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The use of molecular assays to identify plant pathogenic organisms vectored by biological control agents

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Abstract. The planthopper *Prokelisia marginata* Van Duzee (Homoptera: Delphacidae) has been considered for the biological control of the weed *Spartina alterniflora* Loisel (Poaceae) in Willapa Bay, Washington, U.S.A. *Prokelisia marginata* is a stenophagous phloem-feeding insect with the potential to transmit bacterial plant diseases that could be moved by less-specific vectors to other plant species. Initial assays with PCR primers that are putatively specific for phytoplasmas gave positive results in *Spartina*. However, subsequent analyses did not indicate the transmission of the pathogen by the planthopper. We sequenced the 16S ribosomal RNA (rRNA) gene of the bacterial species that gave positive results in PCR. Comparisons with sequences available in GenBank suggested that the positive results using the putatively specific PCR primers were due to the presence of such bacteria as *Pseudomonas*, *Holomonas*, *Vibrio*, and *Acinetobacter*. We did not find phytoplasmas in either *Spartina* or the planthopper *P. marginata*.

Key words: biological control, ecological safety, Homoptera, indirect effects of introductions, invasive plant, pathogen, phytoplasma, *Prokelisia marginata*, *Spartina alterniflora*, vector

Introduction

Ecological safety is an important element of biological control (Thomas and Willis, 1998; Strong and Pemberton, 2000; Pemberton and Strong, 2000). Recent developments in molecular techniques now allow assessment of the infrequently considered consequence that plant diseases can accompany introductions of biological control insects. The introduction of phytophagous insects as control agents can be a source of introduced microbes due to the vectoring of novel plant diseases. Homoptera, which feed by means of stylet insertion into the plant vascular system, vector more than 90% of insect-transmitted diseases in plants (Eastop, 1977). Introduced Homoptera can

carry exotic, and potentially cryptic (Lee et al., 1998), diseases into a new region; they also can spread pathogens already present (see Bezark, 1999). The use of highly specific PCR assays that do not depend on obvious symptomology in plant hosts has great potential to assist in the appraisal of the risk of disease transmission by biological control insects.

Spartina alterniflora (smooth cordgrass) is a tall, dense grass that grows in the intertidal habitat of estuaries in extensive monocultures (Radford et al., 1968). It is native to the East and Gulf coasts of North America and has become a highly invasive weed in some Pacific estuaries. In San Francisco Bay, California and Willapa Bay, Washington, it has spread rapidly, invading mud flats that are largely unoccupied by vascular plants. The Washington Department of Natural Resources, the Washington Department of Agriculture, and both State and Federal Departments of Fish and Wildlife have attempted both chemical control with glyphosate and mechanical control by mowing. These expensive methods have been only modestly successful.

The phloem feeding planthopper *Prokelisia marginata* Van Duzee (Homoptera: Delphacidae) has been studied as a possible biological control agent for *Spartina alterniflora* at Willapa Bay, WA. This insect is native to the East and Gulf coasts of North America as well as to California, where it is a monophagous herbivore of the native California cordgrass, *Spartina foliosa* (Denno et al., 1987). In greenhouse experiments, *S. alterniflora* from Willapa Bay, Washington was shown to be susceptible to stunting and death when exposed to *P. marginata* from San Francisco Bay (Daehler and Strong, 1997).

Of possible relevance for the contemplated biological control of *Spartina alterniflora* by *Prokelisia marginata* in Willapa Bay, WA are nonculturable phloem-limited phytoplasmas. Phytoplasmas were previously known as mycoplasma-like organisms (MLO's) and phytopathogenic mollicutes (Purcell, 1982). Phytoplasmas inhabit plant phloem and, though infections are systemic, pathological effects are seen in plant parts where concentrations of bacteria are highest (Kuske and Kirkpatrick, 1992). The effects of phytoplasma diseases can be severe, including plant death, but not all plant species infected with and supporting phytoplasmas have disease symptoms. Some phloem-feeding leafhoppers, planthoppers and treehoppers acquire these pathogens by feeding upon plants, and plants acquire them only by being fed upon by infected insects (Purcell, 1982).

Preliminary work in the greenhouses at the Bodega Marine Laboratory (BML) suggested that phytoplasmas could be involved with pathology associated with *Spartina alterniflora* from Willapa Bay that was fed on by *Prokelisia marginata*. The symptoms of shortened internode length, reduced root biomass, narrower leaves, shorter leaves, and proportionally fewer large

vascular bundles that we observed (Daehler and Strong, 1997; personal observation) are reminiscent of those produced by phytoplasmas (Kirkpatrick, personal communication). Our assays with PCR primers that are used as diagnostic tools for phytoplasmas (Smart et al., 1996) indicated this bacterial pathogen could be associated with a disease in *Spartina*. Here we present the results of the screening for potential transmissible plant pathogens on a phloem-feeding planthopper, *P. marginata*. We illustrate that the molecular primers available for detecting these pathogens are not sufficiently specific to distinguish the pathogens from related, apparently benign microbes. We also underscore the impossibility of assessment of all risks; it is not feasible to consider every potential pathogen.

Materials and methods

PCR detection for phytoplasmas. We used two pairs of putatively pathogen specific molecular primers to screen for phytoplasmas. A CTAB (cetyltrimethylammonium bromide) method of extraction (Zhang et al., 1998) and amplification of diagnostic DNA method (Smart et al., 1996) were modified from the literature. Differences in extraction technique included: leaf samples were ground in liquid nitrogen, sterile materials were used for all stages of the extraction and pellets were dried in a laminar flow hood.

For PCR testing for phytoplasmas, each reaction had a volume of 30 μl. For each reaction, the following volumes were used: H_2O 17.35 μl; dNTPs 0.45 μl (1.5 mM); 10X $MgCl_2$-free PCR buffer (Promega, Madison, Wisconsin) 3 μl; $MgCl_2$ (Promega, Madison, WI) 3 μl (3 mM); primer P1 (Deng and Hiruki, 1991) 3 μl (5 μM); primer Tint (Smart et al., 1996) or P7 (Schneider et al., 1995); 3 μl (5 μM); Taq polymerase 0.2 μl (5U/μl) (Promega, Madison, WI); DNA 2 μl (approximately 12 ng genomic DNA). Polymerase chain reaction was done using a Perkin Elmer 9600 thermocycler (Norwalk, CT). Amplification cycles were: 95 °C for 5 min.; 35 cycles of 95 °C for 1 min.; 56 °C for 1 min.; 72 °C for 2 min.; 72 °C for 10 min.; 4 °C until samples are removed from PCR. 15 μl of each sample was run on a 1.5% agarose gel. PCR products were stained with ethidium bromide and visualized by UV transillumination. Samples were scored positive if there was a band of the diagnostic length 1.7 kb (P1/Tint) and negative if there was no band at 1.7 kb and a non-diagnostic 200 kb band was present (see Smart et al., 1996). For primer pair (P1/P7), a sample was scored positive if there was a band of the diagnostic length 1.8kb (see Schneider et al., 1995) (Figure 1).

Plant species screened for the presence of phytoplasmas (P1/Tint and P1/P7) are: S*partina alterniflora*, *S. foliosa*, *S. foliosa* × *alterniflora*, *S. anglica* Hubbard and a positive control of Elm Yellows extracted from *Cath-*

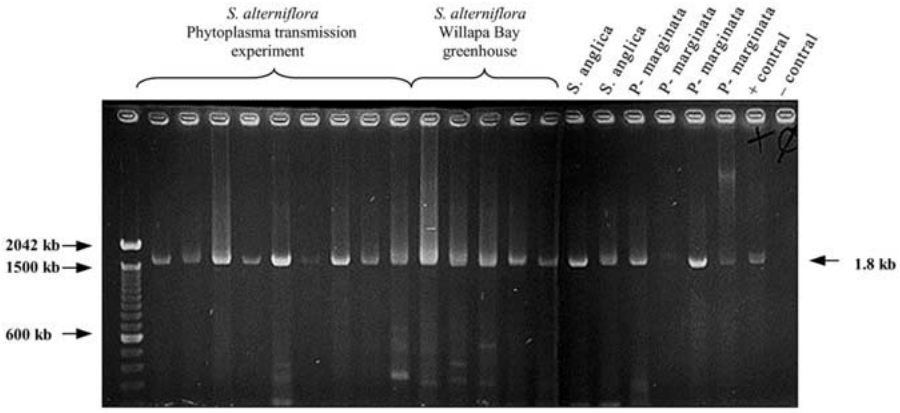

Figure 1. Phytoplasma amplification. Ethidium bromide-stained agarose gel of PCR amplification products using primer pair P1/P7. Diagnostic band is at 1.8 kb. Size standard at left is a 100 bp DNA Ladder (GibcoBRL, Rockville, MD). Species extracted are included above each lane.

aranthus roseum acquired from the Kirkpatrick Laboratory at U.C. Davis (Table 1). Multiple extractions were performed on some plant individuals. *Prokelisia marginata* screened included: from the BML greenhouse, caged on yellowed and stunted plants in the BML greenhouse and from the field (San Francisco Bay) (Table 2). 10 or 30 planthoppers were pooled for each extraction.

Phytoplasma transmission by Prokelisia marginata. The objectives for this experiment were: (1) test the effectiveness of *Prokelisia marginata* to transmit phytoplasmas to *Spartina alterniflora* seedlings by molecular assay, and (2) test for symptoms of disease on infected *S. alterniflora* seedlings.

Spartina alterniflora seed from Virginia (Environmental Concern Inc., St. Michaels, MD) were germinated and grown in an insect free greenhouse. One hundred plants from the Virginia seedlings were randomly selected and grown for three months until they had developed sufficient roots, shoots and leaf tissue to sustain planthopper growth. Prior to the experiment a set of 20 randomly selected plants from the same cohort tested negative for phytoplasma by the molecular assay using PCR primers P1/Tint. At the end of the three months, vigorous plants were sorted into five groups of twenty; within groups, 10 plants were designated as control and the other 10 as experimental.

One hundred and fifty greenhouse reared 3rd instar *Prokelisia marginata*, originally from the San Francisco Bay, were placed in plastic cages on 10 *Spartina alterniflora* plants from Willapa Bay, WA, and one *S. anglica* from Puget Sound. Each plant received about 14 planthoppers. Each plant had previously tested consistently positive for phytoplasmas by PCR using

Table 1. Plant species, with location of origin (original field collection site) and collection and exposure to *Prokelisia marginata*, with number of accessions screened for phytoplasmas and testing positive (primers P1/Tint and P1/P7)

Species	Location of Collection	Location of Origin	Exposed to *P. Marginata*	P1/Tint		P1/P7	
				Screened	Positive	Screened	Positive
S. alterniflora	BML gh	CB	no	4	0	4	0
S. alterniflora	BML gh	CB	yes	100	27	100	13
S. alterniflora	BML gh	CB	no	10	1	10	1
S. alterniflora	BML gh	WB	no	15	1	15	3
S. alterniflora	BML gh	WB	yes	75	12	75	6
S. anglica	BML gh	PS	yes	17	7	17	4
S. anglica	PS	PS	no	5	1	5	0
S. foliosa × *alterniflora*	SFB	SFB	yes	19	2	19	1
S. patens	BML gh	unknown	yes	2	0	2	0
*Catharanthus roseus**	UCD gh	UCD gh	N/A	7	7	7	7

CB – Chesapeake Bay, VA.
PS – Puget Sound, WA.
SFB – San Francisco Bay, CA.
WB – Willapa Bay, WA.
BML gh – Bodega Marine Laboratory greenhouse.
UCD gh – University of California Davis greenhouse.
*Positive control, infected with phytoplasma disease Elm Yellows.

Table 2. Number of amplifications from pooled planthoppers, with location of origin (original field collection site) and collection, screened for phytoplasmas and testing positive (primers P1/Tint and P1/P7).

Species	Location of collection	Location of origin	P1/Tint		P1/P7	
			Screened	Positive	Screened	Positive
P. marginata	SFB	SFB	18	5		
P. marginata	BML gh	SFB	99	2	99	0
*P. marginata**	BML gh	SFB	15	0	15	0

CB – Chesapeake Bay, VA.
PS – Puget Sound, WA.
SFB – San Francisco Bay, CA.
WB – Willapa Bay, WA.
BML gh – Bodega Marine Laboratory greenhouse.
UCD gh – University of California Davis greenhouse.
*From eggs excised from leaves and reared on *S. alterniflora* raised from seed.

primers P1/Tint. In this experiment *P. marginata* were fed on 'infected' plants for a total of 15 days; this is ample time for a competent insect to acquire the pathogen (Purcell, 1982). At the end of the fifteen-day acquisition access period (AAP), the surviving *P. marginata* were pooled and randomly sorted into 10 groups of 10.

Prokelisia marginata were fed on the leaves of each group of 10 experimental plants for a total of seven days, confined inside a ventilated tube two cm in diameter. Each of the 10 experimental plants and 10 control plants was rigged with a plastic cage with one leaf per cage. Two days prior to the start of the incubation access period, number of shoots, number of leaves, chlorosis (cm of yellowing, measured from tip to base of each leaf contained within the plastic cages) and total shoot length were counted and measured for control and experimental plants. Every seven days the same planthoppers were serially transferred to new sets of 10 plants. In this manner, five transfers were performed.

At the end of a 40 day incubation period, the shoot number, leaf number, chlorosis and total shoot length of each group of control and experimental plants were measured. The data were analyzed as a 2-way MANCOVA (multiple analysis of covariance) (SAS 7.0) with treatments of insect pathogen acquisition and latent period. The covariates were the measurements of the response variables before application of the treatments. Finally, all *Spartina* plants and planthoppers were again tested with PCR for the presence of phytoplasmas.

Sequencing of 16S rRNA gene. As the results from the efforts described above were inconsistent for phytoplasmas (Table 1), we sequenced the PCR

product from the phytoplasma reactions. The 16S rRNA gene was sequenced from products of the diagnostic length of amplified DNA (examples of product in Figure 1) from 18 *Spartina* plants and planthoppers (5′ end only, P1).

The DNA was amplified using the protocol described above with primer pair P1/Tint or P1/P7. The reaction volumes were increased to 50 μl. The concentration of the PCR product was visually estimated by running 5 μl of product from each reaction on a 1.5% agarose gel along with Gibco DNA mass ladder as a standard. PCR products from multiple reactions from an individual DNA extraction were combined as needed and directly purified using the Bio-Rad Prep-A-Gene DNA purification kit (Hercules, CA). The concentration of the purified PCR product was estimated as previously described and 15 μl of purified PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized by UV transillumination to check for the presence of non-specific bands. Samples that showed non-diagnostic bands then underwent gel purification to isolate the diagnostic bands. In such cases the bands were excised from a 1% agarose gel and purified using the BioRad Prep-A-Gene DNA purification kit. The final purified PCR generated products were sequenced at the Department of Biological Sciences (DBS) Automated DNA Sequencing Facility at the University of California at Davis using a Perkin Elmer ABI 377 Automated Sequencer. Eighteen sequences were submitted to GenBank with the accession numbers of AF288709 – AF288726. We performed BLAST searches (Altschul et al., 1997) on each of these sequences at the website supported by GenBank and recorded the 10 hits with the greatest similarities.

PCR amplification products were sequenced from extractions of: (1) plants from the phytoplasma transmission experiment. Two dead experimental (inoculated by *Prokelisia marginata*) plants (AF288709, AF288721), two live experimental plants (AF288710, AF288722), two dead control plants (AF288711, AF288713) one live control plant (AF288712), (2) one dead *S. alterniflora* seedling from seed collected in Virginia, raised in greenhouse with no planthoppers (AF288714), (3) five yellowed and stunted *S. alterniflora* from Willapa Bay, WA from BML greenhouse with *P. marginata* present (AF288715, AF288716, AF288717, AF288723, AF288724), (4) two *S. anglica* from BML greenhouse with *P. marginata* present (AF288718, AF288719), (5) one hybrid *S. foliosa* × *alterniflora* collected from San Lorenzo Marsh, San Francisco Bay, CA (AF288725), (6) *P. marginata*. One field collection of 30 planthoppers pooled extraction from San Lorenzo Marsh (AF288720) and one greenhouse pooled sample of 10 *P. marginata* that had been caged on yellowed and stunted Willapa *S. alterniflora* for 2 weeks (AF288726).

Results

Screening for phytoplasmas. Screening for the presence of phytoplasmas produced results inconsistent with the hypothesis and the mechanism of the hypothesis, including: individual plants extracted multiple times produced both positive and negative results, some plants never exposed to planthoppers gave positive results, plants 'inoculated' by planthoppers exposed to plants exhibiting symptoms did not produce positive results, plants that had extractions from both root and culm did not yield consistent results, planthoppers reared from eggs excised from leaves and reared on *Spartina alterniflora* raised from seed tested positive. Multiple overlapping bands were frequently produced from plants that were dead when extracted. This suggests that the amplified products may have been from multiple origins, possibly due to external or internal populations of saprophytic bacteria. We postulated, therefore, that the amplification of product was yielding false-positives. However, there was no indication that the technique produced false-negatives. The positive control lane contained a band of the appropriate weight in every gel with amplified product. This supports the conclusion of Smart et al. (1996) that the primer pairs P1/Tint and P1/P7 are reliable in not producing false-negatives.

Phytoplasma transmission by Prokelisia marginata. There were no overall significant differences between control and experimental plants for treatment effect of pathogen acquisition by *Prokelisia marginata* (Wilks' Lambda $P = 0.9352$). There was an overall significant effect of latent period (Wilks' Lambda $P < 0.0001$) and no overall significant effect of interaction between pathogen acquisition and latent period (Wilks' Lambda $P = 0.2218$). Results of screening of experimental and control plants and planthoppers using PCR and primer pairs P1/Tint and P1/P7 showed no treatment effect.

Sequencing results. Our results from analyses of fragment sequences amplified from the 16S rRNA bacterial gene isolated from *Spartina* and *Prokelisia marginata* did not indicate the presence of phytoplasmas. Sequence fragments with a mean number of 350 nucleotides generated by PCR amplification (using primers P1/Tint and P1/P7) were subjected to BLAST searches of GenBank (Altschul et al., 1997). The results of the searches indicated these sequences showed greatest similarity to 16S rRNA sequences from a number of common genera of gram-negative bacteria including *Halomonas*, *Pseudomonas*, *Acinetobacter* and *Vibrio.*

For sequences AF288709–AF288714 and AF288719–AF288721, all top ten hits were represented by *Pseudomonas spp*., excepting between zero and four hits for each sequence were represented by unknown bacterial species. The Expect values (this parameter indicates the number of hits that would occur by chance when searching the database of a given size, decreasing exponentially with the score assigned between two sequences) were between –110 and –125. For sequences AF288715–AF288717, all top ten hits were represented by *Halomonas spp*., excepting between two and four hits for each sequence were represented by unknown bacterial species. The Expect values were between –106 and –125. For sequences AF288718 and AF288722, both derived from insect extractions, all top ten hits were respectively represented by *Vibrio spp.* and *Acinetobacter spp*. excepting five and three hits represented by unknown bacterial species. The Expect values for the former ranged from –47 to –71 and the later from –41 to –53. All of the lowest scores denote perfectly matched sequences.

Discussion

In this experiment we tested the ability of *Prokelisia marginata* to vector phytoplasma pathogens. Ultimately, by analysis of nucleotide sequences of the evolutionarily conserved 16S rRNA gene, we showed that the bacteria in and upon *Spartina* that were detected by PCR were not derived from phytoplasmas. Seemueller and Kirkpatrick (1996) stress that the alternative methods they describe to identify phytoplasmas are similarly not foolproof. These results indicate that putative positive PCR results must be confirmed by sequencing the PCR products. The primer pair P1/P7 produced fewer positive results than the primer pair P1/Tint (Tables 1 and 2), suggesting P1/P7 has the greater specificity. There was no indication that the use of these primer pairs engendered false-negatives. Some error, consisting primarily of false-positives, in the use of these primers may be acceptable if the original identification of pathogen presence in a new species or population is confirmed via sequencing. Particular care should be applied to interpreting positive results from samples extracted from tissue with any decay present. Although we demonstrated that *P. marginata* did not transmit phytoplasmas, we did not test for other potential pathogens including viruses, fungi and other bacterial species. The decline in *S. alterniflora* from Willapa Bay, Washington (Daehler and Strong, 1997) after exposure to *P. marginata* is probably attributable to either the direct effects of the insect, possibly a toxin, or some unknown pathogenic agent.

The concern of false results of pathogen detection, both positive and negative, can hinder the implementation of biological control by insects.

Ecological safety as well as agricultural safety requires assessment of the implications for introduction of novel pathogens and spread of already-present plant diseases by imported herbivorous insects. The risks associated with the introduction of *Prokelisia marginata* as a biological control agent to Willapa Bay, Washington has been researched far beyond that required under law. This effort also included host-specificity screening including types of the target species, species in the same genus as the target species, species in the same subfamily, species in the same family, species in other families of economic importance and threatened and endangered species. The proposal to introduce the Homopteran biological control agent, *P. marginata*, was submitted for a review to the Technical Advisory Group on the Biological Control of Weeds (TAG) (Grevstad et al., 2000). This review for the introduction of *P. marginata* from California to Washington is not obligatory. All 14 reviewers on the TAG committee recommended the release of *P. marginata* for the control of *Spartina alterniflora* to APHIS (the USDA's Animal and Plant Health Inspection Service) with approval number 46757.

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