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

Sabine Holz[1], Bojan Duduk[2], Carmen Büttner[1], and Michael Kube[1]

[1]Humboldt-Universität zu Berlin, Faculty of Life Sciences, Division Phytomedicine, Lentzeallee 55/57, 14195 Berlin, Germany;
[2]Institute of Pesticides and Environmental Protection, Banatska 31b, P.O. Box 163, 11080 Belgrade, Serbia

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 (AldY) phytoplasma (16SrV-C) frequently infects *Alnus* spp. (alder) and is related to economically important phytoplasma causing Flavescence dorée (FD) (16SrV-C, -D) in grapevine
- 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**)

Figure 1. Dieback and yellowing of *Alnus glutinosa* (black alder) caused by the Alder yellows phytoplasma (16SrV). Shown are mild and severe symptoms (adapted from Marcone et al., 2014).

- In Spreewald habitat, fifty-seven *Alnus glutinosa* (black alder) trees were examined for phytoplasma infection in summer 2013

➢ **How prevalent is AldY phytoplasmas infection rate in asymptomatic black alder in Spreewald?**

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 AldY
- Diagnostic direct & nested PCR for amplification of partial 16S rRNA by applying primer pairs P1/P7 and P1A/P7A
- Restriction fragment length polymorphism (RFLP) analyses on 16S rRNA using *Taq*I (16SrV-group detection) and *Bfa*I (differentiation of 16SrV-C and –A) endonucleases
- Direct & nested PCR for amplification of non-ribosomal marker genes *secY* (preprotein translocase membrane subunit) & *map* (methionine aminopeptidase) for more detailed differentiation of FD strains
- Analyses of selected 16S rRNA 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 AldY phytoplasmas in all fifty-seven examined trees
- Assignment to taxonomic group, 16SrV-C, apart from '*Ca.* P. ulmi' (16SrV-A) (**Fig. 2a**)
- Delineation between AldY and FD strains was not possible based on 16S rDNA (**Fig. 3**)
- Analyses on *map* gene (**Fig. 4**) revealed finer strain differentiation and diverse strains
- Population variations displaying mixed infections were detected in AldY strains (data not shown)
- Strains were assigned to phylogenetic map-clusters closely related to Palatinate grapevine yellows (PGY), AldY or FD strains (**Fig. 4**)
- Common monophyletic origin for FD, AldY and PGY phytoplasmas (**Fig. 4**) is indicated in accordance with other studies
- AldY phytoplasmas infection in black alder is prevalent

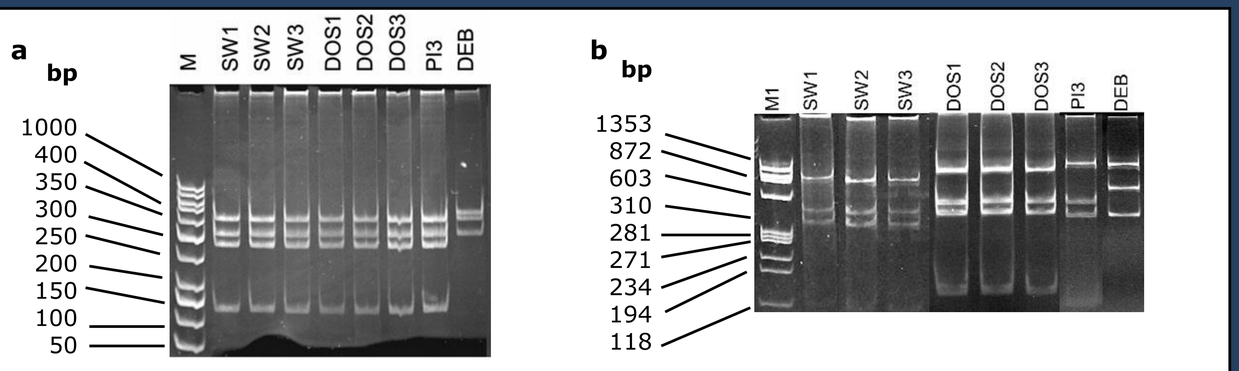

Figure 2. Polyacrylamide gels showing the restriction fragment length polymorphism patterns, obtained with **a**) *Bfa*I endonuclease (8% gel), **b**) *Taq*I endonuclease (7% gel) of 16S rDNA and spacer region amplified with the P1A/P7A primer pair. The image consists of multiple gel images. Obtained 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 (Dossenheim, Germany) and PI3 (Pirot, Serbia); '*Ca.* Phytoplasma ulmi' (16SrV-A) from *Ulmus* sp., DEB (Debeljaca, Serbia); marker, M (GeneRuler 50 bp DNA ladder), M1 (Φ X174, *Hae*III digested).

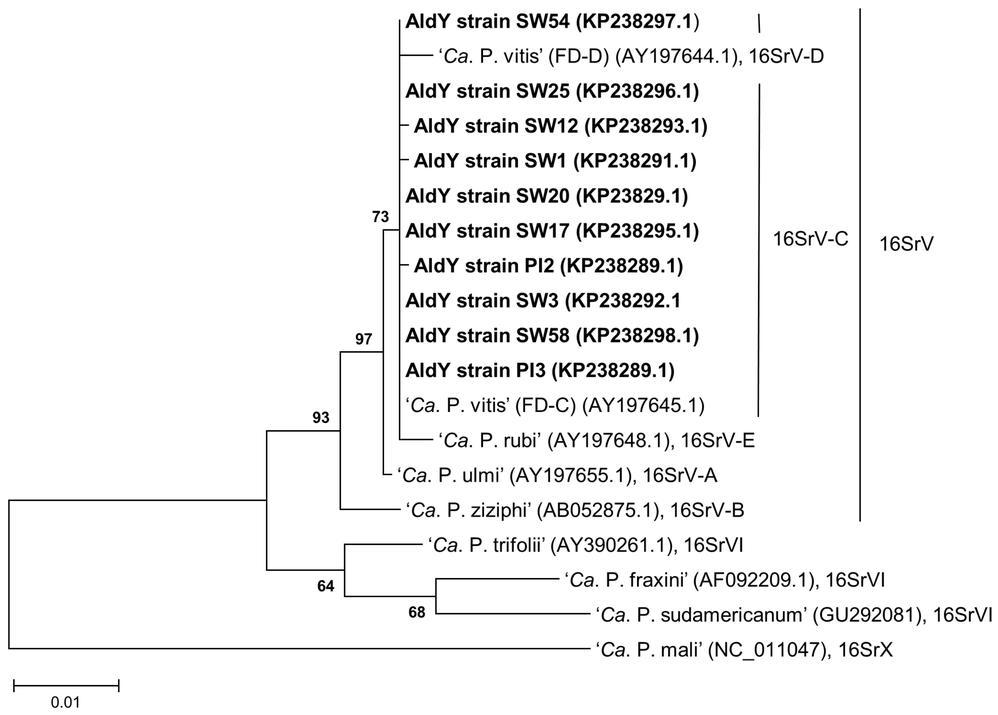

Figure 3. Phylogenetic relationships of eight selected AldY phytoplasma 16S rDNA sequences obtained from *A. glutinosa* (black alder) in Spreewald (SW). Two AldY strains from Serbia (Pirot; PI2, PI3) were used. AldY strains from this study are in bold. Reference sequences were taken from GenBank. The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

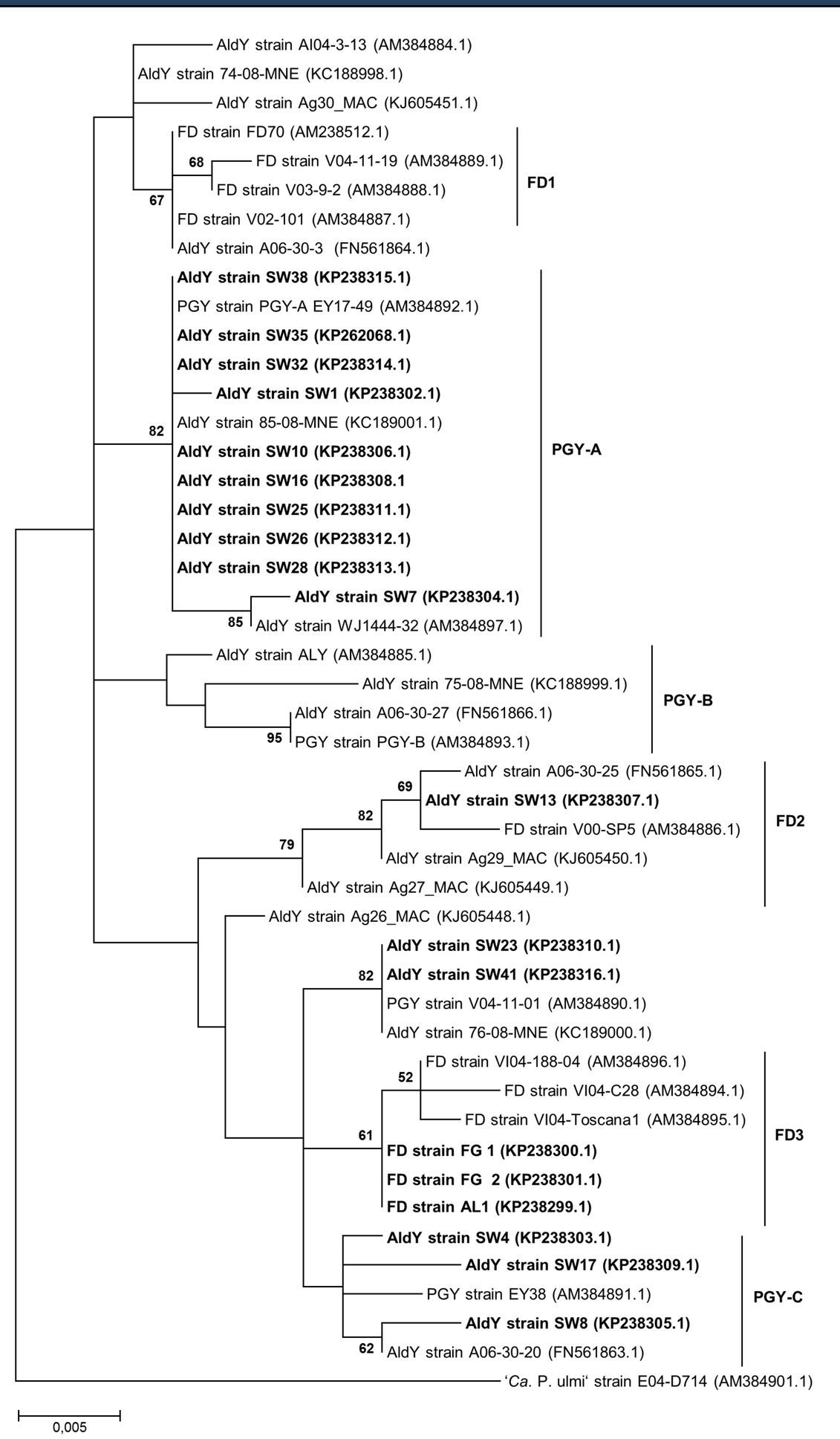

Figure 4. Phylogenetic relationships of sixteen AldY phytoplasma *map* sequences obtained from *A. glutinosa* (black alder) in Spreewald (SW). Three Flavescence dorée strains from Serbia (Aleksandrovac, central Serbia, AL1; Fruska Gora, northern Serbia, FG1 and FG2) were used. AldY strains and FD strains 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: AldY, Alder yellows; FD, Flavescence dorée; PGY, Palatinate grapevine yellows.

SUMMARY

- ✓ **Amplification of 16S rDNA followed by RFLP and sequence analysis allowed for detection of AldY phytoplasmas in all fifty-seven examined trees**
- ✓ **Taxonomic assignment to 16SrV-C**
- ✓ **Analyses on *map* gene revealed diverse strains as well as mixed infections with closely related AldY strains**
- ✓ **Strains were assigned to phylogenetic map-clusters closely related to Palatinate grapevine yellows (PGY), AldY or FD strains**
- ✓ **AldY phytoplasma infection in black alder is prevalent**
- ✓ **Results also indicate presence of an established phytoplasma population in symptomless, chronically infected black alder**

SELECTED REFERENCES

Ahrens, U.; Seemüller, E. (1992) Phytopathology 82, 828–832
Arnaud, G.; Malembic-Maher, S.; Salar, P.; Bonnet, P.; Maixner, M.; Marcone, et al. (2007) Appl Environ Microbiol 73, 4001–4010
Deng, S.; Hiruki, C. (1991) J Microbiol Methods 14, 53–61
Lee, I. M.; Gundersen-Rindal, D. E.; Davis, R. E.; Bartoszyk, I. M. (1998) Int J Syst Bacteriol 48, 1153–1169
Lee, I.-M.; Martini, M.; Marcone, C.; Zhu, S. F. (2004) Int J Syst Evol Microbiol 54, 337–347
Marcone, C (2014) Ann Appl Biol 165, 199-221
Martini, M.; Murari, E.; Mori, N.; Bertaccini, A. (1999) Plant Dis 83, 925–930
Schneider, B.; Seemüller, E.; Smart, C. D.; Kirkpatrick, B. C. (1995) Molecular and diagnostic procedures in mycoplasmology. Academic Press, pp. 1–79.
THIS STUDY: Holz, S., Duduk, B., Büttner, C., Kube, M. (2015) Forest Pathol. doi: 10.1111/efp.12206

ACKNOWLEDGEMENTS

This research was supported by grants KU 2679/2-1 and BU 890/21-1 from Deutsche Forschungsgemeinschaft (DFG), A-2011-77 from the Einstein Foundation, COST Action FP1401 and project 56266384 of the German Academic Exchange Service (DAAD).