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An update on the diversity of *Wolbachia in Spalangia* spp. (Hymenoptera: Pteromalidae)

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Results from 13 additional host populations improves resolution on the diversity of *Wolbachia* bacteria in *Spalangia* spp. (Hymenoptera: Pteromalidae). These bacteria are of interest because they can profoundly affect their host's reproduction. Manipulating *Wolbachia* infections may provide a method to improve the efficacy of biocontrol agents including *Spalangia* spp.

Keywords: biological control; symbionts; survey; recombination

Parasitoid wasps (Pteromalidae: Hymenoptera) are of interest as biocontrol agents for pest flies (Diptera: Muscidae) of livestock confinements; e.g. dairies, cattle feedlots, poultry houses, swine barns. Many of these wasps are infected with *Wolbachia* bacteria that can profoundly affect their host's reproduction. *Wolbachia* induce parthenogenesis in *Muscidifurax uniraptor* Kogan & Legner (Zchori-Fein, Gottlieb, and Coll 2000) and cytoplasmic incompatibility in *Nasonia vitripennis* (Walker) (Breeuwer and Werren 1990) and *Urolepis rufipes* (Ashmead) (Kyei-Poku, Floate, Benkel, and Goettel 2003).

Additional information on the occurrence and effect of *Wolbachia* on members of this parasitoid guild may facilitate their mass-production for use in biocontrol programmes. A recent survey identified infections of *Wolbachia* in 15 of 21 species of these wasps (Kyei-Poku et al. 2006). Most of the species were infected with single isolates of *Wolbachia* as determined by genetic variation in the *wsp* gene. Infections of two and four isolates were detected in *N. vitripennis* and *Spalangia cameroni* Perkins, respectively. The same study showed incompatible crosses of *S. cameroni* to produce fertilised eggs that died during embryogenesis. In addition, the progeny of infected males exhibited prolonged development regardless of whether the female parent was infected or whether the males developed from fertilised or unfertilised eggs (Kyei-Poku et al. 2006).

The current study expands upon previous findings by incorporating new information on species identification and results from additional populations of *Spalangia*. *Wolbachia*-infected populations of wasps from Kazakhstan and Russia were identified in Kyei-Poku et al. (2006) as *Spalangia nigroaenea* Curtis. However, these populations since have been identified using genetic markers as the morphologically similar *Spalangia slovaca* Boucek (Taylor, Moon, Gibson, and Szalanski 2006). Hence, Kyei-Poku et al. (2006) provides no information on *Wolbachia* isolates from *S. nigroaenea*. The current study provides

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information on three populations of *S. nigroaenea* plus a further 10 populations of *S. cameroni*, *S. endius* and *S. nigra* not considered in the earlier work.

With minor exception, methods of *Wolbachia* detection, sequencing and analyses were as described in Kyei-Poku et al. (2006). Specimens were preserved in 95% ethanol at –20°C until removed for DNA extraction. Total genomic DNA was extracted from individual insects using a Qiagen DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA), according to the manufacturer's instructions. DNA was eluted using 30 µL of buffer EB, and then stored at −20°C until needed.

Extracted DNA was tested for the presence of *Wolbachia* using a polymerase chain reaction (PCR)-based assay and the primer pair *wsp* 81F and *wsp* 691R (Braig, Zhou, Dobson, and O'Neill 1998). These primers amplify an approximate 600-bp region of the *Wolbachia*-specific *wsp* gene and commonly are used to characterise and distinguish among infections of the symbiont (e.g. Braig et al. 1998; Zhou, Rousset, and O'Neill 1998; van Meer, Witteveldt, and Stouthamer 1999; Kyei-Poku, Colwell, Coghlin, Benkel, and Floate 2005). PCR amplification of *Wolbachia* was done in a 25-µL reaction mixture using TAKARA Master mix kits (TaKaRa Shuzo Co., Biomedical Group, Shiga, Japan) on a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Foster City, CA). The mixture contained 1 µL of template DNA, 2.5 µL 10 × LA PCR Buffer II, 4.0 µL dNTP mixture (2.5 mM each), 0.5 µL 20 µM forward and reverse primers, and 0.2 µL-LA *Taq* DNA polymerase (5 units/µL) with distilled deionised water (HPLC Grade) added to a final volume.

Each DNA sample was tested concurrently with positive (i.e. the *Wolbachia*-infected fly, *Haematobia irritans* (Muscidae: Diptera)) and negative (HPLC grade distilled deionised water) controls. To minimise nonspecific amplification, a touchdown PCR profile was used with an initial denaturation step at 94°C for 1 min, two cycles consisting of denaturation at 94°C for 1 min, annealing at 62 to 52°C for 1 min, and extension at 72°C for 2 min. During subsequent two cycle sets, the annealing temperature was lowered by 1°C until it reached 52°C. This was followed by 30 cycles consisting of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C. Amplification was completed by incubation for 10 min at 72°C. Twelve µL of each amplified PCR product was fractionated through a 1.0% TAE-agarose gel containing ethidium bromide.

For sequencing, *wsp* amplicons were first purified with a QIAGEN QIAquick® Gel Extraction Kit (QIAGEN Inc. Valencia, CA) according to the manufacturer's instructions. Amplicons were then sequenced directly using an ABI 377 DNA sequencer with the ABI PRISM Dye Terminator Sequencing Ready Kit (Perkin-Elmer, Applied BioSystems, Foster City, CA) and a temperature profile of 25 cycles at 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Results of direct sequencing occasionally produced ambiguous outputs that indicated the presence of multiple *Wolbachia* isolates in the sample. In such cases, amplicons were cloned directly into pGEM-T vector (Promega Corporation) according to the manufacturer's instructions. Sequences then were obtained for three to five clones per sample. Intra-sample sequences that differed by less than 1% were used to generate consensus sequences that were deposited in GenBank.

The similarity of *wsp* sequences obtained in the current study was compared to *wsp* sequences deposited in GenBank and previously examined in Kyei-Poku et al. (2006). The combined set of *wsp* sequences was aligned using the multisequence alignment software program ClustalX 1.53b (Thompson, Gibson, Plewniak, Jeanmougin, and Higgins 1997). The sequence of Braig et al. (1998) (Accession AF020070) was used as a standard such that all base locations refer to the *wsp* coding portion of that sequence.

The aligned *wsp* sequences, bases 82–692 (Braig et al. 1998), were used to generate mid-point rooted, phylogenetic trees. The third hypervariable region of the *wsp* gene (bases

602–628) could not be aligned with certainty and was therefore excluded from analyses (Zhou et al. 1998). Phylogenetic trees were created with neighbor-joining (NJ) and maximum parsimony (MP) analyses using the computer program MEGA, version 3.2 (Kumar, Tamura, and Nei 2004). The analyses used a Kimura two-parameter distance matrix, complete deletion of insertions and deletions with a transition/transversion ratio of 2.0, and the assumption of one substitution rate. Tree robustness was assessed using bootstrapping with 1000 replicates. In cases of multiple infections, isolates were distinguished with 'seqvar' (sequence variant) designations as has been advocated for isolates characterised by one or few genes (Bandi et al. 2003).

Recombination between phylogenetically distinct *Wolbachia* isolates can produce sequences that may skew phylogenetic interpretations (Baldo, Lo, and Werren 2005; Baldo, Bordenstein, Wernegreen, and Werren 2006). The *Spalangia* alignment was therefore scanned with the computer software programs RDP, MaxChi, Chimera, and Geneconv as implemented in RDP2 (Martin, Williamson, and Posada 2005) to test for such recombination events. Only events detected by all four programs are reported.

Infections of *Wolbachia* were detected in 13 of the 14 populations of *Spalangia* examined (Table 1). The 48 *wsp* sequences (ca. 600 bp) that were obtained, generated 25 consensus sequences that were deposited in GenBank (Table 1). The high alignment scores of these sequences in comparison with *wsp* sequences previously deposited in GenBank provided final confirmation of *Wolbachia* in the tested material. These additional sequences were consistent with, and expand upon, findings reported in Kyei-Poku et al. (2006).

Table 1. Populations of *Spalangia* tested in the current study.

Genus and species	Source[1] (no. of wasps tested)	Infected? (+/−)	GenBank Accession No.
Spalangia cameroni Perkins	USA – AK (1 ♀)	+	EU154939, EU154940[2]
	– MN (1 ♀)	+	EU154941[2], EU154942[2], EU154943
	– NB (1 ♀)	+	EU154935, EU154936[2], EU154937[2], EU154938[2]
Spalangia endius Walker	Kazakhstan – (lc) (14 ♀)	+	EU154924[2], EU154927[2]
	Russia – (lc) (12 ♀)	+	EU154925[2]
	USA – AK (2 ♀)	−	
	– FL (2002)[3] (19 ♀)	+	EU154923[2], EU154928[2]
	– MN (21 ♀, 1 ♂)	+	EU154926
	– NB (5 ♀)	+	EU154921[2], EU154922[2]
Spalangia nigra Latreille	USA – MN (1 ♀)	+	EU154919[2]
	– NB (1 ♀)	+	EU154920[2]
Spalangia nigroaenea Curtis	USA – FL (4 ♀)	+	EU154933, EU154934
	– MN (4 ♀)	+	EU154929, EU154932
	– NB (5 ♀)	+	EU154930, EU154931

Accession numbers identify *wsp* sequences deposited in GenBank. State abbreviations are AR, Arkansas; FL, Florida; MN, Minnesota; NE, Nebraska. [1]lc, laboratory colony. [2]Identified as being of recombinant origin. [3]Population previously tested in Kyei-Poku et al. (2006).
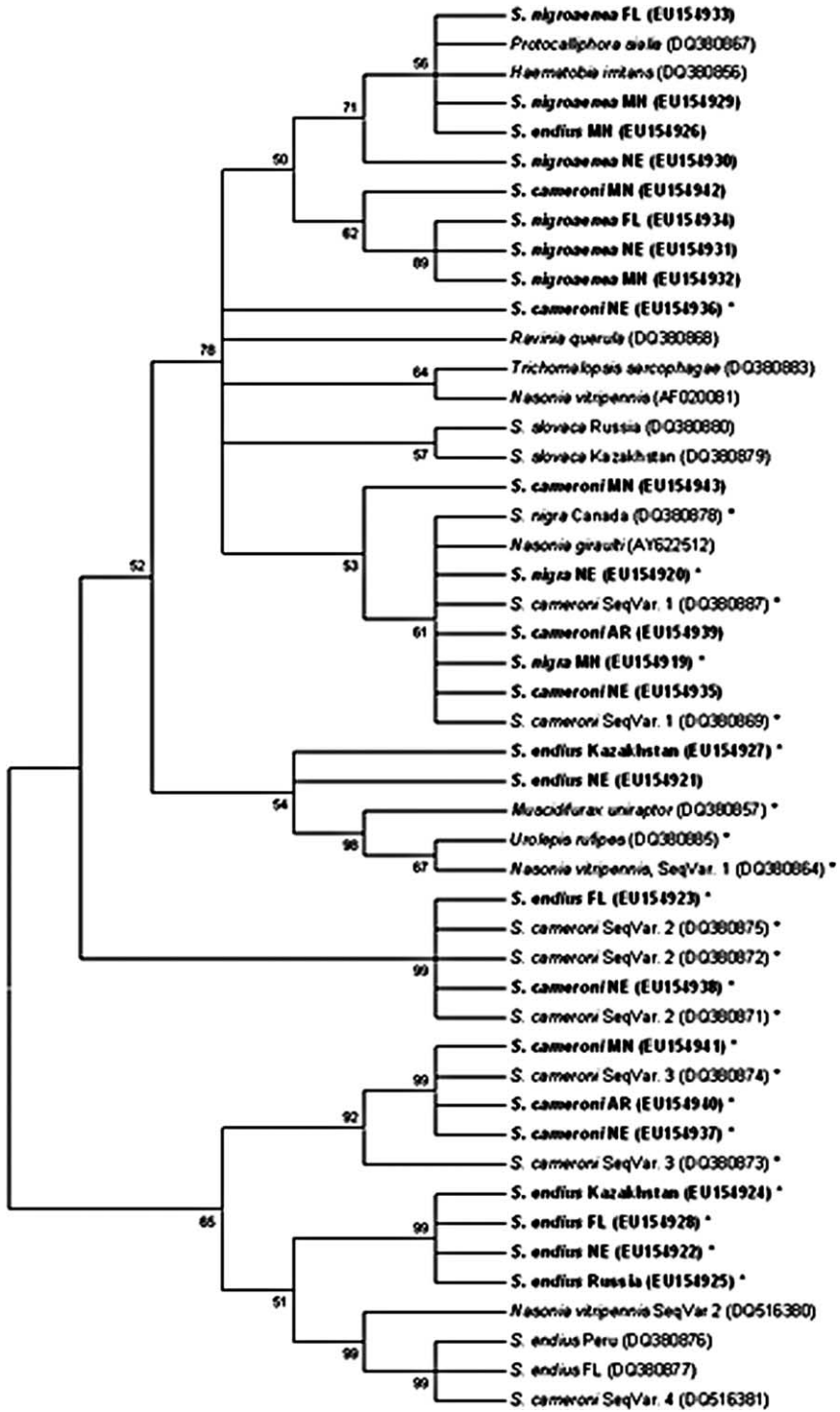
Figure 1 (Continued)

Results for *S. cameroni* support the putative universality of *Wolbachia* infection in this species. Infections were detected in each of three new populations (Arkansas, Minnesota, Nebraska, USA). In combination with results from Kyei-Poku et al. (2006), infections now have been detected in 20 of 21 populations. The sole exception remains a laboratory population from a commercial insectary. Insectaries occasionally use antibiotics to maintain colony health. If antibiotics were applied for such a purpose to this colony, it unintentionally may have eliminated *Wolbachia* infections. In addition, phylogenetic analyses clarify the distribution of sequence variants (seqvars). Kyei-Poku et al. (2006) reported four seqvars from populations of *S. cameroni*. Seqvar 1 was restricted to populations from Israel and Canada. Seqvars 2 and 3 co-occurred in populations from France, Denmark and Peru. Seqvar 4 was detected only once, in a population in Florida, USA. The addition of new sequences for populations from Arkansas, Minnesota and Nebraska, identify the presence of Seqvars 1, 2 and 3 from populations in the USA. This clarifies that all populations of *S. cameroni* may have these co-occurring seqvars. Seqvar 4 was not detected in the current study. Two unique sequences (EU154936, EU154942) identified in the current study from *S. cameroni* were determined to be products of recombination.

Results for *S. endius* identify multiple infections of *Wolbachia* in this species. Kyei-Poku et al. (2006) reported only one isolate, which was detected in populations from Peru and the USA (Florida). The current study identifies a second isolate, which was detected in populations from Kazakhstan, Russia and the USA (Florida, Minnesota). These new sequences form a tight clade distinct from the seqvar reported earlier. Two unique sequences from *S. endius* (EU154921, EU154927) identified in the current study were determined to be products of recombination. It is noted that infections were detected in *S. endius* only with difficulty; i.e. infections were detected in only eight of the 74 individuals tested.

One isolate from populations of *Spalangia nigra* Latreille (Minnesota, Nebraska; USA) was detected in the current study. This same isolate previously was reported to infect a population of *S. nigra* from Ontario, Canada (Kyei-Poku et al. 2006).

Results for *S. slovaca* conform with the findings of Taylor et al. (2006). Originally identified as the morphologically similar species *S. nigroaenea,* molecular markers provide clear genetic separation between the two species. In accordance, isolates of *Wolbachia* obtained from populations of *S. slovaca* (identified as *S. nigroaenea* in Kyei-Poku et al. 2006) are distinct from the two isolates obtained in the current study from populations of *S. nigroaenea*.

Spalangia cameroni, *S. endius* and *S. nigroaenea* have global distributions, probably resulting from secondary distribution through their intentional introduction as biocontrol agents of pest flies or from range expansions associated with the spread of pest flies (Taylor et al. 2006). Thus, it is perhaps not unexpected that populations of *S. cameroni* from Asia (Kazakhstan, Russia), Europe (Denmark), North America (Canada and/or USA), and South America (Peru) share the same set of co-occurring *Wolbachia* isolates. Given inherent difficulties in obtaining *wsp* sequences from *S. endius*, it seems probable that

Figure 1.   Neighbour joining phylogram of *Wolbachia* isolates based on *wsp* sequences (631 bp of DNA). Isolates detected in the current study are identified by bold font. Brackets enclose accession numbers that reference the sequences in GenBank. Numbers on the nodes indicate percentages of 1000 bootstrap replicates. Bootstrap values <50 are not shown. Sequences identified as being of recombinant origin are marked with an '*'. State abbreviations are AR, Arkansas; FL, Florida; MN, Minnesota; NE, Nebraska.

further study also will identify a common set of co-occurring isolates among disparate populations of this species. Similarly, further study likely will detect the presence of the two seqvars identified in North American populations of *S. nigroaenea* in populations from other geographic locations.

Recombinant events were detected for 24 of the 48 *wsp* sequences used for analyses, including sequences isolated from *S. cameroni*, *S. endius* and *S. nigra* (Figure 1). Recombinant sequences were not isolated from *S. nigroaenea* or *S. slovaca*. Widespread recombination in the *wsp* gene has been reported by others (Werren and Bartos 2001; Reuter and Keller 2003; Baldo et al. 2005). Findings of recombination do not negate the conclusions of the current or our previous (Kyei-Poku et al. 2006) study, but rather provide further evidence that use of the *wsp* gene is likely to confound interpretations of *Wolbachia* phylogeny.

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