# Background

- *Genus Candidatus Phytoplasma* comprises wall-less bacteria that are associated to diseases in more than 1,000 species worldwide, including many important crops and forest trees.
- Alder yellows (Ayd) phytoplasma (16SrV-C) infects Alnus spp. (alder) and is related to economically important phytoplasma causing Flavescence dorée (FD) (16SrV-C, -D) in grapevines.
- 20% of infected trees exhibit symptoms such as yellowing, die-back of branches, reduced foliage, small leaves or decline (Fig. 1), while 80% remain symptomless (Fig. 1).

> How prevalent is AldY phytoplasma infection rate in asymptomatic black alder in Spreewald?

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## Materials and Methods

- Random collection of fifty-seven A. glutinosa tree leaf samples.
- DNA extraction by CTAB protocol of pooled leaf samples from trees without symptoms associated to Ayd.
- Diagnostic direct & nested PCR for amplification of partial 16S rRNA by applying primer pairs P1/P7 and P1A/P7A.
- Restriction fragment length polymorphism (RFLP) analysis on 16S rRNA using P1/P7A with 7% gel for detection and P1A (16SrV-C and -A) endonucleases.
- Direct & nested PCR for amplification of non-ribosomal marker genes secY (prereproton translocase membrane subunit) and map (methionine aminopeptidase) for more detailed differentiation of FD strains.
- Analyses of selected 16S rDNA and map sequences followed by phylogenetic tree construction.

## Results

- Amplification of 16S rDNA followed by RFLP (Fig. 2a,b) and sequence analysis of 16S rDNA (Fig. 3) allowed for detection of Ayd phytoplasmas in all fifty-seven examined trees.
- Assignment to taxonomic group, 16SrV-C, -D, apart from ‘Ca. P. ulmi’ (16SrV-A) (Fig. 2a).
- Delineation between Ayd and FD strains was not possible based on 16S rDNA (Fig. 3).
- Analyses on map genes (Fig. 4) revealed finer strain differentiation and the existence of new Ayd phytoplasma groups.
- Population variations displaying mixed infections were detected in Ayd strains (data not shown).
- Strains were assigned to phylogentic map-clusters closely related to Palatinate grapevine yellows (PGY), Ayd or FD strains (Fig. 4).
- Common monophyletic origin for FD, Ayd and PGY phytoplasmas (Fig. 4) was indicated in accordance with other studies.

## Detection, Taxonomy and Genetic Variability of Alder Yellows Phytoplasma in Black Alder in Spreewald Habitat

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## Figures

**Figure 1.** Dieback and yellowing of Alnus glutinosa (black alder) caused by the Alder yellows phytoplasma (Ayd). Shown are mild and severe symptoms (adapted from Marcone et al., 2014).

**Figure 2.** Polyacrylamide gels showing the restriction fragment length polymorphism patterns, obtained with a) Tfil endonuclease (8% gel), B) Taq endonuclease (7% gel) of 16S rDNA and spacer region amplified with the P1A/P7A primer pair. The image consists of multiple gel images. Obtained restriction patterns are identical among all samples from Spreewald and three selected strains are shown. Strain abbreviations: Alder yellows phytoplasma strains from A. glutinosa, SW1–3 (Spreewald, Germany) and DOS1–3 (Dresden, Germany) and PI3 (Pirita, Serbia). ‘Ca. Phytoplasma ulmi’ (16SrV-A) from clovis sp., DEB (Debeljaca, Serbia); marker M (GeneRuler 50 bp DNA ladder), M (b 1374, HaellI digested).

**Figure 3.** Phylogenetic relationships of eight selected Ayd phytoplasma 16S rDNA sequences obtained from A. glutinosa (black alder) in Spreewald (SW). Two Ayd strains from Serbia (PI2; PI3, PI4) were used. Ayd strains from this study are in bold. Reference sequences are in italics. The tree was constructed in Mega 6.06 using the maximum likelihood method with 500 replicates. ‘Ca. Phytoplasma mai’ was used as the outgroup. Abbreviations: Ayd, Alder yellows; FD, Flavescence dorée.

**Figure 4.** Phylogenetic relationships of sixteen Ayd phytoplasma map sequences obtained from A. glutinosa (black alder) in Spreewald (SW). Three Flavescence dorée strains from Serbia (Aleksandrovac, central Serbia, ALI; Pruska Gora, northern Serbia, FD1 and FD2) were used. Ayd strains and FD strains from this study are in bold. Reference sequences were taken from GenBank. The tree was constructed in Mega 6.06 using the maximum likelihood method with 500 replicates and support values below 50% are hidden. ‘Candidatus Phytoplasma ulmi’ was used as the outgroup. Mixed infections were excluded from single variant analysis. Abbreviations: Ayd, Alder yellows; FD, Flavescence dorée; PGY, Palatinate grapevine yellows.

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**Table 1:** Phylogenetic trees showing the restriction fragment length polymorphism patterns, obtained with a) Tfil endonuclease (8% gel), B) Taq endonuclease (7% gel) of 16S rDNA and spacer region amplified with the P1A/P7A primer pair. The image consists of multiple gel images. Obtained restriction patterns are identical among all samples from Spreewald and three selected strains are shown. Strain abbreviations: Alder yellows phytoplasma strains from A. glutinosa, SW1–3 (Spreewald, Germany) and DOS1–3 (Dresden, Germany) and PI3 (Pirita, Serbia). ‘Ca. Phytoplasma ulmi’ (16SrV-A) from clovis sp., DEB (Debeljaca, Serbia); marker M (GeneRuler 50 bp DNA ladder), M (b 1374, HaellI digested).

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