity between the *E. purpurea* and

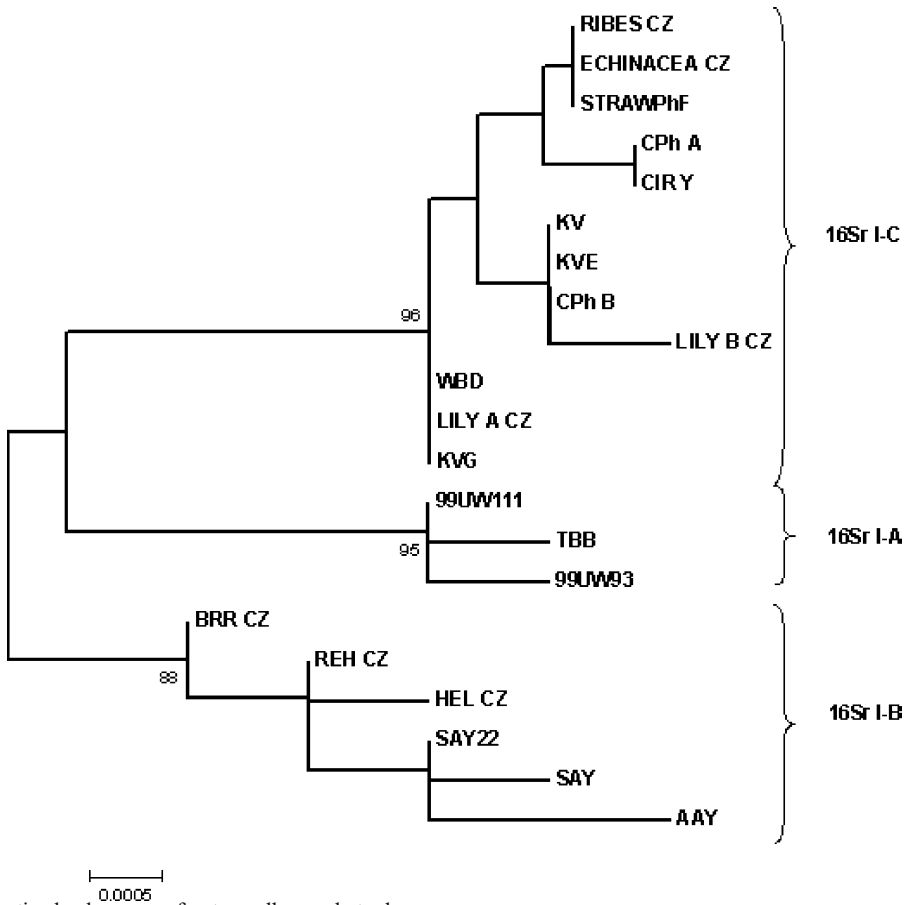

Fig. 5 Phylogenetic dendrogram of aster yellows phytoplasma isolates. The analysis was carried out using the neighbour-joining tree option of ClustalW. The scale bar represents a phylogenetic distance of 0.5%. The numbers on the branches are bootstrap values. Phytoplasmas and GenBank accession numbers are as follows: RIBES CZ: '*Ribes rubrum*'phytoplasma (AY669063), ECHINACEA CZ: Czech '*Echinace purpurea*' phyllody phytoplasma (EF546778), STRAWPhF: Strawberry phylloid fruit phytoplasma (AY102275), CPhA: Clover phyllody phytoplasma operon A (AF222065), CIR Y: Cirsium yellows phytoplasma (AF200431), KV: Mollicutes 16S rRNA gene strain KV (X83870), KVE: Clover phyllody phytoplasma strain KVE (AY265217), CPh B: Clover phyllody phytoplasma operon B (AF222066), LILY B CZ: Lily fasciation phytoplasma operon B (AY953423), WBD: Aster yellows phytoplasma strain wheat blue dwarf (DQ078304), LILY A CZ: Lily fasciation phytoplasma operon A (AY839617), KVG: Clover phyllody phytoplasma strain KVG (AY265218), 99U111: Aster yellows phytoplasma A (AF268408), TBB: Tomato big bud (L33760), 99UW93: Aster yellows phytoplasma A (AF268406), BRR CZ: '*Brassica napus* var. *arvensis*' phytoplasma (U89378), REH CZ: '*Rehmania glutinosa* var. *purpurea*' phytoplasma (AF335107), HEL CZ: '*Helichrysum bracteatum*' phytoplasma (AF515771), SAY22: Aster yellows phytoplasma strain AY1 (AF222063), SAY: Severe strain of western American aster yellows (M86340), AAY: American aster yellows (X68373)

StrawbPhF phytoplasmas, the latter was classified in a new ribosomal subgroup, 16SrI-R, based solely on RFLP differences (Jomantiene et al. 2002). We suggest that this classification is incorrect and is a result of the amplification of clover phyllody rRNA operon A present in the phytoplasma genome. Clover phyllody operons A and B differ only in their restriction sites, resulting in the occurrence of different sized bands after cutting with *Mse*I, *Alu*I and *Hae*III endonucleases. Two or three substitutions between the A and B 16S rRNA operons seem to be common, and were found, e.g., in clover phyllody phytoplasma (Bertaccini et al. 2005) and the onion yellows phytoplasma (Namba et al. 1993). These operon differences could contribute to intra-subgroup sequence variability (see Fig. 5 for classification of operons CPhA/CPhB and LilyA/B), but are statistically insignificant for phytoplasma classification.

Tuf gene RFLP comparison was included in the analysis, since it was previously shown to have more

genetic variability suitable for phytoplasma molecular characterization (Schneider et al. 1997). For instance, *Tas*I and *Hpa*II could discriminate the reference AY from SGP and *E. purpurea* phytoplasmas. Digestion with *Sau*3AI was also identical to the reference strain SGP, and in good agreement with the results of Schneider et al. (1997). Similar results revealed RFLP studies of rp operon sequences amplified by direct PCR and subsequent nested PCR. In both cases, digestion with the *Alu*I enzyme resulted in a pattern identical to the SGP control and in agreement with results reported for other members of the *rp*IC subgroup (Lee et al. 1998, 2004). However, digestion with *Tas*I and *Mse*I showed slight differences in comparison with patterns demonstrated in the literature mentioned above. Amplicons primed with rpF1/rpR1 and digested with *Tas*I revealed only two bands of about 120 bp in contrast with the results of Lee et al. (1998, 2004), where three bands were demonstrated for CPh, SGP and ranunculus phyllody. Digestion with the *Mse*I enzyme is also very similar but not identical to the recently reported profile of phytoplasmas belonging to the *rp*IC subgroup. There was a slight difference in the distribution of the four bands between 120–150 bp.

The role of these genes for finer phytoplasma characterization and/or RFLP classification is a matter of future research after collection of a larger set of data.

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