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Novel microsatellite DNA markers indicate strict parthenogenesis and few genotypes in the invasive willow sawfly *Nematus oligospilus*

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Abstract

Invasive organisms can have major impacts on the environment. Some invasive organisms are parthenogenetic in their invasive range and, therefore, exist as a number of asexual lineages (=clones). Determining the reproductive mode of invasive species has important implications for understanding the evolutionary genetics of such species, more especially, for management-relevant traits. The willow sawfly *Nematus oligospilus* Förster (Hymenoptera: Tenthredinidae) has been introduced unintentionally into several countries in the Southern Hemisphere where it has subsequently become invasive. To assess the population expansion, reproductive mode and host-plant relationships of this insect, microsatellite markers were developed and applied to natural populations sampled from the native and expanded range, along with sequencing of the cytochrome-oxidase I mitochondrial DNA (mtDNA) region. Other tenthredinids across a spectrum of taxonomic similarity to *N. oligospilus* and having a range of life strategies were also tested. Strict parthenogenesis was apparent within invasive *N. oligospilus* populations throughout the Southern Hemisphere, which comprised only a small number of genotypes. Sequences of mtDNA were identical for all individuals tested in the invasive range. The microsatellite markers were used successfully in several sawfly species, especially *Nematus* spp. and other genera of the Nematini tribe, with the degree of success inversely related to genetic divergence as estimated from COI sequences. The confirmation of parthenogenetic reproduction in *N. oligospilus* and the fact that it has a very limited pool of genotypes have important implications for understanding and managing this species and its biology, including in terms of phenotypic diversity, host relationships, implications for spread and future adaptive change. It would appear to be an excellent model study system for understanding evolution of invasive parthenogens that diverge without sexual reproduction and genetic recombination.

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Introduction

Invasive organisms can have major impacts on the environments they invade. They have an effect on biodiversity and are one of the main causes of extinction (Diamond, 1989; Courchamp *et al*., 2003; Raven & Yeates, 2007). They can also deeply affect communities and change ecosystem processes (e.g. Atkinson & Cameron, 1993; Lonsdale, 1999; Mack *et al*., 2000; Levine *et al*., 2003; Sanders *et al*., 2003). Due to increased human movement and climate change, the prevalence of invasive organisms is likely to increase in the future (Hobbs & Mooney, 2005).

Knowledge of the biology, natural history and biological requirements of a given invasive organism is valuable in order to facilitate management and control, and to predict potential distribution. For example, information on genetic variation and reproductive mode of a species can contribute to predicting its longer-term impacts. Due to a typically small number of individuals initially introduced, many invaders experience bottlenecks due to low genetic variation in the introduced population (Lambrinos, 2004). Invasive organisms may undergo rapid evolution to adapt to local conditions, although this is more likely if genetic variation is high for evolution to act upon (Lee, 2002; Kawecki & Ebert, 2004). Furthermore, genetic variation can affect the capacity to colonize and invade (Tagg *et al*., 2005; Crawford & Whitney, 2010) and thus has important implications in terms of the success of invaders (Roman & Darling, 2007; Lucek *et al*., 2010).

Invasive organisms sometimes shift from sexual reproduction to obligate or facultative parthenogenesis in their invasive range (e.g. Moran, 1992; Dybdahl & Lively, 1995; Sunnucks *et al*., 1996). Without any need for mating, parthenogenesis allows populations to increase quickly, with a two-fold reproductive advantage compared to sexual populations (Maynard Smith, 1978). Although rapid increase in population size is an advantage for parthenogens, genotypic variation may be very low; and, in the absence of genetic recombination, natural selection is unable to act on individual genes or chromosomal regions. *In situ* adaptation, therefore, may be considered less likely. Thus, the success of parthenogenetic invasive organisms has been attributed mainly to phenotypic plasticity or to the introduction of different specialized asexual genotypes (Peccoud *et al*., 2008). Nonetheless, evolution by mutation can occur and lead to new phenotypes of ecological significance (Lynch, 1985; Sunnucks *et al*., 1998; Loxdale, 2008).

There are different types of parthenogenesis, with different consequences for population genotypic variation. Automictic parthenogenesis involves meiosis and, therefore, the possibility of recombination (so daughters differ genetically from their mothers), although some forms result in increasing and high homozygosity. In apomictic parthenogenesis, new forms or clones can occur only following mutation and chromosomal rearrangements, and so daughters are genotypically very similar to their mother (Suomalainen, 1962). Genotypic variation is generally expected to be low in apomictic parthenogenesis, although this depends on rates of formation of new asexual lineages from sexual ancestors, population sizes and mutation rates (Loxdale, 2008, 2009). In many parthenogens, individuals capable of sexual reproduction can also be produced.

Molecular tools have revolutionized our understanding of population biology and ecology. Of all the genetic markers available, high resolution microsatellite markers are by far the most commonly used in ecological studies (Selkoe & Toonen, 2006). Microsatellites or simple sequence repeats (SSRs) are repeated motifs of one to six bases found mainly in the non-coding areas of the genome and have several favourable properties as genetic markers (Sunnucks, 2000). They are typically selectively neutral and highly length-variable (Chambers & MacAvoy, 2000), and this polymorphism makes them very applicable to focus on fine-scale questions that were previously impossible to tackle (Zane *et al*., 2002; Selkoe & Toonen, 2006). For example, their variability makes them ideal for distinguishing individual genotypes and assessing levels of gene flow between populations (Lowe *et al*., 2004). They are also well suited for assessing mating strategies and reproductive modes. For example, sexual and asexual populations are readily distinguished (e.g. Gómez & Carvalho, 2000; Wilson *et al*., 2002; Vorburger *et al*., 2003).

The willow sawfly, *Nematus oligospilus* (Hymenoptera: Tenthredinidae), is native to North America (southern Alaska to Mexico) and Eurasia (Ireland to the Himalayas), where it is widespread (Smith, 1979), whilst it has been introduced into Argentina (Dapoto & Giganti, 1994), South Africa (Urban & Eardley, 1995), New Zealand (Berry, 1997; Charles *et al*., 1998) and Australia (Bruzzese & McFadyen 2006; Ede *et al*., 2007). It has spread quickly after each new introduction (Dapoto & Giganti, 1994; Urban & Eardley, 1995; Cowie, 2006; Caron, 2011). Population densities can increase rapidly, resulting in defoliation of willow trees and substantial damage in some areas (Cowie, 2006; Ede *et al*., 2009). On the basis of natural history data such as only females being observed, *N. oligospilus* is thought to be parthenogenetic in its invasive range, whereas both sexes occur in the native range (Charles *et al*., 1998; Koch & Smith, 2000). However, rare and unobserved sexual reproduction is common in parthenogens (Hurst *et al*., 1992). If it can be established that it is indeed parthenogenetic in its invaded range, *N. oligospilus* provides an opportunity to study the ongoing ecology and evolution of an invasive species that has lost sexual reproduction. Effective molecular markers are necessary to establish the reproductive mode, spread and population dynamics of *N. oligospilus*.

Like all Hymenoptera, the ancestral reproductive mode for tenthredinid sawflies is haplodiploidy (arrhenotoky); females are the product of fertilization and have two copies of each chromosome, whilst males arise from unfertilized eggs and carry only a maternal haploid set of chromosomes (Normark, 2003). Parthenogenesis occurs in many species and is obligate

in some (Craig & Mopper, 1993). In some insects, males are unknown (Benson, 1950), while in others, a few males are produced and their role is ambiguous (Craig & Mopper, 1993). Both apomictic and automictic parthenogenesis occur in tenthredinids (Suomalainen, 1962; Knerer, 1993).

Tenthredinid sawflies have been relatively little studied compared to other groups of Hymenoptera. Nonetheless, due to interesting life characteristics, such as their reproductive mode and feeding strategies, and because tenthredinids have undergone recent evolutionary radiation, they can be studied in order to investigate ecological and evolutionary theories concerning host-plant relationships, trophic interactions, speciation and adaptive radiation (Knerer, 1993; Roininen *et al.*, 1996; Price *et al.*, 2005; Nyman *et al.*, 2006a, 2010). However, little has so far been done on population biology and mating systems of this group taking advantage of highly-resolving molecular markers such as microsatellites. Previous population studies on phylogeography have used allozymes (Müller *et al.*, 2004), while studies into heritability of defence traits were conducted using laboratory crosses (Müller *et al.*, 2003). Most other molecular genetic studies on this group have used mitochondrial DNA (Nyman *et al.*, 2006a, 2010; MacQuarrie *et al.*, 2007). Microsatellites developed for *N. oligospilus* could also prove useful for other sawfly species, although microsatellites developed from one species are typically less likely to be useful in genetically more distant species. Success of cross-amplification depends on the group targeted (e.g. Primmer *et al.*, 1996; Zane *et al.*, 2002; Zenger *et al.*, 2003; Wilson *et al.*, 2004; Barbarà *et al.*, 2007; Palma-Silva *et al.*, 2007).

The aims of the present study were twofold. The first was to determine the reproductive mode of *N. oligospilus* with the level of asexual (clonal) diversity by developing and applying microsatellite markers. Since sawflies represent an interesting study group, the second aim was to assess the applicability of the microsatellites so designed in different sawfly species of varying relatedness to *N. oligospilus*.

Materials and methods

Study organisms

Nematus oligospilus individuals from different parts of the invasive range were used for the study. Individuals originated from South Africa, the North and South Island of New Zealand, and from eastern and western Australia. Although widespread, *N. oligospilus* is inconspicuous in its native range (Pschorn-Walcher, 1982). Individuals thought likely to be *N. oligospilus* were collected from Arizona, USA from sites where previous studies have been performed on this species (Carr *et al.*, 1998) and in Montreal, Canada at a specific site where several specimens preserved in insect collections originated (V. Caron, personal observation). Other species chosen for this study, such as *Nematus brevivalvis* Thomson, were closely related to *N. oligospilus* as shown in previous studies (Nyman *et al.*, 2010), while some other species were less closely related, belonging to other nematine tribes (e.g. *Pristiphora* spp.) and other subfamilies (e.g. *Caliroa cerasi* L., *Rhadinoceraea* sp. and *Endelomyia aethiops* F.; table 1).

The tenthredinid species assessed here encompassed different life strategies. Some are gall-formers such as *Euura mucronata* Hartig (Nyman, 2002). *Pristiphora angulata* Lindqvist is a facultative miner of flower buds, while the others are external feeders. Many are parthenogenetic, including *C. cerasi*, *E. aethiops* and *Pristiphora erichsonii* Hartig (Benson, 1950). Species like *N. pavidus* and *Nematus iridescens* Cresson are gregarious; the other species tested are primarily solitary but can occur at high population densities, as seen in *N. oligospilus* in its invasive range (Ede, 2009). Many of the species chosen in this study are economically important. For example, *C. cerasi* (pear or cherry slug) can cause important damage to fruit trees (Carl, 1972) and has been accidentally introduced to several countries including New Zealand and Australia (Naumann *et al.*, 2002). Similarly, *P. erichsonii*, commonly known as the larch sawfly, can become a destructive pest of larch *Larix* sp. (Boévé, 2004).

Microsatellite development

DNA was extracted using the 2 × CTAB extraction protocol (Grosberg *et al.*, 1996) from legs of four *N. oligospilus* individuals collected from different areas in Australia and preserved in 96% ethanol. Microsatellite loci were developed using the FIASCO enrichment protocol (Zane *et al.*, 2002) with minor alterations. The initial digestion did not contain Bovine Serum Albumin (BSA) and the pre-amplified product was cleaned with 0.1 volume of 3M NaOAc and 2.5 volume of 100% ethanol, followed by an overnight incubation at –20°C. The product was then centrifuged at 13,200 rpm (Centrifuge 5415D, Eppendorf, Hamburg, Germany) for 30 min and 70% ethanol washed. The DNA pellet was resuspended in 30 µl of 1 × TE. After cleaning, 400 ng of the pre-amplified PCR product was hybridized to two probes, $(AC)_{17}$ and $(ATT)_{17}$, which were then captured with Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison, WI, USA) as described in Roberts & Weeks (2009). PCR products amplified from the enriched DNA pool were cloned using a TOPO TA Cloning Kit for Sequencing according to the manufacturer's instructions (Invitrogen). Eighty-seven clones were commercially sequenced (Macrogen Inc., Seoul, Korea).

DNA extraction for microsatellite screening

Between 250 and 1300 *N. oligospilus* individuals, most at the larval stage, were tested for each microsatellite locus, while six to ten individuals of each of the other sawfly species were screened. DNA was extracted from individuals preserved in 96% ethanol using the salting-out DNA extraction protocol described in Sunnucks & Hales (1996). DNA pellets were re-suspended in 50 to 100 µl of 1 × TE buffer.

Microsatellite screening

Following sequencing of DNA clones enriched for microsatellites, 43 primer pairs were designed using Primer3 v.0.4.0. (Rozen & Skaletsky, 2000) (table 2). One primer of each pair was labelled with infra-red dyes IRD™700 or IRD™800 (LI-COR Biosciences, Lincoln, NE, USA). PCR reactions contained: 1 µl of 10 × *Taq* buffer, 0.8 µl of 25 mM $MgCl_2$, 1.6 µl of 1.25 mM dNTPs, 0.5 µl of 10 mg ml^{-1} BSA, 0.4 µl of *Taq* Polymerase (Fermentas, Burlington, Canada), 0.5 µl of 1 µM of each primer, 0.3 µl of 1 µM of IRD™700 or IRD™800, 1 µl of DNA template and Milli-Q water for a total volume of 10 µl. Cycling conditions were: 94°C for 4 min, 35 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, with the annealing temperature of the first five cycles reducing by 1°C per cycle, the remaining 30 cycles having an annealing temperature of 50°C, all followed by a final extension of 72°C for 5 min.

Table 1. Sawfly species used in this study with the source, host plant, collector and number of individuals used (*N*)

Subfamily	Tribe	Species name	Source	Host plant	*N*	Collector
Nematinae	Nematini	*Nematus oligospilus* (Förster)	Australia	*Salix* sp. (Salicaceae)	1062	V. Caron
			New Zealand		246	V. Caron
			Bethlehem, South Africa		26	C. Eardley
		Nematus oligospilus (Förster)	Flagstaff, AZ, U.S.A.	*Salix lasiolepis* (Salicaceae)	10	V. Caron
		Nematus sp.	Montreal, Qc, Canada	*Salix* sp. (Salicaceae)	8	V. Caron
		Nematus iridescens (Cresson)	Flagstaff, AZ, United States	*Populus* sp. (Salicaceae)	8	P. Price
		Nematus pavidus (Lepeletier)	Stow, England	*Salix* sp. (Salicaceae)	4	V. Caron
			Harpenden, England		4	V. Caron
		Nematus brevivalvis (Thomson)	Kevo, Finland	*Betula pubescens* (Betulaceae)	6	L. Kapari
		Eitelius dentatus (Lindqvist)	Parikkala, Finland	*Salix phycifolia* (Salicaceae)	8	H. Roininen
		Euura mucronata (Hartig)	Kilpisjärvi, Finland	*Salix glauca* (Salicaceae)	3	T. Nyman
			Abisko, Sweden		5	T. Nyman
Nematinae	Pristiphorini	*Pristiphora angulata* (Lindqvist)	Oulu, Finland	*Spirea chamaedryfolia* (Rosaceae)	8	T. Nyman
		Pristiphora erichsonii (Hartig)	Ellsworth, ME, U.S.A.	*Larix* sp. (Pinaceae)	1	C. Linnen
			Joensuu, Finland		7	T. Nyman
Heterarthrinae	Caliroini	*Caliroa cerasi* (L.)	Melbourne, Vic, Australia	*Prunus* sp. (Rosaceae)	8	V. Caron
		Endelomyia aethiops (Fabricius)	Joensuu, Finland	*Rosa* sp. (Rosaceae)	8	T. Nyman
Blennocampinae	Phymatocerini	*Rhadinoceraea* sp.	Keret, Russia	*Veratrum album* (Melanthiaceae)	8	H. Roininen

Multiplex reactions were prepared using the Qiagen PCR multiplexing kit (Qiagen, Hilden, Germany) when possible, to a maximum of 13 loci per PCR, following the manufacturer's instructions. PCR products were electrophoresed with LI-COR Global IR2 two-dye DNA sequencers (models 4200 and 4300).

Mitochondrial DNA (mtDNA) analysis

To estimate genetic similarities within *N. oligospilus* and among species, Cytochrome oxidase I mtDNA was sequenced using primers HCO1490 and LCO2198 (Folmer *et al.*, 1994). Seventy-four *N. oligospilus* individuals were assayed from 18 sites (14 in Australia, three from New Zealand, one from South Africa), and the mtDNA was also sequenced for one to eight individuals of the other sawfly species. The PCR reaction contained 2.5 µl of 10 × *Taq* buffer, 2 µl of 25 mM $MgCl_2$, 4 µl of 1.25 mM dNTPs, 12.25 µl of 1 mg ml^{-1} BSA, 1.25 µl of *Taq* Polymerase (Fermentas), 1.25 µl of 10 µM of primer HCO and LCO, 0.5 µl of DNA template to a total of 25 µl. Cycling conditions were: 95°C for 5 min; 30 cycles at 95°C for 45 s, 50°C for 45 sec, 72°C for 1 min; 72°C for 10 min. PCR products were precipitated with 3M NaOAc and ethanol as described above for microsatellite development. Dried DNA pellets were commercially sequenced (Macrogen Inc.).

Analyses

Microsatellites were genotyped visually in Saga Generation 2 software (LI-COR). The number of alleles and genotypes, and expected heterozygosity were calculated in GENALEX 6 (Peakall & Smouse, 2006). Mitochondrial DNA sequences were edited and aligned in Geneious Pro 5.3.4 (Biomatters Ltd, Auckland, New Zealand). Maximum likelihood phylogenetic trees were computed in MEGA version 5 (Tamura *et al.*, 2011). The best model of molecular evolution was chosen using the maximum likelihood fits of different nucleotide substitution models. The substitution model with the lowest Bayesian information criterion (BIC) was the General Time Reversible model with gamma distributed rates and was used to compute a tree for the whole dataset with 100 bootstrap re-samplings. To assess if there was a relationship between percent divergence and the number of loci amplified, a linear regression was done with *p*-distance expressed as a percentage sequence difference between each haplotype and that of *N. oligospilus* from the invasive range, in SYSTAT 13 (Cranes Software International Ltd, Bangalore, India). To determine if microsatellite evolution had a directional bias and if smaller and larger alleles of heterozygotes were equally likely to mutate, Pearson's chi square tests for goodness-of-fit were used. A mutation was assumed to have occurred when a rare multilocus genotype differed at only one allele compared to a very common genotype (Wilson *et al.*, 2003).

Results

Microsatellite development

Of 87 clones sequenced, 79 (91%) contained microsatellite sequences. Six unclear sequences that contained microsatellites were discarded. Where clones yielded similar sequences, only one of each group was developed as a locus. Fifteen clones contained two microsatellite sections. AC repeats were by far the most common, with 61 clones containing between five and 23 repeats. GA, GAA or GAAAA repeats were also relatively common, with ten sequences ranging between four and 100 repeats. AAT and ATT were shorter and rarer; only six sequences contained them. Many of the microsatellite sections were impure (table 2). Four sequences containing

minisatellites were also detected. All 43 loci could be successfully amplified using *N. oligospilus* DNA as PCR template. Two loci (WF1 and WF43) yielded undefined products and were discarded after initial screening. WF19 also gave inconsistent results and was eventually discarded.

Reproductive mode and genotypic diversity in N. oligospilus

All *N. oligospilus* from the invasive range screened shared a single allele at 23 (56.1%) of the 41 loci tested. Of the remaining 18 loci showing heterozygosity, few alleles were found; for 14 loci, there were only two alleles, while WF2, WF4, WF35 and WF40 had three. Furthermore, a maximum of three genotypes per locus were identified in invaded range *N. oligospilus* (table 2). Of these, 18 heterozygous loci, seven presented the same heterozygote in all invaded range individuals, so 11 were useful to discriminate among genotypes of invasive range individuals. One main genotype was discerned from the invasive range. There were 16 other closely related genotypes, of which 14 were different from the common genotype at only one allele of one locus, while the other two genotypes were different at two loci. Differences between alleles were apparently due to single mutations with alleles losing or gaining one repeat. The mutational process did not have apparent directional size bias (Pearson $\chi^2 = 1.0$, df = 1, $P = 0.317$) and the smaller allele was not significantly more likely to undergo mutation than the larger allele (Pearson $\chi^2 = 0.818$, df = 1, $P = 0.366$). Low variation was also seen in mtDNA sequences; all *N. oligospilus* sequenced from South Africa, New Zealand and Australia had identical mtDNA sequences (fig. 1).

Except for a single individual, all *N. oligospilus* in this study proved to be diploid (indicated by heterozygous loci) and are thus likely to be female. One apparent haploid individual (thus most likely a male) was detected during screening; the individual was collected as a larva, so could not be sexed physically. The lack of haploids combined with the distribution of a very common genotype and an array of rare, but closely related genotypes (despite considerable polymorphism) suggest that obligate or facultative parthenogenesis ('functional parthenogenesis') is by far the most likely reproductive mode for *N. oligospilus* in its invaded range. The low genotypic diversity is unlikely to be caused by low power in the genetic assay; the loci can readily distinguish different genotypes when they are present in other species (next section) and, with 18 multi-allelic loci, would detect new sexually-produced genotypes in the sampled invaded range with extremely high certainty.

Other sawfly species

Many of the loci developed for *N. oligospilus* also amplified successfully in other sawfly species. Only two loci were specific to *N. oligospilus*. Most loci amplified in up to five of the species tested, and a few loci amplified in eight or more, including WF5 which amplified in all species tested (table 3). When they did amplify, most microsatellites had alleles unique to each species. Twenty-one loci revealed intraspecific variation, often in multiple species (table 3). Microsatellite loci useful in discriminating *N. oligospilus* genotypes were not more likely to differentiate between individuals of other species. Allele diversity was the highest for *Nematus* sp. (Canada) (53), followed by *N. brevivalvis* (47) and *N. pavidus* (33), despite low samples sizes (6–8), as compared with >1300 *N. oligospilus*. There was considerable genotypic diversity in these other species; most individuals bore different genotypes, with the exception of *N. iridescens* and *Nematus* sp. (Arizona, USA). This reinforces the low genotypic diversity of *N. oligospilus*, in which individuals nearly all had the same genotype (table 3). The majority of individuals of most sawfly species screened were heterozygous at some loci, indicating they were likely to be female and therefore would reproduce parthenogenetically. In two exceptions, *N. brevivalvis* and *P. angulata*, genotypic patterns were consistent with the presence of haploid males and diploid females.

The different sawfly species screened with the new microsatellite markers were related to *N. oligospilus* to different extents as estimated from mtDNA sequences (fig. 1). Members of the tribe Nematini formed a distinct group, while the tribe Pristiphorini formed a sister group. The three other species, *Rhadinoceraea* sp., *C. cerasi* and *E. aethiops*, were placed at the base of the tree in a less-defined cluster. The most closely related to *N. oligospilus* was *Nematus* sp. collected in Canada, while *Nematus* sp. collected in Arizona was in a sister clade with *N. brevivalvis*. *Nematus pavidus* COI sequences were monophyletic but fell in two distinct branches. *Euura mucronata* and *Eitelius dentatus* had modest support for grouping within *Nematus*.

The percentage COI sequence divergence between invaded range *N. oligospilus* and other taxa was strongly negatively correlated with the percentage of loci amplifying in the other taxa ($R^2 = 0.92$, $F_{1,11} = 176.8$, $P < 0.001$) (fig. 2). Successful amplification of loci in *Nematus* sp. varied between 29% for *N. iridescens* and 95% for the *Nematus* sp. from Canada, while *Nematus* sp. from Arizona, USA, had 56% success. The three more divergent species, *Endelomyia aethiops*, *C. cerasi* and *Rhadinoceraea* sp., each had only one locus amplifying (2.4%), while the success rate for *Pristiphora* species was ~13%.

Discussion

Reproductive mode and clonal diversity in N. oligospilus

Some of the most successful invaders are parthenogenetic (e.g. Corrie *et al.*, 2002; Jokela *et al.*, 2003; Mergeay *et al.*, 2006; Budde *et al.*, 2011). *Nematus oligospilus* is reported to be parthenogenetic in its invasive range (Urban & Eardley, 1995; Koch & Smith, 2000), which is supported by the present data. Males are thought non-existent, but could be merely rare or morphologically unrecognized. The microsatellite markers suggest that *N. oligospilus* daughters are diploid and bear no recombined copies of their mother's microsatellite genotypes. Microsatellites indicated extremely rare haploid males in the invasive range – a single one was found in the >1300 individuals screened here. Although male(s) thus occur, their role is unknown; sexual reproduction has not been detected in this study according to the genotypic data. Given the number of variable loci, any new sexual recombination producing the screened individuals would have been detected very easily. *Nematus oligospilus*, therefore, can be recognized as a functionally parthenogenetic organism within its invasive range. Apomictic and automictic parthenogenesis can be hard to discern when using few molecular markers (Rabeling *et al.*, 2009), especially when recombination levels are very low (Kellner & Heinze, 2011). In this study, the large number of microsatellite markers used showed no recombination, and heterozygosity was maintained; *N. oligospilus* is, therefore, unlikely to be automictic, and hence more likely to be

Table 2. Characteristics of microsatellite loci isolated from *Nematus oligospilus*. Locus name, primer sequence, core repeat in sequenced clone, number of individuals tested (N), number of alleles, number of genotypes, number of base pairs lost or gained when compared to main genotype (M), observed (H_o) and expected (H_e) heterozygosity, range of allele size, and F_{IS}

Locus		Primers (5′–3′)	Repeat	N	N allele	N genotype	M (bp)	H_o	H_e	Allele size	F_{IS}
WF2	F	ATGTATCGGGAGTCCAATGC	$(CTT)_5 \sim (AC)_7$	1264	3	4	−2	0.704	0.467	209–211	0.506
	R	TCGATAACGATATCTGTGTTACGAG									
WF3	F	CAGGAAGTAACTAGTGGCTCAAAC	ACC GAG $(AC)_4$	641	1	1	0	0	0	137	–
	R	CTCGTCATCTTCGTGATTCG									
WF4	F	TGTTCGCTGGTTGGAATAGC	$(AC)_5$ GA $(AC)_8$	364	3	2	+2	0.997	0.503	204–210	−0.986
	R	TATCGCAGGAAAACCGTACC									
WF5	F	TAACCAATAGGGGCGAAGTG	$(AC)_{12}$	398	1	1	0	0	0	199	–
	R	AGCTTGGGGTAGGTTTTTCC									
WF6	F	TGGATCTGTGGGACACAATG	$(GA)_2AA$ $(GA)_5 \sim (AC)_{11}$	1158	1	1	0	0	0	204	–
	R	CTCGTCGGGCAAGACTCTG									
WF7	F	TGTTGGCAGCATTTCTGTTC	$(AC)_7$ AGAT $(AC)_3$	526	1	1	0	0	0	228	–
	R	AGCCAGACTTGGCTCATTTC									
WF8	F	TGGACGAACTTGAGTTGCAG	$(AC)_{23}$	468	1	1	0	0	0	286	–
	R	CGAGATATCGACAAAACTGTGG									
WF9	F	GAGCACCCCTCACGTACC	$(AC)_{10}$	1119	2	2	−6	0.999	0.500	204–210	−0.998
	R	CAATTCCTATCCTGCGATCC									
WF10	F	GGCTTAGGCTCCCACTTCTC	$(AC)_2$ (TC) $(AC)_6$	1285	1	1	0	0	0	124	–
	R	AACTGCATTCCACGTTTTACG									
WF11	F	GATTTTCCCGATCGTACTAACC	$(AC)_6$ GTACC $(AC)_3$	1276	1	1	0	0	0	142	–
	R	TGCACTCGCGTATTGTTCTC									
WF12	F	CATGAAGAAAATCGCCCTTC	$(AC)_{10}$ $(AG)_{13}$	1258	1	1	0	0	0	185	–
	R	CCCCACTTCCCAAAATTCTC									
WF13	F	CCTCTGACTATTTTCATTTCACG	$(AC)_{14}$	1255	2	3	+2	0.006	0.008	127–129	0.197
	R	TCATTTCTAAATCGAGGGAAGC									
WF14	F	GGATGGACTGAAAGCCAGAG	$(AC)_3$ TC $(AC)_5$	1194	1	1	0	0	0	171	–
	R	TTGGACCCAAGCTAAACTCAC									
WF15	F	CACTACCTTGGAAGATTTGTTG	$(AC)_{12}$	1222	2	2	+6	0.999	0.500	264–270	−0.998
	R	AGGATTTGACCCTGGACCTC									
WF16	F	GTGGCTGCAGTAGTCAGTGG	$(AC)_5$ GAAT $(AC)_2$ AG $(AC)_3$ AG $(AC)_2$	1215	1	1	0	0	0	295	–
	R	AGCAGGACAGCAAAACCTTG									
WF17	F	GCCCAGTTGGTCAAATCC	$(AC)_3$ AA $(AC)_6$	253	2	1	0	1.000	0.500	185–187	−0.992
	R	CATTTCTTGGCGTCGTGTAG									
WF18	F	AACAGGCCGTGATTCGAG	$(AC)_2$ CCCACC $(AC)_5$	505	1	1	0	0	0	132	–
	R	CATGGCGAGAGACTGATACG									
WF19	F	AGTGGAAAATACTCGCAGAGC	$(AC)_{16}$	293	2	1	0	1.000	0.500	147–164	−1.000
	R	CGTCCACACGTCGCATAC									

Table 2. (*Cont.*)

Locus		Primers (5′–3′)	Repeat	*N*	*N* allele	*N* genotype	M (bp)	H_o	H_e	Allele size	F_{IS}
WF20	F	TTTGCAGGCATGAGAGTCAG	ACC $(AC)_7$	1268	2	2	+2	0.001	0.001	136–138	0.000
	R	AAGTGAACGTAATTAGATGCAGATG									
WF21	F	CATCAAATTTCACGTGGACAG	$(AAT)_3$ AAC $(AAT)_8$	1265	2	2	−3	0.999	0.500	334–337	−0.998
	R	TCTTCTTCCGTATGCTTCGAG									
WF22	F	AATAATTGGAGCAATATGTCG	$(AC)_2$ AT $(AC)_2$ CCACGC $(AC)_3$ ATAC	368	1	1	0	0	0	263	–
	R	ACGAGCGTGGTGAGAAAATG									
WF24	F	GGTCGTTTGAACGGAACATAG	$(AC)_2$ CC $(AC)_3$ AG $(AC)_3$	367	1	1	0	0	0	283	–
	R	TCTGCTTAGATGGATGGATCG									
WF25	F	TTGTTCGGGAAGGTAAGTCAG	$(CTT)_6 \sim (CTT)_6$	1002	2	2	−6	0.001	0.001	513–519	0.000
	R	CGAGAATCCTGTTGCGTGAG									
WF26	F	CGTCAGCTTGTCTTTTCGAG	$(AC)_8$	1274	2	1	0	1.000	0.500	417–419	−1.000
	R	CGGTGGTTGTACGCTCGTC									
WF27	F	GAAAGCTGAATGCGGTATTTTC	$(AC)_2$ GC $(AC)_7$ AG $(AC)_3$	1274	2	2	−2	0.999	0.500	120–122	−0.998
	R	CAACGATAATAAAGTGTCCGATG									
WF28	F	GGACAGATGTGTGGAAATCG	$(AC)_2$ A $(AC)_4$ T $(AC)_3$	313	2	1	0	1.000	0.500	200–250	–
	R	ACGCCTGCAGAAGTTAGGAC									
WF29	F	CCATATCAGAGAGCCGTTC	$(AC)_{11}$	540	1	1	0	0	0	169	–
	R	GGAACGTGAGGGACACTGAG									
WF30	F	GGCTTTAGTGAGCTGAGATAGTCG	$(CTT)_5 \sim (AAC)_7 \sim (CTT)_{10}$	1198	2	2	−2	1.000	0.508	403–415	−0.969
	R	CCGATCAGTCGATCCGTTAG									
WF31	F	ACCTATGAGTCTGGCGGATG	$(ATT)_3$ AGA $(ATT)_2$	295	1	1	0	0	0	173	–
	R	TTGGGATCTTTGGCTAATGC									
WF32	F	GGGCAACGTAATGTGGAAAG	$(AC)_8$	1246	2	2	−2	0.998	0.500	163–165	−0.995
	R	CAACCCCTGCAATAAACACC									
WF33	F	GAGGCAGAAGCAGTTTTTGG	$(AC)_4$ AA $(AC)_7$	466	1	1	0	0	0	420	–
	R	CGTACCGACTTTCCGTCTTC									
WF34	F	TGCGTGTTATCTGACTCAAGG	$(AC)_5$ GCACCCAT $(AC)_3$	1272	1	1	0	0	0	276	–
	R	ACGTTACCCGGAAGATCAAG									
WF35	F	CACGTTGCTATCCACTTTAC	$(AC)_{14}$	1274	3	2	−2	1.000	0.501	185–193	−0.995
	R	AAATCATAGAGGCTGACCAC									
WF36	F	CCTCGCTAATCAATCCAC	$(AC)_2$ CC $(AC)_2$ CCACCC $(AC)_3$ AG $(AC)_2$	484	1	1	0	0	0	200	–
	R	GCGATATGGAGGAGATAAAG									
WF37	F	CGATCTTCAATTCGGTCTAC	$(AC)_2$ AT $(AC)_4$ $(GC)_2$ $(AC)_4$	1195	1	1	0	0	0	279	–
	R	TTCTCATGTTCTTCAACG									
WF38	F	AATACCAGAAACAACCGAAG	GCTTCT $(GCTTT)_2$ GCTTC	1295	1	1	0	0	0	121	–
	R	TTTCTACAGGCGTAGAGTGG									
WF39	F	GAAGCGTGTGGCATAATC	$(GAAA)_4$	1259	1	1	0	0	0	219	–
	R	CTGCTCAACCAGTCTTTAC									
WF40	F	TTTTCTGGGTGCAGGTTATTG	$(AC)_{11}$	1169	3	2	±2	0.999	0.520	176–184	−0.920
	R	GGAAAATAATGATACAAACTCTGTTG									
WF41	F	CGCTTGTTAGAGCCTTGGTC	$(AT)_3$ $(AC)_{10}$	1258	2	1	0	1.000	0.500	285–287	−1.000
	R	CGGTTTGTGCAGCTTACAATG									
WF42	F	GCGAATATTTTGGTGCTATCC	$(AC)_8$	302	1	1	0	0	0	200	–
	R	CCAGCAGGCTATAGACTACGTTC									

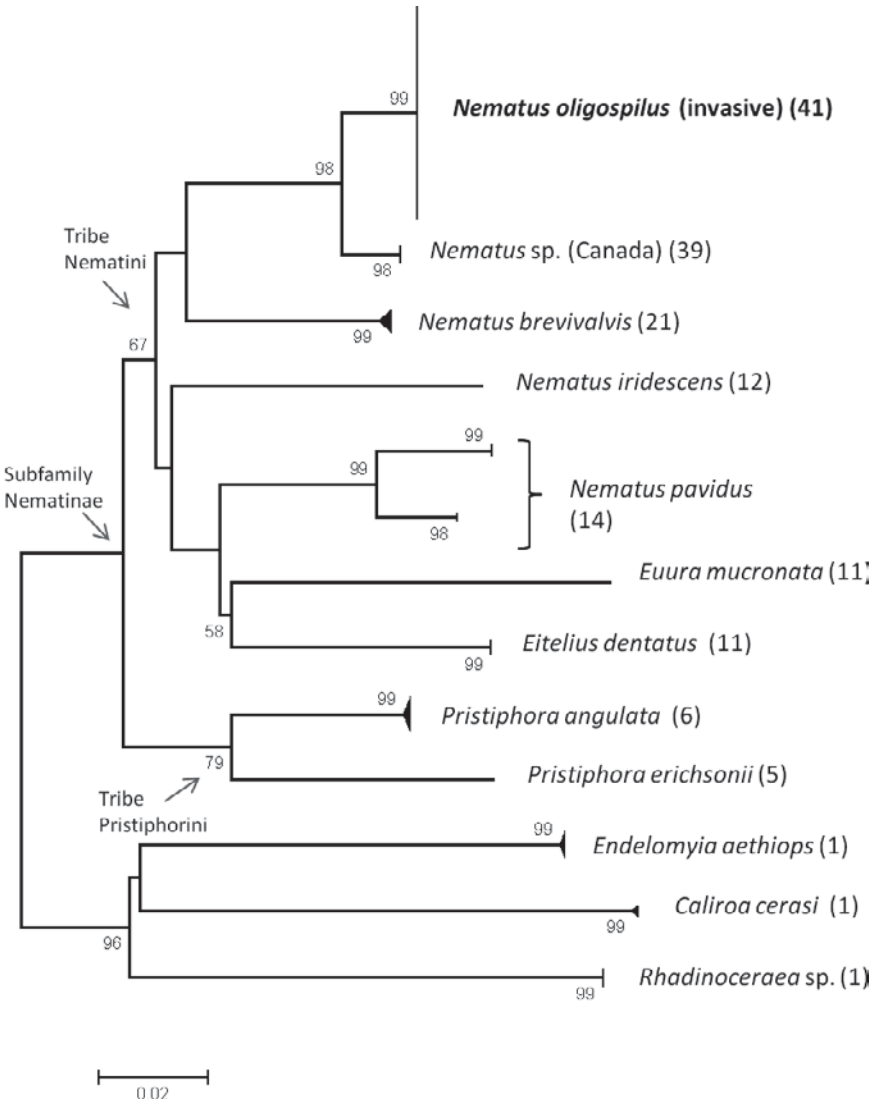

Fig. 1. Maximum likelihood phylogeny of the sawfly species estimated applying the GTR-G substitution model for the mitochondrial DNA cytochrome-oxidase I sequences. The number of microsatellite loci amplifying for each species is given in parentheses.

apomictic (i.e. reproduces parthenogenetically by mitotic copying of the maternal genome). Parthenogenesis being the sole reproductive mode helps explain the fast population growth observed in previous studies in the Southern Hemisphere (Dapoto & Giganti, 1994; Urban & Eardley, 1995; Charles & Allan, 2000; Ede *et al*., 2007).

In its native range, *N. oligospilus* has sexual females and males (Koch & Smith, 2000). Thus, the parthenogenesis experienced in the Southern Hemisphere is geographical, where parthenogenesis occurs only in some of the range of the organism. The native range of *N. oligospilus* is extensive, covering most of North America and Europe all the way to the Himalayas (Smith, 1979; Liston, 1995), and it is likely to represent a species complex (Schmidt & Smith, 2009). We cannot discern if the species has a complete absence of sexual reproduction until native populations are assessed thoroughly. Two potential *N. oligospilus* populations were assessed in this study: *Nematus* sp. from Arizona and Montreal. When comparing mtDNA sequences, and considering the relatively modest proportion of microsatellite loci that amplified, *Nematus* sawflies collected in Arizona are likely to be a different species to *N. oligospilus* found in the Southern Hemisphere. The COI sequence divergence of 7.2% is in the range usually seen between members of different species of flying insects (e.g. Hebert *et al*., 2003, 2004; Park *et al*., 2011). In contrast, the *Nematus* sp. collected in Montreal had lower mtDNA sequence divergence (2.6%) and high microsatellite locus cross-amplification, and could be conspecific with *N. oligospilus*. Although, it is unknown if the source of *N. oligospilus* is parthenogenetic or if sexual reproduction occurs, the high genotypic diversity of *Nematus* sp. from Montreal indicates sexual reproduction in a close relative.

Genotypic (clonal) diversity is very low in the invasive range of *N. oligospilus*. The combination of microsatellites and mtDNA sequences in this study showed that the invasive sawfly found in South Africa, New Zealand and Australia are

Table 3. Characteristics of microsatellite loci isolated from *Nematus oligospilus* screened on different sawfly species (number of alleles, number of genotypes, range of allele lengths). Empty cells indicate no amplification

Locus		*Nematus* sp. (USA)	*Nematus* sp. (Canada)	*Nematus iridescens*	*Nematus pavidus*	*Nematus brevivalvis*	*Euura mucronata*	*Pristiphora angulata*	*Pristiphora erichsonii*	*Eitelius dentatus*	*Endelomyia aethiops*	*Caliroa cerasi*	*Rhadinoceraea* sp.
WF2	Allele #	1	1										
	Genotype #	1	1										
	Range	247	211										
WF3	Allele #		1										
	Genotype #		1										
	Range		137										
WF4	Allele #	1	1	1	3	1	4	2		1			
	Genotype #	1	1	1	2	1	3	2		1			
	Range	200	205	196	185–212	179	202–208	197–204		194			
WF5	Allele #	1	1	1	2	3	1	2	1	1	Undefined product	1	1
	Genotype #	1	1	1	2	4	1	3	1	1		1	1
	Range	196	184	140	164–166	190–200	178	196–198	210	198		178	280
WF6	Allele #		1										
	Genotype #		1										
	Range		201										
WF7	Allele #	1	2			2							
	Genotype #	1	1			1							
	Range	102	222–229			202–209							
WF8	Allele #												
	Genotype #												
	Range												
WF9	Allele #	1	1										
	Genotype #	1	1										
	Range	193	198										
WF10	Allele #	1	2		1	1		1	1	1			
	Genotype #	1	2		1	1		1	1	1			
	Range	118	124–127		103	107		103	102	108			
WF11	Allele #	1	1	1		4				1			
	Genotype #	1	1	1		4				1			
	Range	141	142	129		136				123			
WF12	Allele #		2										
	Genotype #		2										
	Range		178–190										
WF13	Allele #												
	Genotype #												
	Range												
WF14	Allele #		1										
	Genotype #		1										
	Range		172										
WF15	Allele #	1	2			2							
	Genotype #	1	1			1							
	Range	370	252–258			241–247							

WF16	Allele #	2	1			2				
	Genotype #	1	1			1				
	Range	267–280	293			264–276				
WF17	Allele #		1							
	Genotype #		1							
	Range		186							
WF18	Allele #		1		2	1				1
	Genotype #		1		2	1				1
	Range		132		132–135	129				132
WF19	Allele #		Undefined product	Undefined product						
	Genotype #									
	Range									
WF20	Allele #		1		2	1				
	Genotype #		1		2	1				
	Range		129		134–136	125				
WF21	Allele #	2	1	1	2	3	3	5	2	
	Genotype #	1	1	1	2	3	3	4	1	
	Range	305–317	339	300	282–284	343–347	303–309	276–286	276–279	
WF22	Allele #	1	1			1				
	Genotype #	1	1			1				
	Range	270	263			267				
WF23	Allele #	2	1	1	2	3	1			
	Genotype #	2	1	1	2	3	1			
	Range	230–232	232	239	226–230	237–241	235			
WF24	Allele #		1							
	Genotype #		1							
	Range		284							
WF25	Allele #		1							
	Genotype #		1							
	Range		508							
WF26	Allele #	2	2	2	4	4	3			2
	Genotype #	1	1	1	2	2	4			1
	Range	410–412	398–420	288–296	238–246	384–412	454–460			495–501
WF27	Allele #	2	2		5	1				2
	Genotype #	1	1		5	1				1
	Range	203–209	128–130		122–131	116				108–110
WF28	Allele #	2	2							
	Genotype #	1	1							
	Range	250–280	198–247							
WF29	Allele #	1	2	1		1	1			
	Genotype #	1	1	1		1	1			
	Range	169	169–171	162		166	166			
WF30	Allele #		1							
	Genotype #		1							
	Range		512							

Table 3. (*Cont.*)

Locus		*Nematus* sp. (USA)	*Nematus* sp. (Canada)	*Nematus iridescens*	*Nematus pavidus*	*Nematus brevivalvis*	*Euura mucronata*	*Pristiphora angulata*	*Pristiphora erichsonii*	*Eitelius dentatus*	*Endelomyia aethiops*	*Caliroa cerasi*	*Rhadinoceraea* sp.
WF31	Allele #	1	1										
	Genotype #	1	1										
	Range	176	174										
WF32	Allele #		2										
	Genotype #		1										
	Range		162–163										
WF33	Allele #	2	1		3	2	2			1			
	Genotype #	1	1		3	2	2			1			
	Range	410–418	412		396–407	416–424	404–405			413			
WF34	Allele #		1										
	Genotype #		1										
	Range		276										
WF35	Allele #		3			2							
	Genotype #		4			1							
	Range		180–189			170–175							
WF36	Allele #	1	1	1	1	5	1			1			
	Genotype #	1	1	1	1	5	1			1			
	Range	186	199	187	179	189–200	180			188			
WF37	Allele #		2										
	Genotype #		2										
	Range		253–273										
WF38	Allele #	1	1										
	Genotype #	1	1										
	Range	109	117										
WF39	Allele #	1	1	1	2	6	1						
	Genotype #	1	1	1	2	5	1						
	Range	198	219	198	186–188	191–208	189						
WF40	Allele #		3										
	Genotype #		3										
	Range		166–194										
WF41	Allele #	1	2	1	3	1	1	3	2	2			
	Genotype #	1	2	1	2	1	1	2	1	1			
	Range	295	290–292	296	282–286	282	279	298–302	288–290	288–290			
WF42	Allele #	1	1	1	1	1	1	1	1	1			
	Genotype #	1	1	1	1	1	1	1	1	1			
	Range	195	202	210	190	205	196	204	220	184			
	Total no. loci	**23**	**39**	**12**	**14**	**21**	**11**	**6**	**5**	**11**	**–**	**1**	**1**
	N	**10**	**8**	**8**	**8**	**6**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
	No. aleles	**30**	**53**	**12**	**33**	**47**	**19**	**14**	**7**	**14**	**–**	**1**	**1**
	No. genotypes	**2**	**8**	**1**	**6**	**6**	**6**	**8**	**1**	**1**	**–**	**1**	**1**
	Ho	**0.151**	**0.220**	**0.024**	**0.079**	**0.152**	**0.043**	**0.053**	**0.024**	**0.073**	**–**	**–**	**–**
	He	**0.113**	**0.146**	**0.012**	**0.131**	**0.168**	**0.060**	**0.051**	**0.012**	**0.037**	**–**	**–**	**–**
	F_{IS}	**−0.144**	**−0.401**	**−1.000**	**0.423**	**0.043**	**0.337**	**−0.075**	**−1.000**	**−1.000**	**–**	**–**	**–**

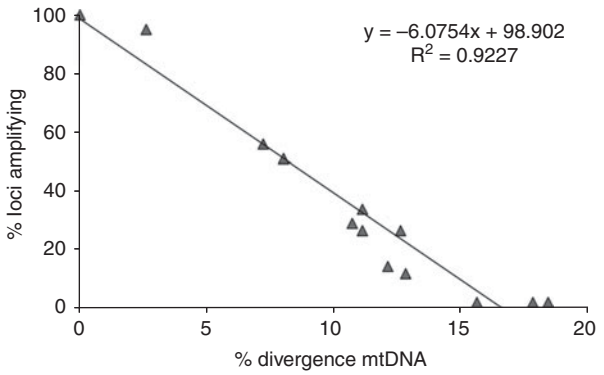

Fig. 2. Relationship between the percentage of loci amplifying successfully and the percentage of interspecific sequence divergence as compared to the mitochondrial DNA cytochrome-oxidase I gene from *Nematus oligospilus* in the invasive range.

extremely genetically similar. They have apparently identical mtDNA sequences (certainly in the COI region), and microsatellite loci showed very similar (often identical as far as could be discerned) alleles and genotypes. Differences seen among *N. oligospilus* clones in the invasive range could be due to either the introduction of different clonal lineages or to mutations arising since introductions. Success of some invasive species has been attributed to the introductions of multiple clones, as in the pea aphid, *Acyrthosiphon pisum* Harris (Peccoud *et al.*, 2008). In the case of *N. oligospilus*, the low number of closely related clones and one widespread 'main' genotype suggest one or very few colonization events. In apomictic parthenogens, genotypes can be altered by mutations and chromosomal rearrangements (Loxdale, 2010). Slippage mutation during the replication of DNA is the main mutational process occurring in microsatellites (Schlotterer & Tautz, 1992). In *N. oligospilus* loci having more than one genotype, the difference between alleles of the main genotype and the novel alleles were the equivalent of losing or gaining a microsatellite repeat with equal probability. This shows that the differences in clonal lineages arose following non-directional mutations, at least roughly consistent with the stepwise mutation model, in which mutations are by single repeat slips rather than larger ones and unbiased in direction (Chambers & MacAvoy, 2000). Further work is necessary to assess the spatial and temporal distributions of those clones, as well as their dispersal and host relationships.

Other sawfly species

Cross-amplification success of the new microsatellite markers was high for some other sawfly species tested. Relative to *N. oligospilus*, the proportions of additional genotypes discovered per individual screened were higher in some species, even though few individuals were screened for each species and most originated from a single site. Species such as *C. cerasi* are also thought to be parthenogenetic in Australia since males have not been collected (Naumann *et al.*, 2002), while other species such as *N. brevivalvis* were inferred here to be sexual, showing more allelic diversity in a few individuals than *N. oligospilus* over a large part of its invasive range as here sampled.

As shown in other studies of broad taxonomic groups, here the more closely related to *N. oligospilus* a species was, the more successful microsatellite amplification (Zenger *et al.*, 2003; Primmer *et al.*, 2005). Overall, the genus *Nematus* and the tribe Nematini had high proportion of successful amplification. The Pristiphorini tribe had lower success, but most loci that did amplify revealed multiple alleles. A review on the success of cross-amplification of microsatellites shows that different taxonomic groups have different success rates. As a generalization for arthropods, over 60% of loci developed for a species will amplify in congenerics. This decreases rapidly for different genera in the same family (Barbarà *et al.*, 2007). Since largest genera in the Nematinae subfamily tend to be paraphyletic (Nyman *et al.*, 2006b), it would not be judicious to predict the success of microsatellite markers at the level of genus; instead, the present results suggest that COI mtDNA sequence divergence is a very good predictor. In general though, microsatellite markers developed in this study are likely to be generally useful within the tribe Nematini, and still useful but less so for members of other tribes and subfamilies.

Due to their fairly high cross-amplification, carefully chosen microsatellite markers from the suite as here developed would allow the simultaneous study of several species, allowing comparison between species having different life strategies and reproductive modes (and indeed elucidating those modes in the first place). Furthermore, some genera such as *Euura* have been more thoroughly studied than others and already form strong model systems (Craig *et al.*, 1992; Roininen *et al.*, 1996; Hjalten & Price, 1997; Nyman, 2002; McGeoch & Price, 2005; Price & Hunter, 2005). Microsatellites used in conjunction with such models could be useful to test further ecological and evolutionary theories.

Conclusions

To manage an invasive species effectively, knowledge of its biology and ecology are essential. Reproductive mode and genotypic diversity can contribute to predicting impacts, distribution and population densities of invasive species. The present study showed that *N. oligospilus* is functionally parthenogenetic within its invasive range, with populations comprising only a small number of very closely related clones, which might go some way to explaining the rapid population growth seen in the introduced range (Caron, 2011). In Australia and other Southern Hemisphere countries, *N. oligospilus* would appear to be an excellent study system for understanding evolution of invasive parthenogens that diverge without sexual reproduction. Furthermore, the microsatellites as developed represent useful tools for new and more highly-resolving approaches in well-established model systems, but also to study multiple species simultaneously.

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