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Review

Applying molecular-based approaches to classical biological control of weeds

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ABSTRACT

The use of molecular techniques is rapidly growing as the tools have become more diverse and powerful, more widely available, and easier to implement. Molecular analyses are able to elucidate information about target weeds that is critical to improving control success, such as taxonomic clarification, evidence of hybridization and cryptic species, better development of test plant lists, population structure and origin of invasions. Similarly, molecular approaches can improve our knowledge of biological control agents, providing taxonomic clarity, identification of immature arthropods and fungal pathogens, and description of genetic variability in agents. Molecular tools also allow easier identification of host associations and provide a tool for post-release evaluation and tracking of agents. This review provides an overview of how to use molecular approaches in biological control of weeds, with the aim of assisting the adoption and facilitating fruitful collaboration between scientists studying the biology and ecology of agents and their targets and those with skills using molecular approaches. We describe the current molecular techniques relevant to classical biological control of weeds, instruct how to collect field materials for molecular analyses, and give recent examples of the use of molecular methods in biological control of weeds, with comments on the most appropriate methods for analysis of molecular data.

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1. Introduction

A critical goal of scientists engaged in biological control of weeds is to reduce the risk of non-target and indirect effects by releasing fewer, more host-specific, and more effective agents (Strong and Pemberton, 2001; Louda et al., 2003; Balciunas, 2004). Molecular-based approaches have much to offer in attaining this goal (Briese, 2005; Goolsby et al., 2006), providing both pre- and post-release information that is simply not available, or not readily available, using other means. Molecular data can clarify taxonomy and evolutionary relationships, uncover evidence of closely related species that cannot be morphologically distinguished (cryptic species) and hybridization events, elucidate methods of reproduction and complex life cycles of pathogens, determine population structure and origins of target weeds and agents, and identify arthropods at immature life stages which cannot otherwise be distinguished from similar species.

Molecular methods, like biological control, have their limitations. Molecular genetic data are most useful when good taxonomic, morphological, ecological, historical and demographic information are available to complement them and provide the context for understanding results. Molecular methods can also be costly and time consuming, and at times, gaps in biological control programs can be addressed without resorting to them. But DNA based molecular markers have the advantage that they are not normally influenced by environmental stimuli or plasticity, unlike most morphological and other phenotypic data. In addition, the variety of molecular methods is continually expanding so that some questions can now be more easily answered, and improvements in protocols and equipment are making these tools cheaper and more accessible to those that do not specialize in their use.

There have been previous reviews of the use of molecular approaches for biological control and invasions (e.g. Nissen et al., 1995; Unruh and Wooley, 1999; Antonini et al., 2008; Rector,

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2008; Le Roux and Wieczorek, 2009), but no recent ones specific to classical biological control of weeds, a discipline which presents some unique challenges worth discussing. Also, due to very recent advances in the molecular field, an update is required. In addition to this paper, there are publications that provide good examples of uses of diagnostic markers for the biological control of arthropods (Gariepy et al., 2007), the importance of molecular tools to native range studies (Goolsby et al., 2006), how to sample for plant genetic diversity (Ward and Jasieniuk, 2009), and how molecular tools help in the analysis of risk to non-target plants (Sheppard et al., 2005; Berner et al., 2009).

This review has three goals: (1) to describe the current molecular techniques that could be used during the development and practice of classical biological control of weeds, and to suggest which tools are best suited to address which questions, (2) to describe how to properly collect field materials for molecular analyses, and (3) to give recent examples of the use of molecular methods in biological control of weeds, with comments on the most appropriate methods for analysis of molecular data.

2. The current toolbox of molecular techniques

Readers may wish to skim through this section, and return later to for more in-depth descriptions of techniques and citations that apply to their specific research needs. Tables 1 and 3 provide a quick reference of which molecular techniques are typically used to answer questions in biological control of weeds. Techniques are applicable to plants, arthropods and microorganisms, unless otherwise indicated in Table 1.

The methods in Sections 2.1.3–2.3 typically utilize the polymerase chain reaction (PCR), a technique to amplify a single or a few copies of a piece of DNA into thousands to millions of copies. PCR relies on an enzymatic replication performed by a heat-stable DNA polymerase (after which the method is named). The DNA polymerase assembles a new DNA strand from added DNA building blocks (nucleotides) by using single-stranded DNA (the target region) from the organism of choice as a template and DNA oligonucleotides (also called primers), which are required for initiation of DNA synthesis. The technique also relies on alternately heating and cooling the target region DNA to a defined series of temperature steps. First, a high temperature step physically separates the two strands of the DNA double helix, then, at a lower temperature step, the chosen primers anneal (DNA pairing by hydrogen bonds to a complementary sequence) to the single stranded DNA, and the polymerase creates a complement of the target DNA starting at the end of each primer. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions. As PCR progresses, the short DNA strands generated are themselves used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

2.1. Molecular markers requiring no prior knowledge of the target organism genome

2.1.1. Karyotype and cytogenetics

Ploidy determinations in plants have traditionally been done only by classical chromosome counts from mitotic plates of contracted and well-spread chromosomes that are usually obtained from root tips of young germinations (Sessions, 1996) or less commonly from flower buds (Reveal and Styer, 1974). For insects, embryos are the best sources of mitotic divisions, but the midgut or salivary glands may be used (Sessions, 1996). Chromosome counting is laborious, particularly if many individuals need to be evaluated and if chromosomes are small and in high numbers. More recently, flow cytometry is commonly used for determining the DNA content of nuclei and deducing ploidy level, because it permits sensitive measurements of fluorescence intensity of large numbers of stained nuclei within seconds (Galbraith et al., 1983). For plants, fresh leaves are recommended but roots may also be used, and for insects, haemolymph cells from larvae are utilized. Many plant species contain individuals with different ploidy levels and different numbers of chromosome. The ability to count chromosome numbers in these species can help determine origins, population structure and reproductive barriers of invasive plant lineages.

Identification of alien chromosomes, of chromosome segments shared among several species and of meiotic pairing in hybrids, is also important when inferring origin and evolution in weeds, particularly polyploids. FISH (Fluorescent In Situ Hybridization;

Table 1
A comparison of genetic techniques useful for biological control of weeds programs[a].

Molecular technique	Common applications	Variability	Dominance[b]	Cost
No prior knowledge of genome required				
Karyotype/cytogenetics	Ploidy level/matching chromosomes	Low[c]	Not applicable	$-$$
Allozymes/proteins	Population genetics	Low[c]	Co-dominant	$
RFLPs	Population genetics	Low-moderate[d]	Co-dominant	$
RAPDs/ISSRs/UP-PCR/DAF	Population genetics	Moderate-high[d]	Dominant	$
AFLPs	Population genetics	High[d]	Dominant	$-$$
Prior knowledge of genome required				
SSRs	Population genetics	Moderate-high[d]	Co-dominant	$$-$$$
DNA sequencing				
Chloroplast		Low-moderate[e]	Uniparental	$$
Mitochondrial	Insect/fungal population genetics, phylogenetics	Low–high[e]	Uniparental	$$
Nuclear	Population genetics, phylogenetics	Low-moderate[e]	Co-dominant	$$
SNPs	Population genetics/genotyping	High[d]	Co-dominant	$$$
SSCP/DSCP/DGGE	Determine existence of differing alleles	Not applicable[d]	Co-dominant	$-$$
RT-qPCR	Detection and quantification specific of DNA	High[d]	Co-dominant	$$-$$$
ESTs/Microarrays	Find/quantify expressed genes	High[d]	Dominant and co-dominant	$$-$$$

$ = relatively inexpensive; $$ = moderately expensive; $$$ = relatively expensive.

[a] Includes information adapted from Le Roux and Wieczorek (2009), Sunnucks (2000).

[b] Co-dominant means both alleles of a diploid individual can be distinguished, dominant means it is not possible to distinguish between the two alleles present in a diploid individual, and uniparental means that there is only one allele, because only one copy of the genome is present in an individual.

[c] Marker typically used for interspecific studies.

[d] Marker typically used for intraspecific studies.

[e] Marker used for both inter- and intraspecific studies.

Fregonezi et al., 2004; Basu and Zwenger, 2009) and GISH (Genomic In Situ Hybridization; Iqbal et al., 2002) are the two cytogenetic techniques commonly employed. The first uses a genomic region or gene and the second the total genomic DNA from a donor species as a probe on cytological preparations to identify genome structure and evolution of the targeted weed. Using FISH, Fregonezi et al. (2004) revealed ultra-structural similarities within chromosome arms of weeds in the Asteraceae family that exhibited karyotypic differences.

2.1.2. Allozymes and proteins

Allozymes are the different protein forms encoded by various alleles at one locus. The allozyme method consists of separating enzyme molecules in a crude or purified homogenate by electrophoresis through a supporting matrix (generally starch or acrylamide) (Murphy et al., 1996). Allozymes have particularly been useful in plant population studies, but may also be applicable to arthropods. As most enzymes are temperature labile, fresh or frozen materials are required. For plants, leaves are recommended, although other parts can be used. The banding profile obtained for a particular allozyme marker may change depending on the type of tissue (e.g. root vs. leaf) because a gene that is being expressed in one tissue might not be expressed in the other. In contrast, insects are often used whole. The allozyme method has been applied in numerous population genetics studies, including measurements of outcrossing rates, subpopulation structure and population divergence (Kephart, 1990; Murphy et al., 1996). In several cases, these markers have proved their efficiency in determining the dynamics of introduction and spread of invasive weeds (see review by Novak and Mack, 2005).

Deciphering the Mendelian genetic variation of the observed phenotype (banding pattern or zymogram) is the most difficult step in the allozyme method (Kephart, 1990; Murphy et al., 1996). Phenotypes are interpretable as genotypes given prior knowledge of the ploidy and the quaternary structure of the enzymes. Some of the enzymes are made of one copy of the polypeptide chain (monomer), two copies (dimer) or four subunits (tetramer). Allozymes are co-dominant markers (alleles or variants from each parent can be observed in the offspring, whereas for dominant markers, such as RAPDs discussed below, only one parental allele will be observed in the offspring) that have high reproducibility. Zymograms can be readily interpreted in terms of loci and alleles, or they may require segregation analysis of progeny of known parental crosses for interpretation. Sometimes, however, zymograms present complex banding profiles arising from polyploidy or duplicated genes and the combination of multiple and differing enzyme copies may complicate interpretation. Overall, allozymes are simple, quick, and relatively cheap to use although for some species considerable optimization of techniques may be required for certain enzymes.

2.1.3. RFLPs

Restriction Fragment Length Polymorphisms (RFLPs) involve the use of restriction endonuclease enzymes that are site specific. If the individual's DNA contains the short nucleotide sequence that is recognized by the restriction enzyme, the DNA will be cut at that location. The resulting DNA fragment or fragments can be separated by standard agarose gel electrophoresis and made visible by fluorescent staining (Dowling et al., 1996). Any differential cutting of DNA between different samples indicates that variation is present. The starting material can be any part of the organism. RFLPs can be applied to the entire genomic DNA, mitochondrial DNA (mtDNA) or plastid DNA (e.g. cpDNA) or only to a region within the genome. RFLPs are co-dominant markers and provide a relatively simple and cheap method for analyzing unpublished sequence data. However, when the RFLP method is not PCR-based, high quantity and quality of total starting DNA is necessary.

2.1.4. RAPDs, ISSRs, UP-PCR, DAF

Several single, arbitrary primer-based DNA amplification techniques (RAPDs, ISSRs, UP-PCR, and DAF) are available and can be grouped under the general acronym MAAP (Multiple Arbitrary Amplicon Profiling) (Caetano-Anolles et al., 1994).

MAAP involves the use of a short, arbitrarily chosen primer which anneals to genomic DNA. The PCR method is then used for amplification of multiple genome regions whose length is determined by where the primer annealed. MAAP is general, so that a primer used for one species can be used for others, even if evolutionary distances between the species are large. When the genome region at the priming sites varies in length, the amplified fragments will also be of variable length. If sequence variation at the priming site affects annealing, then the fragment may not amplify in some samples. Under carefully controlled PCR, these two factors explain most of the observed variation that is revealed through a simple agarose gel electrophoresis (Hoelzel and Green, 1998). If gel electrophoresis does not provide sufficient resolution between fragments, automated capillary electrophoresis coupled with laser-induced fluorescence detection can be used, although it is a more expensive method. With fluorescence methods, many more fragments may be detected, thus requiring software programs based on advanced analysis algorithms that can rapidly and accurately identify common and different MAAP fragments among large number of samples. The required quantity of DNA extracted from an organism can be very low as in most PCR-based techniques. However, the generation of reproducible and comparable banding patterns is very dependent on the quality of the extracted DNA (degraded DNA may result in missing bands that would be present if the sample were better preserved; longer pieces of DNA are also more likely to degrade before shorter pieces) and PCR conditions such as annealing temperature. All these markers are dominant, and technically easy and cheap to use.

Random Amplification of Polymorphic DNAs (RAPDs), first reported by Williams et al. (1990), consists of using a single 10-nucleotide base primer and low stringency annealing conditions (allowing the primer to anneal to multiple locations in the genome) in the PCR. The method Inter Simple Sequence Repeats (ISSRs) as described by Wolfe et al. (1998) is nearly identical to RAPDs except that ISSR primer sequences are designed to anneal to common microsatellite regions (see Section 2.2.1). No prior knowledge of the microsatellite sequences is required. The ISSR process amplifies the regions between those microsatellites using PCR and the annealing temperatures used are higher (and thus more stringent) than those used for RAPDs markers. Protocols and the most current ISSR primers can be found at http://www.biosci.ohio-state.edu/~awolfe/ISSR/ISSR.html.

Another method similar to RAPDs is the Universally Primed-PCR (UP-PCR) (Bulat et al., 1994) although it differs by the use of UP primers which are relatively long (15–18 nucleotides) and designed for fingerprinting at relatively high annealing temperatures. UP-primers primarily target intergenic, more variable areas of the genome and for this reason the method is especially suitable for detection of intraspecific variation. Caetano-Anolles et al. (1991) developed DNA Amplification Fingerprinting (DAF). Of all MAAP procedures, DAF utilizes the shortest primers, down to five nucleotides in length. DAF products are routinely separated through thin polyacrylamide gels that are stained by silver nitrate. All these dominant markers can be converted into co-dominant ones through the Sequence Characterized Amplified Region (SCAR) approach (Paran and Michelmore, 1993). Basically, PCR products that appear specific to samples can be gel extracted and cloned prior to

sequencing (see Section 2.2.2). Sequence information is used to design primers for PCR amplification of the respective SCARs.

2.1.5. AFLPs and SAMPL

Amplified Fragment Length Polymorphisms (AFLPs) described by Vos et al. (1995) is a method based on the selective amplification of sets of restriction fragments obtained after cleavage of genomic DNA with a pair of restriction enzymes (usually, one that cuts frequently and one rarely). Short nucleotide sequences that complement the cut end of the digested DNA are attached to each end of these fragments. PCR primers designed to complement the sequence of the adaptors are used to amplify the fragments in a pre-selective PCR. The number of fragments amplified is intentionally limited by including one or two arbitrarily chosen extra nucleotides at the 3'end of the primer, which may or may not complement the DNA fragment. Then, a selective PCR is used with 1–4 additional arbitrary nucleotides attached to the same primers, allowing amplification of only a subset of the many thousands of restriction fragments as observed in RFLPs. As in the MAAP approach, products were traditionally separated through a high resolution (agarose or acrylamide) electrophoresis gel, but nowadays are typically run under an automated fluorescence-based multicapillary electrophoresis system.

Selective Amplification of Microsatellite Polymorphic Loci (SAMPL; Witsenboer et al., 1997) is similar to AFLPs but differs in using a combination of one adaptor specific primer (like AFLP) and one primer that anneals to a microsatellite (see Section 2.2.1) in the selective PCR. This can reveal more genetic variation than a typical AFLP, as microsatellite loci are hyper-variable regions in the genome.

2.2. Molecular markers requiring prior knowledge of the target organism genome or of a closely related organism

2.2.1. SSRs

Microsatellites, or Simple Sequence Repeats (SSRs) are short, tandemly repeated sequence motifs consisting of iterations of 1–6 nucleotides in length (e.g. GTGTGTGT or CTACTACTA) that have been detected in the genomes of every organism analysed so far (Tautz, 1989). They are highly polymorphic DNA markers with discrete loci and co-dominant alleles (Schlötterer, 1998), and can exhibit length variation even between closely related individuals. The most common mutations are changes of a single repeat unit and the observed mutation rates range from 10^{-2} to 10^{-6} events per locus per generation (Li et al., 2002). Primers flanking the repeats are used for PCR and the sizing of the products (allele) on high resolution gels or fluorescence-based multi-capillary electrophoresis systems allows for the determination of the number of repeats. But before each microsatellite locus can be amplified, sequence information for the flanking DNA is required to allow the design of specific primers. Therefore, for many organisms, the isolation of microsatellite loci and their flanking regions still remains the first step in their analysis. This step can be time consuming and relatively costly. In some cases, the development of microsatellite markers can be based on DNA sequence information deposited in databases, or microsatellites developed in model organisms that cross-amplify in closely related species. Several protocols exist for the isolation of microsatellites from genomic DNA such as the creation of genomic libraries (collections of DNA fragments) enriched for microsatellite sequences. This strategy is devised to increase the opportunity for marker discovery, notwithstanding it typically provides a few hundred sequences (Schlötterer, 1998). The recent emergence of next generation sequencing, such as pyrosequencing (see Section 2.2.2), could lead to larger amounts of sequences at lower cost and hence maximize the chance of discovering microsatellites (Castoe et al., 2010). Concomitantly, new bioinformatic tools have been developed for microsatellite detection and primer design from such larger sets of sequences (Meglecz et al., 2010).

2.2.2. Sequence data

Sequencing technologies are extensively described in Hillis et al. (1996). Current methods can directly sequence only relatively short (300–1000 nucleotides) DNA fragments in a single reaction. PCR products can be directly sequenced or sequenced after cloning (cloning is a process in which just one copy of a fragment of DNA is inserted into and amplified by a bacterium as the bacterium reproduces. This process is useful for selecting a single copy of DNA for sequencing when there are multiple copies present that may differ in their sequences within an individual). Pyrosequencing is a new method for quickly obtaining sequences of short DNA segments (300–500 nucleotides), which can be assembled with software to create whole or partial genome sequences. PCR products can be easily shipped to private companies worldwide for DNA sequencing. Depending upon the taxa and the questions addressed, different genomes and regions can be targeted, each with varying rates of evolution and modes of inheritance (Table 2).

DNA is either inherited from one or both parents. A single copy of a portion of DNA in an individual is called a haplotype (e.g. mitochondrial or chloroplast DNA, or just one parent's contribution of nuclear DNA), while the combination of both parental contributions is a genotype. Genotypes and haplotypes (or even phenotypic data) can each be used to construct phylogenies, which estimate and describe evolutionary relationships among taxa. The DNA sequences of a group of organisms are aligned among individuals, and methods such as maximum parsimony or likelihood utilize evolutionary models to derive the phylogeny, typically done at the interspecific level or higher, where gene flow is not expected between taxa. For analyses at the intraspecific level, where gene flow would be expected, haplotype networks are typically utilized. Position of a haplotype in a network provides information; e.g. haplotypes that are internal in the network may be older, and haplotypes at the tip of the network may be more recently evolved. A formal construction of a network of all observed haplotypes can be performed with the software TCS and the implemented algorithm (Clement et al., 2000), followed by nested clade analysis, an approach which uses gene genealogies and their geographic distributions to separate population structure from population history (though see Beaumont et al., 2010 for known problems with nested clade analysis).

The utilization of a small section of DNA for identification to species is often called DNA bar-coding (Hebert et al. 2003), and the method is not without controversy (Rubinoff et al. 2006). The DNA sequence used may not have adequate resolution to distinguish closely related species, or the various genomes in an organism may have different evolutionary histories (e.g. mitochondrial vs. nuclear). Also, the DNA sequence obtained is often compared to those in publicly available databanks (e.g. GenBank), and the reference sequence may come from a misidentified specimen or be a sequence of questionable quality with many misread nucleotides. Regardless, when used with appropriate caution and resolution, DNA sequence comparison can be a very effective tool for identification of unknown organisms.

2.2.3. Single nucleotide polymorphism (SNP)

A SNP is DNA sequence variation occurring when a single nucleotide in the genome differs between two individuals or between paired chromosomes in an individual. They are the most abundant of all DNA variants known so far in animal and plant genomes (Gupta et al., 2001). Some freely available plant SNP databases already exist and are listed in Basu and Zwenger (2009). Several approaches are used for the search and typing of SNPs as outlined by

Table 2
Commonly used genomic regions in biological control of weeds studies.

	Lineage/genomic region		
	Mitochondrion	Chloroplast	Nucleus
Insect			
	Cytochrome oxidase I and II (*COI* and *COII*) Cytochrome oxidase b (*Cytb*) NADH dehydrogenase I 12S 16S		Ribosomal DNA internal transcribed spacer (ITS) 1 & 2 and surrounding regions
Plant			
	Cytb	tRNA intergenic spacers (e.g. *trnT-trnF*)	Ribosomal DNA internal transcribed spacer (ITS) 1 & 2 and surrounding regions
	16S	Ribulose 1,5-bisphosphate carboxylase (*rbcL*) RNA polymerase C1 and C2 (*rpoC1* and *rpoC2*)	
Fungi			
	COI		Ribosomal DNA internal transcribed spacer (ITS) 1 & 2 and surrounding regions
	Cytb		Translation elongation factor subunit 1a (*tefl*) RNA polymerase subunit 2 (*rpb2*) actin (*act*)

Kwok (2001), but they are relatively costly although very powerful in terms of data supplied. Before deciding on a particular protocol to use, Kwok recommended considering the following factors: (1) scope of genotyping (numbers of SNPs to be screened, number of samples to be tested, as well as how many genotyping projects are to be conducted at the same time), (2) level of molecular biology expertise in the lab, and (3) cost of capital investment and consumables.

2.3. Analytical methods complementary to sequencing and gel analysis

2.3.1. SSCP, DSCP, DGGE

Several methods have been developed for simple gel analysis to determine if sequence differences exist in PCR products of identical length, indicating if sequencing would be fruitful to perform. They are mainly based on the notion that the physical melting properties or conformation of DNA molecules depend on the sequence itself. Single-Stranded Conformation Polymorphism (SSCP) separates DNA based on the conformation that each single-stranded DNA segment will take when it is quickly chilled and folded onto itself (Dean and Miligan, 1998). Double Strand Conformation Polymorphism (DSCP) is detected when mutations alter the curvature of the helical axis of double stranded DNA molecules, resulting in changes in their electrophoretic mobility in a gel (Saad et al., 1994). Both methods are sensitive with shorter fragments (<300 nucleotides). Denaturing Gradient Gel Electrophoresis (DGGE) is based on the fact that physical melting properties of double stranded DNA depend on the sequence itself and that homozygous (both DNA strands have the same nucleotide sequence) and heterozygous (some nucleotide differences exist between strands) DNA molecules travel at different speeds through a gel matrix (Dean and Miligan, 1998). Because DGGE relies on samples experiencing non-uniform denaturing conditions in gels, it requires a more sophisticated and costly gel apparatus than the two other methods.

2.3.2. Real-time qPCR (RT-qPCR)

Real-time quantitative PCR is becoming a well-established technology for studying gene expression and also genetic variation. This technique is based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule in real time during each PCR cycle and requires a specialized PCR machine (Higuchi et al., 1993). This has been recently applied to the detection of SNPs in combination with HRM (High-Resolution Melting) technology (Grievink and Stowell, 2008). HRM was recently introduced as a homogeneous closed-tube system that allows SNPs mutation scanning and genotyping without the need for many costly labelled primers. It relies on a new generation of generic heteroduplex (heterozygous)-detecting double-stranded DNA binding dyes. Heteroduplex products are identified by the presence of a second low-temperature melting transition. The entire experiment, including real-time and post-PCR analysis, can be done on one instrument in either a 96- or 384-well format and can be completed within 1 h. Several technical platforms exist on the market.

2.3.3. ESTs and microarrays

More recently, functional genomics, through the analysis of the transcriptional state of an organism, has appeared as an approach that can provide information on the genetic components responsible for making a weed an invasive species (as reviewed by Basu and Zwenger, 2009; Broz and Vivanco, 2009). Basically, identification of gene transcripts is done via Expressed Sequence Tags (EST). An EST is a short sub-sequence of a transcribed complementary DNA (cDNA) sequence. First, mRNA is isolated from the organism and reverse transcribed into cDNA. The cDNAs can be PCR amplified and cloned to create an EST library. An EST is produced by one-shot sequencing of the clone resulting in sequences of approximately 500–800 nucleotides, representing portions of expressed genes. Basu and Zwenger (2009) report that more than 3 million sequences from more than 200 plant species have been deposited in a publicly available EST database, and some are weeds (http://www.ncbi.nlm.nih.gov/dbEST). However, the method that has become a standard in global gene expression experiments is the microarray (Lee and Tranel, 2008). In microarray experiments, the probes are hundreds or thousands of selected DNA sequences representing different genes. The probes are attached to a solid support, called a chip. The target molecules are generated from cDNA from the tissue of interest (e.g. shoot tissue after herbicide treatment, dormant seeds), either fluorescently or radioactively labelled. The pools of the labelled cDNA are incubated with the probes immobilized on the chip. Through DNA association kinetics, labelled cDNA molecules hybridize with their corresponding probes. Non-hybridized cDNA is washed away. The remaining fluorescence at a given spot on the chip is proportional to the abundance of that transcript in the sample RNA pool. There are several different microarray chips or platforms that are commercially available. These platforms differ in the origin of probes (spotted cDNA or oligonucelotide arrays), the density or number of

probes, and how these probes are attached to the chip (Lee and Tranel, 2008).

2.4. Software

Software programs available for analysis of molecular data are too numerous to list and are well covered in other publications (Excoffier and Heckel, 2006) and various websites and discussion forums such as:

Phylogeny Programs; http://evolution.genetics.washington.edu/phylip/software.html;
Softlinks Phylogenetic and Population Genetic Software: http://research.amnh.org/users/koloko/softlinks/; and
The Genetic Software Forum: http://gsf.gc.ucdavis.edu/.

In some examples below, the commonly used software is noted.

3. How to properly collect field materials for molecular analyses

3.1. Collecting plant material

3.1.1. Collection protocols for plant genetic evaluation

Regardless of the study organism involved, there is no general consensus regarding the number of individuals that should be sampled per population for genetic purposes (Muirhead et al., 2008), though the answer clearly depends on the research question being asked. For plants, it is difficult to estimate the accurate collection size, as a sampled patch does not necessarily represent a clearly defined biological unit (Gaskin, 2006), but 15–30 units per population are routinely sampled, with a range of 3–60 samples (Ward and Jasieniuk, 2009). Total sample size has to be greater in selfing than in cross-pollinating species if trying to capture the range of genetic variation (Brown and Biggs, 1991). The minimum distance between collected plants ranges from 10 to 15 m (Marrs et al., 2008a; Wang et al., 2010), but clearly depends on the reproductive biology, i.e., clonal vs. sexual. Both in the native and introduced range of a species, the minimum distance between populations is usually suggested to be 50 km.

Leaf tissue is often collected for DNA samples, but seed collections are also common, with DNA being extracted from plants grown from those seeds. For leaf collections, young leaves will have a better ratio of DNA to tissue volume, and may contain fewer contaminants from disease or feeding. For species with high levels of PCR inhibiting secondary compounds such as polysaccharides and polyphenols, less chemically-defended tissue (e.g. petals in some species) may be appropriate. If not available, etiolated material and/or specialized DNA extraction protocols may be necessary (e.g. Peterson et al., 1997; Rether et al., 1993; Michiels et al., 2003). Testing of extractions and down-stream application success is advised before making large field collections. Depending on the leaf size, 2–5 young leaves per sample (1–20 g total) representing a total surface of 4–50 cm^2 is sufficient material for DNA analysis (Chase and Hills, 1991; Amsellem et al., 2000; Marrs et al., 2008a). For isozyme studies, higher quantity of materials might be required.

For guaranteeing the "phytosanitary" quality of collected material, which can be especially important for applications such as AFLPs and RAPDs, and for export permits, leaves must be entire, with no evidence of disease (e.g. leaf spots) or herbivory by arthropods (e.g. leaf mines or galls).

Field-collected dry seeds are usually stored in paper coin envelopes (small paper bags). If the main purpose of collecting seeds is to provide material for genetic or enzymatic analyses, fully mature, disease-free seeds are highly recommended. It also is important to check seeds extracted individually from seed capsules, berries, spikelets, or follicles, for presence of arthropods prior to storage in paper bags. During transportation and storage, avoid microorganism development by keeping moisture content low. At the lab, freezing of material at −20 °C for 48 h is advised to kill undesirable seed-inhabiting arthropods, unless such treatment affects seed viability.

At each sampling location, for each population, it is useful to collect herbarium voucher specimens. Depending on the research question, either one voucher per population or one per individual collected will be needed. Each record should be accompanied with GPS coordinates, collection date (to assist with timing of future collections), and perhaps a description of the population, plant community, potential biological control agents found, name of collector and abiotic data.

3.1.2. Plant DNA preservation

When collections are made far from the genetic lab, the most common technique is to use a desiccant such as silica gel/crystals for preserving DNA. Tissue and silica can be kept in sealable plastic or paper bags such as mailing envelopes. Certain desiccants contain an indicator that changes color when unable to absorb more moisture; indicating that plant tissue is at risk of degrading or attack by fungi. Older silica formulations that contain blue moisture indicator crystals of cobalt chloride (a carcinogen) should be avoided. Desiccant should not be reused to dry samples for genetic analyses because cross-contamination may occur. The size and thickness of the material will determine how much desiccant to use and how many times it will have to be replaced to insure complete drying. Using at least a 10:1 w/w ratio of silica to tissue is a good starting point. Quicker drying leads to better preservation. Blotting leaves before placing in contact with the silica gel will reduce the number of times that the desiccant should be replaced, which is especially important for aquatic plants or weeds collected in wet conditions. Silica dried tissue is stable for at least a few years at room temperature, or longer if refrigerated or frozen. In very humid climates, silica may not stay dry during fieldwork. An alternative is then to store plant material in a NaCl/CTAB solution (Storchova et al., 2000).

FTA® paper (Whatman, Inc.), a recent alternative to storage in silica gel, is a simple technology ideally suited for field workers. FTA paper is a cellulose-based matrix containing chemicals for cell lysis and nucleic acid preservation at ambient temperature. The chemicals are activated when biological fluid contacts the surface. An additional feature of this chemical treatment is bacterial and viral inactivation which allows transportation of biosamples at ambient temperature without the requirement of a biohazard shipping label. Storage of the plant DNA is achieved by pounding the fresh leaf material directly onto the FTA paper. FTA cards can be stored at room temperature for years (Lin et al., 2000; Mbogori et al., 2006).

When collecting for gene expression analysis, an alternative to collecting fresh, green plant tissue is to use an RNA stabilization solution. Small plant parts can be placed in a 5 mL vial containing this solution (Broz et al., 2009). RNA stabilization solutions eliminate the need to immediately process or freeze samples by effectively deactivating all enzymatic activity (endo and exonuclease activity) for 24 h at 37 °C, 7 days at 18–25 °C, 2 weeks at 4 °C and indefinitely at −20 °C.

3.2. Collecting arthropods

3.2.1. Collection protocols for insect genetic evaluation

The number of arthropod individuals to collect depends on the research questions asked, and ease of collection in the field. In general, as for plants, 15 to 30 individuals is common, and fairly robust

for most population-level analyses. Depending upon the species, adults or larvae are collected with sweep-nets or individually with forceps or aspirators. Typically, individuals will be placed directly into separate vials pre-filled with 95% ethanol. For species that are too small to count in the field such as many mites, infested plant tissue is placed directly into vials of ethanol and later sorted into individual tubes (e.g. Evans et al., 2008). For genetic diversity samples, however, it is important to sample fairly broadly across the site so as not to sample large pockets of related individuals.

3.2.2. Non-destructive DNA extraction from preserved and living arthropods

There is often a need for DNA from rare specimens or even historic museum specimens that were preserved long ago and not always in good condition. However, a major constraint on the use of historical or ancient specimens remains not only in the post-mortem degradation of DNA to sub-amplifiable levels but also in the destructive nature of the sampling procedure. This is of particular concern with minute arthropods where even limited sampling may destroy important morphological characters. Development of non-invasive DNA extraction methods for preserved arthropods has recently made considerable progress (e.g. Rowley et al., 2007; Hunter et al., 2008) because DNA from specimens can be induced to leak into extraction buffers (Gilbert et al., 2007). Gilbert and co-workers were able to amplify 220 bp of mtDNA and 250–345 bp of nuclear DNA in 71% or 79% of assayed specimens, respectively. Recently, Thomsen et al. (2009) and King et al. (2009) demonstrated that it was possible to obtain, non-destructively, DNA from an ancient insect up to 26,000 years old. Shokralla et al. (2010) obtained amplifiable quantities of caterpillar DNA from mescal, the alcoholic beverage famous for the "worm" that is placed in the bottle of many brands, and so gave evidence that DNA from a preserved specimen can leak into its preservative medium, allowing the medium itself to be directly PCR amplified. We caution that successful DNA extraction and amplification cannot be expected from all such specimens due to collection and storage conditions. DNA fragment size may be relatively short due to DNA degradation and hence the choice of molecular markers or analytical approach might be limited.

Another important application of the non-destructive method for DNA extraction concerns the identification of living material, and in particular, cryptic insect species, biotypes or host races. In the biological control field, such application would be helpful in particular to check: (i) the host races or populations used in host-specificity testing, (ii) for cryptic lineages when conducting inter-crosses, and (iii) for cryptic materials in quarantine before shipment. Individual identification via faeces, egg membranes, hairs and feathers as non-invasive sources of DNA in avians and vertebrates appeared over the years to be of prime importance in conservation genetics and in ethology (Taberlet and Luikart, 1999). Surprisingly, genetic studies of arthropods have not benefited substantially from such studies. This is because extracting amplifiable DNA from arthropod faeces is challenging due to: (i) trace amounts of available DNA, (ii) the potential cocktail of exogenous and endogenous nucleases and PCR inhibitors, and (iii) possible cross-amplification of DNA contaminants, as exemplified by the only report of the use of a non-invasive method in insects (Fumanal et al., 2005). These authors directly amplified a short mitochondrial targeted region from faeces of the root gall weevil, *Ceutorhynchus assimilis*, a potential biological control agent of whitetop, *Lepidium draba*. By combining this method with Double Strand Conformation Polymorphism (DSCP) typing, they were able to assign parental morphocryptic populations to their lineages before conducting inter-crosses. This study should open new perspectives for non-invasive methods applied to biological control agents.

3.2.3. Arthropod DNA preservation

The best long-term approach to preserving arthropod DNA is ultra-cold (−80 °C) freezing of fresh tissue. A −20 °C freezer can also be used, though DNA preserves longer at colder temperatures. For field collections, immediate freezing is not available, and killing and preserving insects in 95% ethanol and later storing them in a refrigerator or freezer (Dillon et al., 1996) provides good preservation of DNA, and is the most common preservation technique. Denatured ethanol should not be used due to possible presence of additives. Some researchers suggest acetone as a preservation chemical (De Biase et al., 2003; Bisanti et al., 2009) as it allows arthropod storage at room temperature for several years (Fukatsu, 1999), and even a longer period at 4 °C storage. When using acetone, external morphological structures are preserved, but genitalia extraction and manipulation are difficult, owing to very rapid and efficient tissue dehydration. Acetone is more volatile than ethanol, but specimens in both liquids should be checked for evaporation periodically. Protocols for storing and analyzing minute arthropods, such as eriophyid mites are described in Monfreda et al. (2010).

3.3. Collecting microorganisms

3.3.1. Field collections

Fungal pathogens are the microorganisms almost exclusively considered for classical biological control of weeds (Evans et al., 2001); hence bacteria, nematodes, viruses and virus-like organisms will not be considered here. In common with plants and arthropods, the number of samples collected at a site will depend not only on what is being investigated, but also the type of host plant (e.g. life history, size and density). However, for initial studies 15–30 samples per site, each collected from a separate plant, is reasonable for population level analysis. In addition, a number of samples from one infected plant at a site should be collected (5–10), in order to compare with the level of genetic variation within the site population. Samples for molecular analysis should not be handled directly. Infected plant material can be dried in a plant press between layers of herbarium drying paper (or newspaper) or in paper bags if the plant material is not bulky (Bruckart et al., 1986). It is important that the drying process begin as soon as possible after collection, though often samples are collected in plastic bags and pressed at the end of the day. In hot climates, these bagged samples must be kept in a cool box to prevent deterioration. The drying paper must be changed daily until the specimens are dry. For root pathogens, root material with no soil can be placed into sealed bags and maintained in a portable electric powered refrigerator at 5–10 °C until being processed within 24 h (Caesar et al., 2010).

3.3.2. Techniques used for isolating fungi

The techniques used to isolate the fungi from dried plant specimens will depend on whether the fungus is biotrophic (can only survive on its host), or necrotrophic (can be grown *in vitro*, e.g. on agar based media). If the collector has microbiological training then isolations of the necrotrophic pathogens onto agar can be made in the field from freshly collected material (Waller et al., 2002). Nevertheless, collections of dried voucher specimens are still required. Isolations onto agar can also be made from dried specimens, but the survival of necrotrophic pathogens in a dried state varies considerably, from a few weeks to years. It is the pure mycelium from the agar cultures that is routinely used for molecular analysis (Yoon et al., 1991). However, successful DNA extraction of plant pathogens has even been done directly from dry herbarium samples stored for many decades (Choi et al., 2009). For biotrophic pathogens such as rusts, which are the most frequently used pathogens in biological control of weeds, spores

picked directly from the leaves are used in the molecular analysis. Contamination by environmental microorganisms and hyperparasites can be avoided by carefully selecting young, recently erupted spore masses (or even spores still covered by the plant epidermis or within the spore mass outer structure). Pathogens can be isolated from soil around contaminated roots using a soil, serial dilution, plate method (Kondo et al., 2002).

4. Examples of use of molecular-based approaches in biological control of weeds

4.1. The target weeds

Taxonomic information about the target weed is often needed at several levels, from the intra-specific detail of the structure of invasive and indigenous populations, to the specific level identification used to design appropriate surveys, to the higher classification and phylogeny of the weed's relatives in order to help design appropriate host-specificity test plant lists. Molecular-based methods can assist with all of these.

4.1.1. Weed species identification

Weed biological control is based on the use of host-specific natural enemies. It is therefore central to any program to understand the species concept for the weed in both the source and target areas. Delimitation of a species is often based on morphological data which may not accurately provide information on population structure, origins, historical isolation and potential hybridization. Surveys for biological control agents are usually made primarily on the same species as the target weed, but there have been several examples where botanists have applied multiple plant names to an invasion. Leafy spurge in North America has been named *Euphorbia esula*, *E. agraria*, and *E. pseudoesula* (Crompton et al., 1990) and Dalmatian toadflax has been called many names, including *Linaria dalmatica* and *L. genistifolia* (USDA NRCS, 2010). Additionally, many plants are treated as having sub-species. For biological control programs, we need to understand the validity and distribution of subspecific taxa. It is also important to know how they relate to the target population, and possible source populations, which may be surveyed for species-specific, and sometimes sub-species-specific, biological control agents.

Recent programs have been able to incorporate molecular-based methods to address these issues, and to avoid some of the associated problems. An introduced species of privet, *Ligustrum* spp., became a major threat to forest ecosystems in the Indian Ocean island of La Réunion in 1969 (Lavergne et al., 1999). It was believed to have arrived from Mauritius where this exotic species was introduced ca. 1895 and had already had a serious impact on the remnant native vegetation. Exact information about the geographic origin of introduced material of this species was lacking, in part because *Ligustrum* is a taxonomically difficult genus. Molecular techniques were utilized alongside traditional techniques and historical research to elucidate the taxonomy and exact area of origin of the introduced *Ligustrum* (Shaw and Milne, 1999; Milne and Abbott, 2004). Dried leaf samples were collected during natural enemy surveys and native material of *L. robustum* ssp. *walkeri* from Sri Lanka, *L. robustum* ssp. *robustum* from north eastern India (Assam and Meghalaya) and the closely related *L. perrottetii* from southern India (the Western Ghats) were compared with introduced material from La Réunion and Mauritius using chloroplast RFLP markers and RAPDs. Sri Lankan and introduced material was monomorphic for the same chloroplast DNA (cpDNA) haplotype that was absent from south and northeast Indian *Ligustrum*. Sri Lankan and introduced material was also clearly distinguished from Indian *Ligustrum* by RAPDs. RAPDs also indicated that *L. robustum* ssp. *walkeri* in Sri Lanka is more similar to south Indian *L. perrottetii* than to northeast Indian *L. robustum* ssp. *robustum*. It was concluded that material introduced and established on Mauritius and La Réunion is derived from the Sri Lankan subspecies *L. robustum* ssp. *walkeri*. These results were supported by comparisons between the natural enemies collected from each region, and as a result, studies of potential biological control agents were concentrated in Sri Lanka.

Another example is the reed genus *Phragmites*, a cosmopolitan genus of Poaceae found throughout the world. Today four species are recognized, one of which, common reed, *P. australis*, includes all temperate subspecies and varieties. The distribution and abundance of *P. australis* in North America has increased dramatically over the past 150 years, and it was suspected that this represented an invasion by an alien population, probably from Europe (Tewksbury et al., 2002). Differences in plant morphology and ecology appeared to support this view, but firm conclusions could not be reached. Saltonstall (2002) tested the hypothesis that an alien strain of *Phragmites* is responsible for the observed invasion in North America by sequencing two non-coding cpDNA regions for samples collected worldwide. In addition, modern North American populations were compared with DNA from historical herbarium collections. Results indicated that an introduction had occurred, and the introduced type has displaced native types as well as expanded to regions previously not known to have *P. australis*. Native types apparently have disappeared from New England and, while still present, may be threatened in other parts of North America. Saltonstall et al. (2004) refined these conclusions by describing the main indigenous population in North America as a separate subspecies, *P. australis* ssp. *americanus*, based on characters of the leaf sheaths, ligules and glumes, supported by cpDNA haplotypes. More recently, Paul et al. (2010), using microsatellites, found evidence of limited hybridization between native and introduced lineages in North America.

In parallel, studies started to compare the associated natural enemies of common reed in North America and Europe, with a view to a possible biological control program. It was confirmed that the natural enemy fauna is much richer in Europe than in North America, even though the latter included more than 20 species accidentally introduced from Europe (Tewksbury et al., 2002). The scope for biological control now lies in possible agents from Europe, that are specific at the subspecies level or, more realistically, which prefer or are better adapted to the European subspecies than to the indigenous North American subspecies (Blossey et al., 2002). The European subspecies retains its leaf sheaths in winter, while ssp. *americanus* sheds them. European stem boring moths lay their eggs under the leaf sheaths to overwinter, and can be expected to suffer much higher mortality on ssp. *americanus* (Hafliger et al., 2006).

Molecular methods of species identification can also be important to field surveys, as pathogen and other agent collections may have to be done at a time when plants are not readily identifiable (e.g. rosette stage). In this case, DNA sequences from the immature plants can be compared to accessions in GenBank or other databases, to verify identification. Normally this will clarify whether the plant being surveyed was the target species and if not, the plant can often be identified to genus if the relevant sequences are available in GenBank.

4.1.2. Population structure and origins of weed invasions

Knowing the genetic population structure of an invasive weed, within both the introduced and native ranges, can provide useful information for a biological control program in three main ways. First, information about the structure of neutral genetic variation (variation not associated with adaptive value) within and between sites can reveal whether multiple introductions have occurred

leading to multiple genetic variants in the introduced range, or alternatively, if there were strong bottlenecks in population size that led to reduced variation. This enables research on host use and efficacy to include the range of variation present (Gaskin et al., 2005). Second, quantifying population structure, particularly in the native range, may reveal distinct geographic regions that prove useful in finding agents adapted to different genotypes of the target weed. Third, and related, it can be useful to know from where invasive weeds were introduced, as these areas may be useful to target when collecting candidate biological control agents. It has been suggested that natural enemies from the area of origin might be locally adapted to the particular genotypes of a plant that have invaded a new range. While this may be true for some cases (e.g. Goolsby et al., 2006) it should be regarded as a hypothesis that can be tested (Hufbauer and Roderick, 2005), as, particularly for pathogens, there is evidence that new host-natural enemy associations can be the most damaging (Evans and Ellison, 2004).

For the first two items above, essentially what is needed is a way to measure and describe how variation is partitioned among populations. Markers for elucidating intraspecific population structure in plants (Sections 2.1 and 2.2, above) generally must be more variable than DNA sequence data, which generally is used at higher levels (species and above, rather than within species; Table 3). Even determining chromosome numbers can help narrow potential origins when there is sufficient variation in ploidy level (e.g. Hufbauer et al. 2004). Allozymes, ISSRs, AFLPs, SSRs, and SNPs all can be useful at the intraspecific level, though allozymes are less utilized due to their lack of variation, and SNPs less utilized due to cost (Table 1). Variation in these markers can be analyzed in two main ways: traditional analysis of molecular variance (AMOVA) approaches based on partitioning molecular variation among different hierarchical levels of organization (Excoffier et al., 1992) and more recent assignment tests based on multi-locus genotypes (reviewed in Manel et al., 2005). Using AMOVA, collection sites are essentially assumed to be populations, and the questions that are asked include, "Do sampled populations differ from each other?" and "Is there substantial variation within populations?" Using assignment tests based on multi-locus genotypes the questions that arise include, "How many populations do the samples represent?" and "Are individuals found in one location actually from another location?" Excoffier and Heckel (2006) provide a useful overview of the software programs available for these analyses.

Using population genetic data to address the third item, origins of invasions, is potentially much more difficult for several reasons. There must be population genetic structure within the native range of the species to be able to determine that introduced populations are likely to have come from one or more particular regions. If a strong association with human altered habitats has led to panmixia in the native range then all sample sites within the native range would share the same allele frequencies, and it would not be possible to pinpoint areas of origin of an invasion. One must have genetic markers that have the resolution to reveal population structure that exists within the native range of a species (see above discussion of choice of markers). Sampling, especially of the native range, should be as complete as possible, avoiding the situation where there is an unsampled "ghost population" (Guillemaud et al., 2010; Estoup and Guillemaud, 2010) that has served as the source of an invasion.

Methods for discerning origins of invasions are rapidly changing, and are reviewed by Estoup and Guillemaud (2010). As population genetic data first were gathered to study invasive weeds, analyses typically relied upon creating a matrix of genetic distances between populations and using this to construct a dendrogram of relationships (e.g. Takezaki and Nei, 1996). Sequence data can be used to generate trees based on parsimony (e.g. PAUP, Swofford, 2002) if there is sufficient variation. More recently, individual-based clustering methods have been used, as implemented in the programs STRUCTURE (Pritchard et al., 2000), BAPS (Corander et al., 2003) and Geneland (Piry et al., 2004). These have several advantages in that, rather than lumping all of the individuals from a single location together, the multi-locus information from single individuals are used and make it theoretically possible to distinguish individuals that were recent colonists to a location. The latest development in population genetic analysis of biological invasions is approximate Bayesian computation (ABC, Beaumont et al., 2002), which is particularly powerful in that it enables the user to compare different hypothetical invasion scenarios directly, and can incorporate both population genetic data and prior knowledge about the history of invasions (e.g. when a species was first observed at different locations). ABC can be implemented in DIYABC (Cornuet et al., 2008), a program developed to enable users with little background in programming to use the method. The major disadvantage of this approach is that it can be computationally quite intensive.

There has been an explosion of population genetic research on invasive plants that has helped to clarify the variability of the populations present in the introduced range including: knapweeds, *Centaurea diffusa* and *C. stoebe* (Marrs et al., 2008a, b; Hufbauer and Sforza, 2008); Canada thistle, *Cirsium arvense* (Slotta et al., 2006); kudzu, *Pueraria lobata*, (Pappert et al., 2000); medusahead, *Taeniatherum caput-medusae* (Novak and Sforza, 2008); and hairy fogfruit, *Phyla canescens* (Xu et al., 2010). Often, there is evidence for multiple introductions of an invasive plant (e.g. Gaskin et al., 2005; Marrs et al., 2008a, b; Xu et al., 2010) and the area of origin in the native range can be narrowed down (e.g. Goolsby et al., 2006), but definitively documenting areas of origin remains challenging (Estoup and Guillemaud, 2010; Paterson et al., 2009; Xu et al., 2010). One general pattern to note in data on population genetic markers, particularly highly variable ones such as microsatellites and ISSRs, is that in both native and introduced populations, most variation occurs within populations rather than between populations or between regions. In *Phyla canescens*, 73% of variation in ISSRs was found within population, 21% occurred between population and 6% between regions (Xu et al., 2010). What is more enlightening to know is whether or not statistically significant variation exists between populations as it may suggest that different populations will respond differently to different biological control agents.

4.1.3. Hybridization in weeds

Human-mediated movement of species or genotypes that have been historically isolated can lead to novel hybrid combinations within a plant invasion. Hybridization events are thought to increase invasiveness in some cases (Ellstrand and Schierenbeck, 2000; Whitney et al., 2006; Abbott et al., 2009) by providing a relatively rapid mechanism for increasing genetic diversity and producing novel gene combinations on which natural selection can act (Stebbins, 1959; Lewontin and Birch, 1966). This increase in genetic diversity can be especially important for successful invasion when an introduced species has lost genetic variation due to a founder effect. Regardless of how common hybridization may seem in invasion histories, however, most new hybrid combinations will probably not be well adapted or more invasive than parental types (Donovan et al., 2010).

When novel hybrids are created post-introduction (e.g. Gaskin and Schaal, 2002; Gaskin et al., 2009), or if hybrids represent a significant portion of a plant invasion or even if they have the potential to increase in frequency within an invaded environment (e.g. Moody and Les, 2007; Gaskin and Kazmer, 2009), they should be included in host use and efficacy testing of biological control agents. This applies whether the hybrid is the result of the crossing between or among invasive, alien taxa that are closely-related (e.g.

Table 3
Typical stages in the biological control of weeds process, and molecular techniques that may be used to address key needs and topics.

Project stage	Key needs	Specific topics that may need to be addressed	Suggested molecular techniques[a]
1. Initial information	Understanding the target weed	Weed species identification	DNA sequencing
		Hybridization in weeds	SSRs, DNA sequencing (nuclear), AFLPs, ISSRs
		Establishment/re-assessment of test plant lists (phylogenetics)	DNA sequencing
		Determining method of reproduction in weeds	SSRs, AFLPs, ISSRs
2. Exploration	Determining weed origins	Population structure and origins of weed invasions	SSRs, AFLPs, ISSRs, DNA sequencing, SNPs
	Identifying natural enemies	Agent species identification	DNA sequencing
3. Agent selection	Host-specificity and efficacy	Identification of immature stages of arthropod agents	DNA sequencing
		Identification of host associations from arthropod gut analysis	DNA sequencing
		Establishment/re-assessment of test plant lists (phylogenetics)	DNA sequencing
		Population structure and genetic variability in agents	SSRs, DNA sequencing (mitochondrial), AFLPs
		Arthropod agent hybridization	SSRs, DNA sequencing, AFLP, RFLP
		Identification of endosymbionts/microorganisms associated with arthropod agents	DNA sequencing, real-time qPCR
		Elucidation of fungal agent life cycles and sexuality	DNA sequencing
		Determination of whether plant is infected with pathogen before symptoms are present	DNA sequencing, real-time qPCR
4. Rearing and culturing for release	Colony or culture quality	Population structure and genetic variability in agents	SSRs, DNA sequencing (mitochondrial), AFLPs
		Identification of endosymbionts/microorganisms associated with arthropod agents	DNA sequencing, real-time qPCR
5. Monitoring and evaluation	Rapid identification of agents	Agent species identification	DNA sequencing
		DNA-barcoding of arthropods	DNA sequencing
		Identification of immature stages of arthropod agents	DNA sequencing

[a] Our suggestion of use of SSRs dependent on previous development of SSRs for taxon of interest.

Russian thistle, *Salsola* spp., Ayres et al., 2009; saltcedar, *Tamarix* spp., Gaskin and Kazmer, 2009), or where an invasive alien taxon has crossed with a closely-related native taxon. Examples of the latter are hybridization of alien and native watermilfoils, *Myriophyllum* spp. (Moody and Les, 2007) in North America determined by nuclear DNA sequence data, and hybridization between invasive European purple loosestrife, *Lythrum salicaria*, with the native North American *L. alatum*, determined by AFLP data (Houghton-Thompson et al., 2005). Considering that hybrids may provide a genetic "bridge" allowing host-specific insects to expand their host range from one parental species to another (Floate and Whitham, 1993), the identification and testing of hybrids formed from natives and aliens should be of particular importance in the assessment of non-target risk.

Hybrids are usually discovered through morphological observations of intermediate phenotypes, but this method fails if hybrids, and subsequent generations derived from these hybrids, exhibit extreme or parental characteristics, making them effectively cryptic. Natural back-crossing of hybrids with parental lineages will further complicate identification by creating introgressed plants that may represent a continuum of phenotypes and genotypes (Abbott, 1992; Thompson et al., 2010). Molecular tools can be particularly effective in identifying cryptic hybrid individuals. Earliest methods utilized karyotypic information (when parental species differed in chromosome number) and visual analyses of enzyme or PCR product fragments (e.g. Milne and Abbott, 2000). Eventually, genetic distances derived from multi-locus molecular data were used to provide visual evidence of hybrids in dendrograms or scatterplots (e.g. Saitou and Nei, 1987), or assignment of hybrids into classes such as parental, F1, F2 and backcrosses (e.g. Nason and Ellstrand, 1993), but detecting hybrids relied on the presence of species specific markers, which do not always exist (Vaha and Primmer, 2006). Most recently, Bayesian-based statistical methods, implemented in software such as STRUCTURE (Pritchard et al., 2000) and NewHybrids (Anderson and Thompson, 2002), have been developed that do not require that any alleles be unique to either parental type, or any *a priori* knowledge of allele frequencies in parental types. Individuals are probabilistically assigned to parental or hybrid classes, even when levels of differentiation between parental types is quite low; $F_{st} \geqslant 0.05$ (Latch et al., 2006).

Analysis of hybrids, with Bayesian or other methods, is most commonly performed with co-dominant data (for which both alleles of a diploid individual can be distinguished) such as SSRs, SNPs, nuclear DNA, and RFLPs (e.g. Moody and Les, 2002; Williams et al., 2005; Mercure and Bruneau, 2008; Zalapa et al., 2010), but the more recent Bayesian methods can now also be used with easier to obtain dominant markers (Falush et al., 2007; Anderson, 2008) such as RAPDs, AFLPs and ISSRs (e.g. Tiebre et al., 2007; Lubell et al., 2008; Gaskin et al., 2009; Blair and Hufbauer, 2010).

4.1.4. Determining method of reproduction in weeds

Effective biological control of weeds must limit the spread and density of the invasion. Invasive plants can increase their numbers by seed spread or through vegetative means (e.g. bulbils and growth and fragmentation of lateral rhizomes, suckers, roots and stems), and some species use combinations of methods. Understanding the primary method of spread and increase in density provides information for the selection of biological control agents. Agents that attack seeds would do little to control the spread and density of a weed that reproduces primarily through asexual means. Reproductive mode can also have a great effect on genetic diversity, population structure, effective population size, and evolutionary response to environmental change, all of which directly or indirectly affect biological control methods. While it is relatively easy to determine what types of reproductive strategies exist for a given weed species, it is often less easy to distinguish the contributions of each strategy when multiple strategies exist. Earlier studies relied on comparing age, morphological similarity of above

ground shoots, or physical inspection of root connections (which can be broken due to natural causes over time) to identify genetically identical plants. Molecular methods have greatly enhanced such studies, as hyper variable markers (usually AFLPs, ISSRs, or SSRs) can usually distinguish genetically identical plants from those that resulted from sexual reproduction. Error in molecular analysis can appear to describe multiple genotypes when only one is present, so researchers have used methods such as similarity thresholds (Douhovnikoff and Dodd, 2003), a character incompatibility method (Mes, 1998; Van Der Hulst et al., 2000), and statistical tests for both clonal identity and clonal propagation (Geneclone 1.0; Arnaud-Haond and Belkhir, 2007).

Distinguishing reproduction via seed from clonal reproduction becomes difficult or impossible when a species is apomictic or selfing and highly homozygous, but is relatively easy in obligate outcrossing species. A recent AFLP study showed that for an invasive aquatic, primrose-willow, *Ludwigia hexapetala*, some 95% of plants were genetically identical, indicating that management should target vegetative dispersal and growth (Okada et al., 2009). Gaskin (2006) studied the obligate outcrossing species whitetop, *Lepidium draba*, with AFLPs and found that 55–85% of shoots in a patch were connected via roots underground, and genetic individuals could be at least 38 m across, indicating that seed and flower feeding agents would do little to stop the spread of a patch once a plant is established. Jesse et al. (2010) used allozymes to determine that patches of multiflora rose, *Rosa multiflora*, were dominated by one genotype, and Dong et al. (2006) used ISSRs to find that plants of Canada goldenrod, *Solidago canadensis*, separated by two or more meters were always genetically distinct, indicating a strong bias of sexual reproduction in relatively small scale recruitment. Similarly, Bodo Slotta et al. (2010) used microsatellites to show that Canada thistle, *Cirsium arvense*, also reproduces frequently by seed. Other examples of studies of invasive weed reproduction methods include Li and Ye (2006), Hollingsworth and Bailey (2000), Pappert et al. (2000), Ren and Zhang (2007), and Li and Dong (2009).

Burdon and Marshall (1981) suggested that the mode of reproduction could be used to predict which weed species are more likely to be successfully controlled using biological control, because asexually reproducing plants have lower genetic variation than outcrossing plants. Chaboudez and Sheppard (1995) reanalyzed associations between mode of reproduction and biological control success and found little correlation, and stated that the outcome of biological control is dependent on many factors, including life history, genetics of the control agent and environmental conditions.

4.1.5. Establishment/re-assessment of test plant lists (phylogenetics)

Until recently, test plant lists for the delineation of host range of candidate arthropods and pathogens for classical weed biological control generally have been developed using the centrifugal-phylogenetic method proposed by Wapshere (1974). The method assumes that closely related plant species are morphologically and biochemically more similar than unrelated plants and are, therefore, more likely to possess cues that are recognised by co-evolved specialist herbivores. Species closely related to the target, therefore, should be at greater risk of attack than species more distantly related. Evidence on the evolution of insect-plant associations (e.g. Futuyma, 2000), as well as a review of the incidence of non-target attack by introduced biological control agents on native North American plant species (Pemberton, 2000), have confirmed these assumptions. Because molecular plant phylogenies were rare 35 years ago when Wapshere first proposed his method, relationships based on Linnaean taxonomy were used, and generally have persisted in use by the weed biological control research community. However, some Linnaean taxonomic classifications (e.g. genus, family, order) do not necessarily describe evolutionary history, which is often better estimated when the analysis includes molecular-based data. Taxonomic groupings may be monophyletic (including the most recent ancestor and all of its descendents), paraphyletic (including the most recent ancestors and some, but not all of its descendents) or polyphyletic (including organisms not most closely related to each other), while the clades derived from phylogenetic analyses are strictly monophyletic.

Molecular phylogenies that include target weed taxa may not have yet been constructed, or if they exist, do not include all taxa of interest to the biological control researcher. In these cases, new phylogenetic estimates must be constructed, typically from existing (e.g. GenBank) and new DNA sequence data. The sequence marker (i.e., gene region) to be used depends on the taxonomic level being investigated, with more rapidly evolving markers useful for describing interspecific relationships, and more slowly evolving markers for higher taxonomic levels (see Table 2). This process often requires testing of many markers until finding those with sufficient variation, but a survey of markers used in publications at the taxonomic level of interest serves as a good starting point. After DNA from taxa of interest are sequenced, various phylogenetic programs (e.g. PAUP, Swofford, 2002) can be used to construct the phylogeny, though this process is somewhat complicated by the many options available in phylogenetic analysis, and may require the advice of someone trained in the subject matter.

As molecular phylogenies become available, our understanding of relationships (i.e., tribal or family groupings) often changes, resulting in revised classifications (e.g. in the Brassicaceae after Al-Shehbaz et al. (2006), or the recently ascertained paraphyletic association of the Hydrophyllaceae and Boraginaceae and placement of the former taxon within the latter (Angiosperm Phylogeny Website, 2008)). Especially when the target weed has many native congeners or native genera in the same family, accurate phylogenies can greatly facilitate the selection of test species, saving time and resources (see examples in Kelch and McClay, 2003). With the proliferation of molecular-based phylogenies since the 1990s, it has been suggested that test plant species should strictly be selected and categorized according to their degree of phylogenetic separation from the target weed rather than by taxonomic circumscription (Briese, 2005; Kelch and McClay, 2003). These should be refined by biogeographical overlap and ecological similarity (Briese, 2005), or secondary criteria (rarity, economic importance, etc.) within a phylogenetic framework (Kelch and McClay, 2003).

The Scrophulariaceae are a large, diverse family of temperate and tropical distribution, containing several invasive species. It had been suggested for many years that this family may not be monophyletic, and widely differing circumscriptions in traditional classifications pointed in the same direction (Olmstead and Reeves, 1995; Olmstead et al., 2001). Recent DNA sequence phylogenetics have confirmed that Scrophulariaceae is not a natural grouping (Oxelman et al., 2005), and its former components are actually distributed among seven independent lineages within the order Lamiales, now referred to as the families Scrophulariaceae, Calceolariaceae, Linderniaceae, Orobanchaceae, Plantaginaceae = Veronicaceae, Phrymaceae and Stilbaceae (Tank et al., 2006; Schäferhoff et al., 2010). This major overhaul of plant systematics has spurred the re-examination of the test plant list of the long-running toadflax, *Linaria* spp., biological control program for North America (Sing et al., 2011). According to the new classification, *Linaria* has been transferred to Plantaginaceae, which has significantly expanded from its former conscription to include most of the well-known genera of the former Scrophulariaceae (Albach et al., 2005). Because many of the genera and species of concern in testing also were transferred with *Linaria* to Plantaginaceae (e.g. all genera within the tribe Antirrhineae), the revision generally has not caused a huge realignment of focus on what critical species to test. Instead, there has been a shift in focus away from

clades that are no longer within the same family as *Linaria* (e.g. hemi-parasitic genera such as *Castilleja* and *Pedicularis*, now in the family Orobanchaceae), resulting in greater efficiencies in host-specificity testing.

Apart from helping to establish more biologically meaningful test lists, accurate phylogenies can be used to interpret results of host-specificity tests. An example is the biological control project against garlic mustard, *Alliaria petiolata*, for which four weevil species are currently being studied as potential biological control agents (Gerber et al., 2009). Three of these, two stem-miners *Ceutorhynchus alliariae* and *C. roberti*, and a root-miner *C. scrobicollis*, develop on three European plant species (apart from garlic mustard) under no-choice conditions; *Nasturtium officinale*, *Peltaria alliacea*, and *Thlaspi arvense* (Gerber et al., 2009 and unpublished data). According to classical taxonomy based on morphological characteristics, *P. alliacea* and *T. arvense* belong to the tribe Lepidiae, while the target weed *A. petiolata* belongs to the tribe Arabideae (in Hegi, 1986). However, according to the new molecular phylogeny proposed for the Brassicaceae (Al-Shehbaz et al., 2006) both *P. alliacea* and *T. arvense* are in the same tribe (Thlaspideae) as *A. petiolata* (Al-Shehbaz et al., 2006; Bailey et al., 2006). In addition, the genus *Thlaspi* was found to be polyphyletic, i.e., Old and New World *Thlaspi* species were found to be clearly separated based on DNA sequence data (Koch and Al-Shehbaz, 2004), and New World species were placed in the genus *Noccaea*. Correspondingly, no agent development occurred on a native North American species in the genus *Noccaea*. Host suitability for the potential agents on *A. petiolata* therefore confirms the new classification within the Brassicaceae. Overall, females of *C. scrobicollis* laid more eggs and produced more offspring on test species closely related to *A. petiolata* than on more distantly related plants (Hinz et al., 2008).

In another study, Briese and Walker (2002) separated the plant species to be tested with the leaf-beetle *Deuterocampta quadrijuga* for control of blue heliotrope, *Heliotropium amplexicaule*, into seven categories, comprising the target weed and members of six clades with increasing molecular phylogenetic distance from the target weed. Overall host suitability was based on the rank order of larval survival to five days, the level of larval feeding, the level of adult feeding, and adult oviposition on test species. Kruskal–Wallis analyses of variance showed that the suitability of test species declined with increasing phylogenetic distance. Briese and Walker (2008) found similar results for the root-feeding flea beetle, *Longitarsus* sp. on heliotrope. However, there are also cases, in which potential agents show a disjunct host range. For instance, it has been shown for *Ceutorhynchus cardariae*, a gall-inducing weevil considered as a biological control agent for whitetop, *Lepidium draba*, that molecular phylogenetic distance of the test species to the target weed was not correlated to host preference or suitability under no-choice conditions (Hinz et al., 2008). Other factors, such as secondary metabolite profiles and morphological characteristics are currently being investigated.

Recently, Berner (2010) suggested combining host range data (e.g. disease severity of a pathogen biological control agent on tested plant species) with genetic relationship data from known plant phylogenies to better predict realized host range of candidate agents. A relationship matrix derived from DNA sequences is analyzed with mixed model equations (MME) to produce Best Linear Unbiased Predictors (BLUPs) of susceptibility for each test species. The author argues that as long as DNA sequence data are available for a plant species and known disease reaction data (or host acceptance data for arthropods) are incorporated into the model, BLUPs can be generated without actually testing the species with an agent. This could be particularly helpful for rare or difficult to grow test plant species. Moreover, Berner (2010) describes how this method can be used to validate existing, and develop new, test plant lists.

4.2. Arthropod agents

4.2.1. Arthropod species identification

An issue in candidate agent taxonomy that sometimes creates problems is the lack of morphological characters available to correctly identify specimens used in host choice experiments. As a consequence, feeding behaviors appearing to indicate polyphagy can be artifacts due to the existence of closely related, cryptic species. While the basic taxonomic knowledge of a species or a complex of species is often good enough to allow careful study of the feeding behavior of potential control agents, the use of molecular tools can clarify species identity and identify relevant population variation in difficult groups.

The stem-boring flea beetle *Psylliodes* cf. *chalcomerus* is a potential agent for yellow starthistle, *Centaurea solstitialis*. More than 10 populations that feed on different host plants, all putatively belonging to this taxonomically difficult complex, were sampled from Spain to Russia. Specimens were DNA sequenced for mitochondrial markers. The populations initially seemed to belong to three distinct genetic groups of unclear taxonomic status with distinct trophic ecologies (De Biase et al., 2005; Antonini et al. 2008), although they were morphologically indistinguishable (Konstantinov, pers. comm.). These groups seemed also to reflect a feeding specialization, at least at the local level, with two groups each feeding on single host plant species (*C. solstitialis* and *Onopordum acanthium*) and the third group feeding on several plant species. Thus, the molecular data revealed genetic differentiation that must be taken into account during host-specificity testing. Whether the three groups can be considered separate species remains under consideration.

Molecular data have recently been collected to clarify the relationships and species status of root-boring *Trichosirocalus* weevils associated with Carduinae (a subtribe of Asteraceae containing mostly thistles). Taxonomy based on morphology in this group is ambiguous. Preliminary COI mtDNA sequence data reveal a clear difference between *T. horridus* (associated with nodding thistle, *Carduus nutans*) and *T. briesei* (specific to Scotch cotton thistle, *O. acanthium*). In contrast, *T. mortadelo* (feeding on *C. nutans* and slender winged thistle, *C. pycnocephalus*) is not substantially differentiated from *T. horridus* suggesting that it may not be appropriate to consider them two separate species (De Biase, Cristofaro and Smith, unpublished data).

4.2.2. Identification of immature stages of arthropod agents

Species-level identification of natural enemies is critical at all stages of biological control, and can be particularly challenging with immature life stages of insects that can be difficult or impossible to identify morphologically (Rauth and Hufbauer, 2009). Molecular genetics can help to identify immature stages, aiding the entire process of development and implementation of a biological control program. During foreign exploration, molecular approaches can make it possible to identify field-collected larvae, which often provide more reliable information on host-plant use than field collected adults (Antonini et al., 2008). The ability to identify immature stages can also be useful during host-specificity testing. While many host-specificity tests take place in isolation in a laboratory with little risk of contamination with other species, others take place in field cages or open-field trials and naturally occurring populations of other species may contaminate an experiment despite efforts to keep them out. This is particularly problematic if larval development is not complete and no adults emerge. In such cases molecular diagnostic tools can be invaluable in species identification. Molecular tools for identification of larval stages of candidate agents and their close relatives have recently been used in the program against garlic mustard, *Alliaria petiolata*

(Rauth and Hufbauer, 2009; Rauth et al., 2011) and yellow starthistle, *Centaurea solstitialis* (Antonini et al., 2008; Rector et al., 2010).

Rector et al. (2010) in a straightforward application of DNA-barcoding, used a portion of the mtDNA COI gene to identify all larvae collected during an open-field experiment to test the host association of the weevil, *Ceratapion basicorne*, (a potential agent for yellow starthistle) and allied species living in southern France. All sequenced larvae were compared with DNA sequences obtained from adults that had been identified morphologically by taxonomists, and their association to host plants were congruent with previous knowledge. A few larval samples remained unidentified owing to the lack of reference sequences from other species whose adults were not available. The study demonstrated the pros and cons of the approach, emphasizing that using molecular markers can optimize the use of resources for experiments. The study demonstrated the usefulness of the DNA-barcoding approach in linking life stages of an organism (Antonini et al., 2009; but see also Ahrens et al., 2007).

4.2.3. Population structure and genetic variability in arthropod agents

Methods for assessing genetic variability of agents are similar to those noted for weeds above (Section 4.1.2). Which markers to choose depends on the question asked, as well as access to material. Most population level analyses require information on both alleles as can be determined with co-dominant loci (Table 1). Dominant markers are used to a lesser extent in animal studies compared to studies of plants, fungi and bacteria (Bensch and Akesson, 2005). Maternal inheritance, and rapid, nearly neutral evolution of mitochondrial markers (especially the commonly used mtDNA COI) make them well suited for reconstructing intraspecific phylogeographical patterns (i.e., comparisons of evolutionary relatedness and geographical distribution).

Knowing the extent and structure of the genetic diversity of a natural enemy in its native range can provide useful information to a biological control program in three main ways. First, it is useful for determining how many individuals, from how large an area, should be collected to rear for host-specificity testing. The simplest case is when the candidate agent is considered to be one metapopulation and the target species is its only host (Michalakis and Olivieri, 1992; Antonini et al., 2008). A good example is given by the retrospective population genetic study of the European weevil *Ceutorhynchus scrobicollis*, a candidate for the control of garlic mustard, *Alliaria petiolata*, using AFLPs (Rauth et al., 2011). This study found that *C. scrobicollis* in the vicinity of Berlin, Germany, where individuals have been collected for host-specificity tests, constitute a metapopulation with gene flow among all collection sites. Models indicate that sampling 81 individuals would capture 99% of the genetic diversity in AFLP loci of *C. scrobicollis* populations in this area. Since more than 4100 individuals were collected for the rearing colony used to conduct host-specificity tests, the test results should more than adequately represent the potential host range of the candidate.

Second, knowing the extent and structure of the genetic diversity of a natural enemy could contribute to determining its dispersal capacity. Rauth et al. (2011) in their work on the weevil, *C. scrobicollis*, based on AFLP data, found that although most *C. scrobicollis* remain in their natal sites, they could also disperse. Dispersal distances, either natural or human-assisted, were on average 30 km, but distances of up to 65 km were recorded. This would have likely been difficult to determine with traditional methods.

Third, data on population structure of candidate agents can reveal cryptic variation within candidate species. Phytophagous insect species and fungi often are comprised of genetically distinct lineages specialized on different host species or genotypes, often termed host races (Fox and Morrow, 1981; Mopper and Strauss, 1998; Via, 1999; Hufbauer and Roderick, 2005; Mallet, 2008). It is critical to delineate such variation early in a biological control program. Selective use of specialized lineages might increase the efficacy or safety of biological control (e.g. Goolsby et al., 2006), while using mixed lineages might decrease efficacy, or increase the risk of non-target effects. There are a growing number of examples of important intraspecific variation: Briese et al. (1996) used allozymes to provide evidence for host races in weevils in the genus *Larinus* and Fumanal et al. (2004), using mtDNA COI and DSCP, demonstrated that a purportedly generalist weevil species, *C. assimilis*, is actually several host races, one being strictly specific to the target weed whitetop, *Lepidium draba*. This opens the door for exploring the potential for its use in biological control.

Initially it was thought that the stem-boring weevil, *Mecinus janthinus*, an agent of toadflaxes, *Linaria vulgaris* and *L. dalmatica*, would accept both hosts in North America (Jeanneret and Schroeder, 1992). Collections for initial releases mostly came from *L. vulgaris* in Europe with only a single shipment from *L. dalmatica* (I. Tosevski and A. Gassmann, unpublished). Despite there having been more collections from *L. vulgaris*, the weevil established primarily on *L. dalmatica*, and several hypotheses involving climate were brought forward to explain this result (De Clerck-Floate and Miller, 2002; McClay and Hughes, 2007). Recently, however, genotyping based on the mtDNA COII gene revealed that the *M. janthinus* that established on *L. dalmatica* in North America probably originated from the single shipment from *L. dalmatica* (I. Tosevski and A. Gassmann, unpublished). Investigations on the genetic variability of *M. janthinus* prior to release would have revealed the occurrence of host races or cryptic species and could have prevented the release of *M. janthinus* specialized on *L. vulgaris* onto *L. dalmatica* (and vice versa), thus saving time and resources. Recently, other candidate or previously released agents of *Linaria* spp. have been found to consist of distinct host races, or cryptic species, specializing on either *L. vulgaris* or *L. dalmatica* (Legarreta-Monroy, 2007; Hernandez-Vera et al., 2010).

4.2.4. Arthropod agent hybridization

Molecular-based methods may be useful to address questions relating to hybridization of introduced biological control agents, both between two introduced agents and between an introduced agent and an indigenous species. Our state of knowledge is not adequate to predict in detail the implications of such hybridization, but the likelihood of producing less vigorous offspring (and hence less effective biological control agents) is certainly real, and the possibility of breakdown of host-specificity mechanism cannot be ruled out.

One on-going example is the tamarisk/saltcedar beetle, *Diorhabda* spp., which until recently was being released and established in the USA against several species and hybrids of tamarisk, *Tamarix* spp. Initially researchers believed they were dealing with different strains of one species, *D. elongata*, but each strain was separately tested for host-specificity. A detailed traditional morphological study concentrating on characters of the genitalia by Tracy and Robbins (2009) has shown that five morphologically diagnosable sibling species of the *D. elongata* species group are specialized feeders upon tamarisk. Members of the *D. elongata* group were thought to be strongly reproductively isolated and would interbreed rarely, if at all, in the open field whether in the Palaearctic or Nearctic. Four of these species, previously classified as *D. elongata*, have been released into the open field in the USA: *D. carinulata*, *D. elongata*, *D. carinata*, and *D. sublineata*. *Diorhabda carinulata* and *D. elongata* are confirmed as established in the USA. These conclusions were supported by crossing experiments (D. Thompson unpublished) and mtDNA (COI) and AFLP analyses (D. Kazmer, unpublished).

Using morphological methods (Tracy and Robbins, 2009) and DNA analyses (D. Kazmer, unpublished), it has been shown that

the 'Tunisia ecotype' beetles propagated successfully in cages, were in fact *D. elongata* x *D. sublineata* hybrid beetles, generated inadvertently in outdoor cages at Temple, TX, USA late in 2005. These hybrid beetles were released as the 'Tunisian ecotype' in 2006 no-choice and choice field tests at the south Texas sites, but the field populations did not persist (Moran et al., 2009). Similar hybrids generated via cross-mating (D. Thompson, unpublished) were fully fertile for several generations in some backcrosses. Tracy and Robbins (2009) conclude that laboratory or cage produced *Diorhabda* hybrids should not be considered as candidates for tamarisk biological control because of known and potential problems of hybrid breakdown in the field which would probably lead to low persistence and low efficacy. Further investigation is needed with genetic techniques to search for various types of hybrids and backcross hybrids and genetic introgression between *Diorhabda* species in the native and introduced range, to better understand and interpret the field situation.

4.2.5. Endosymbionts/microorganisms associated with arthropod agents

Molecular tools also can be used to improve the efficacy of biological control by identifying infections by endosymbionts. Many arthropod species are infected with endosymbiotic bacteria (e.g. *Wolbachia*, *Arsenophonus*, *Cardinium*, *Rickettsia*, *Spiroplasma*), which alter their hosts' biology (Bourtzis and Miller, 2006; Werren et al., 2008). Infections may severely skew sex ratios to female (via feminization, parthenogenesis induction, male-killing) or male (via egg-sperm incompatibilities) (Floate et al., 2006; Werren et al., 2008). Furthermore, *Wolbachia* can directly affect host physiology by reducing fecundity, and changing behavior (e.g. dispersal) and longevity (Floate et al., 2006). These effects could reduce the vigor and/or efficacy of weed biological control agents and perhaps prevent their field establishment, especially for obligate sexually-reproducing species. In illustration, *Wolbachia* in the leafy spurge flea beetle, *Aphthona nigriscutis*, may explain host sex ratios of 80–100% female (Kazmer, 2001) and reduced genetic diversity in host beetle populations (i.e., mtDNA; Roehrdanz et al., 2006).

Many biological control researchers are unaware of the implications that endosymbiotic bacteria may have for biological control programs. This partially can be explained by: (1) the relative infancy of studies on endosymbionts, (2) the paucity of publications targeting the biological control community, and (3) the absence of molecular tools needed to study endosymbionts in many programs of biological control (Floate et al., 2006). In one survey of arthropod agents and pests, *Wolbachia* infections were detected in 46% of the 105 species tested (Floate et al., 2006). However, only five of the tested species were used in classical biological control of weeds, only 1–2 individuals from one population of each species was tested, and of these, infection was only detected in one species, *A. nigriscutis*. This suggests that a broader, more intensive survey for *Wolbachia* and other endosymbionts within weed biological control agents is warranted, especially given the implications of infection to agent efficacy, and even host-specificity. The symbiont *Regiella insecticola* has been associated with host-specialization in populations of the pea aphid, *Acyrthosiphon pisum* (see review by Oliver et al., 2010). In addition, endosymbionts can affect the diversity and distribution of their hosts' mtDNA. This has implications for the use of mitochondrial markers to identify source populations, points of introduction, dispersal of biological control arthropods, and even for species' identification (Hurst and Jiggins, 2005).

The benefits of detecting endosymbionts within arthropod agents using molecular methods not only provide a possible explanation of study results or of failure in classical weed biological control, but also may provide solutions to the problem. Once detected, studies may be done using infected and uninfected host populations to determine endosymbiont impact on agent parameters, including fecundity and efficacy. Laboratory colonies, which are especially prone to the spread and fixation of endosymbionts (Floate et al., 2006; Oliver et al., 2010), can be cleansed of the bacteria through treatments with antibiotics or heat (e.g. Kyei-Poku et al., 2003). Another approach would be to find an endosymbiont-free population of the agent for release, in cases where infections are undesirable.

4.3. Fungal agents

4.3.1. Identification of fungal pathogens

It is common practice to employ molecular techniques alongside morphological taxonomy in the identification of pathogens with potential for biological control. For obligate fungi that cannot be grown in culture the biggest challenge is to acquire enough pure material (without the contaminating DNA of its host) for DNA extraction. For fungi that can be grown in culture the standard technique involves DNA extraction from a pure mycelia culture, preferably when not sporulating. Spore production can be discouraged by using a rich medium, whereas for morphological identification, a low nutrient medium is used to induce sporulation. One target region of DNA is amplified using specific primers and this can then be sequenced. The sequence data are then compared for the closest match with the sequences held in open access sequence databases such as GenBank. The most useful fragment in the identification of fungi are the two ITS (Internal Transcribed Spacer) regions of the nuclear ribosomal repeat unit, which contains both relatively conserved and quickly evolving regions. Other gene regions can be targeted once morphological examination has provided a provisional identification and when ITS is not informative. The Translation Elongation Factor subunit 1α (TEF) and Intergenic Spacer (IGS) regions can be useful in the identification of species that are distinguished by variation in host range, but are morphologically indistinguishable (O'Donnell et al., 1998; Pantou et al., 2001; Goker et al., 2009; Seier et al., 2009).

By using molecular methods, it is also possible to identify genetically close fungal species that attack plant species potentially outside of the expected host range of the biological control agent. This information has been used to modify the plant host-specificity test lists. An undescribed species of a leaf spot, *Septoria* sp., isolated from the weed Himalayan balsam, *Impatiens glandulifera*, was found to be very closely related to species of *Septoria* from other hosts, and these host plant species will be included in the test list (R. Tanner, pers. comm. 2009). In many cases, fungi that have been identified for their potential use as biological control agents are new to science, and hence can only be identified to genus level at best. This isn't surprising since in-depth pathology studies tend to have targeted agricultural crops in the past.

4.3.2. Population structure and genetic variability in fungal agents

Methods for assessing genetic variability of fungal agents are similar to those noted for weeds and arthropods. Knowing the extent and structure of the genetic diversity of a natural enemy in its native range can provide useful information to a biological control program. The leaf rust *Phragmidium violaceum* is native to Europe and was introduced into Australia for the control of the invasive alien weed European blackberry, *Rubus fruticosus*. The rust has had significant impact on some of the genotypes of the weed present in Australia, under some environmental conditions (Mahr and Bruzzese, 1998). Additional strains of the rust have recently been introduced to help provide a more comprehensive management of this genetically diverse target weed. A "trap garden" was established in France, containing a representative selection of the blackberry genotypes present in Australia, to source new strains of the

rust (Scott et al., 2002). Molecular analysis using SAMPL showed that the strains recovered from the trap garden were genetically different from those already released and established in Australia and New Zealand (Gomez et al., 2006). A suitable molecular technique was required to follow the fate of the new strains released, since they could not easily be distinguished from those already present in the field. Although the SAMPL technique could achieve this, the method is technically demanding and thus other avenues were explored. Initial efforts to develop a simple PCR-based diagnostic tool using sequencing, SCAR and ISSR (Morin et al., 2006), were not successful (L. Morin, pers. comm.). Reliable and robust polymorphic microsatellite markers were then developed (Molecular Ecology Resources Primer Development Consortium, 2010). These markers have been successfully used to demonstrate that alleles of the new strains released were present in the rust population two years after their release at representative sites (L. Morin, pers. comm.).

4.3.3. Elucidation of fungal life cycles and sexuality

A single fungal species can have two taxonomic descriptions, and consequently two names. This is because many fungi exist in two forms; the anamorphic (asexual) and teleomorphic (sexual) states, with completely different spore types. In rust fungi this is further complicated, because rusts can have up to five spores stages, and potentially three names (referring to the uredinal, telial and aecial stages) and sometimes, two host plant species involved (heteroecious rusts). The DNA sequences of the different spores stages of one species will be the same and hence molecular techniques can be used to elucidate suspected connections between spore states. Once spores states have been linked as the same species, the fungus is known by the teleomorphic name. Ellison et al. (2006) used DNA analysis to assess the relationship between the rusts *Aecidium lantanae* (aeciospores) and *Prospodium tuberculatum* (teliospores and urediniospores), found together on the leaves of lantana, *Lantana camara*. The hypothesis that two fungi are the same species was tested using the internal transcribed spacer (ITS) region of 18S-26S nuclear ribosomal DNA. The results showed that the aeciospores were genetically different from the two rust types of *P. tuberculatum*, and hence *A. lantanae* is not the 'missing' spore stage of *P. tuberculatum*.

4.3.4. Host-specificity testing of systemic fungal pathogens with long incubation periods

The period of time between infection and disease expression of a fungal pathogen normally lies in the range of three days to three weeks. Coevolved biotrophic pathogens often can have longer latent periods, up to many months. Molecular techniques can be used to aid host range testing, and shorten timing of risk assessments. Fungal specific primers can be used to establish whether a plant is infected with a fungus before symptoms are apparent. Boneseed, *Chrysanthemoides monilifera*, is a perennial woody shrub from the Western Cape of South Africa that has become an intractable weed problem in Australia. A systemic rust, *Endophyllum osteospermi*, that produces pycnia and aecia in witches' brooms on *C. monilifera* in South Africa, is being screened for potential release in Australia. The rust has an unusually long incubation period of up to two years from infection to symptom expression, with the rust growing systemically in the host for this period. This posed a serious constraint to host-specificity screening. Fortunately, few species required host-specificity testing and Wood (2008) has developed a novel, non-molecular based screening procedure to enable the risk assessment to be undertaken. However, the rust should soon be approved for release in Australia, and because of the prolonged incubation phase, plans are already in place to use DNA sequencing to monitor establishment and spread of the rust (Morin and Hartley, 2008).

4.4. Post-release evaluation of agents

Molecular methods can also be used for post-release evaluations of biological control agents. Variability of samples preserved during collections in the native range and variability of populations established in the introduced range could be compared to shed light on evolutionary change or population structure post-release. Additionally, post-release evaluations can be useful to detect organisms that were inadvertently introduced into biological control programs, and in evaluating host use after the fact (e.g. the beetle *Brachypterolus pulicarius* on toadflax, *Linaria* spp., MacKinnon et al., 2005, 2007; Hufbauer and MacKinnon, 2008). Post-release studies have been used to better understand differential success of agents on different target weeds (e.g. *Mecinus janthinus* on *Linaria* spp., described in Section 4.2.3) or in different climates, and the effects of hybridization (e.g. *Diorhabda* spp. on *Tamarix*, described in Section 4.2.4). Identification of immature stages of biological control agents can be done using molecular methods more quickly and reliably than by using morphological characters (Section 4.2.2). This is also true for post-release evaluation, particularly when checking for infestation of non-target plants.

5. Future trends

5.1. Access to foreign genetic material

The molecular-based approaches and their uses described in this review depend upon access to genetic material from organisms. Under the Convention on Biological Diversity (CBD; http://www.cbd.int/), countries have sovereign rights over the biological resources, including genetic resources, within their boundaries (CBD Art. 2), and expect to share in the benefits arising from their use. The Nagoya Protocol on Access and Benefit Sharing (UN, 2010) sets out how this process should operate, but the detailed implementation will depend upon legislation and regulations that will be developed by each country.

Many of the research techniques described above require access to genetic material from countries other than the invaded country, and some need samples from a diversity of countries. In future, access to any genetic material required for such studies will need to be agreed with each country from which genetic material is required, based on prior informed consent, mutually agreed terms of use, and potentially some form of benefit sharing (Cock et al., 2010). Non-commercial research can share benefits with source countries through shared research activities (Biber-Klemm and Martinez, 2006). In some cases, molecular-based methods in weed biological control are used for small pieces of work targeted to specific questions, and this limits the scope for shared research (e.g. to establish the area of origin of an introduced weed, plant samples are needed from several or many countries in the indigenous range of the weed, which are then compared in one laboratory). On the other hand, studies linked to teasing out the genetic ecology of plants or potential biological control agents in collaboration with one principle source country are appropriate, and may lead to increased knowledge of the source country biodiversity, new skills in the source country, and joint publications. Although the Nagoya protocol recognizes the need to facilitate non-commercial research, and the needs for access for food and agriculture, it is important that the biological control community is aware of and follows the new procedures.

5.2. Molecular methods

Determining the trophic range of arthropods has been a hot topic in the field of ecology at several levels of ecological organization

(Duffy et al., 2007). Among many of the available approaches, gut content analysis is a very popular one (e.g. Johnson and Nicolson, 2001), which has been enhanced by PCR amplification and sequencing of DNA extracted from gut contents. The technique has been widely used to identify animal prey (King et al., 2008), and more recently, also to identify food plants of herbivore species (Bradley et al., 2007; Matheson et al., 2008; Jurado-Rivera et al., 2009), but successful examples from biological control of weeds are lacking. A preliminary study of the flea beetle species complex, *Psylliodes* cf. *chalcomerus*, was carried out to identify plant remains within gut contents of individuals feeding on yellow starthistle, *Centaurea solstitialis*, and Scotch thistle, *Onopordum acanthium*. Unfortunately the preliminary experiments gave unsatisfactory and negative results hampering any further progress on the issue owing to time and money constraints. Very recent applications of Next Generation Sequencing (pyrosequencing; Section 2.2.2) are opening new horizons for the analysis of herbivore diets (e.g. Valentini et al., 2009a, b) although high cost is currently a limiting factor. The proposed approach is focused on faeces analysis, but we predict it is likely to be suitable for gut content analyses. The pyrosequencing method represents a very promising approach both for studying the trophic range of phytophagous insects and for setting up protocols for non-invasive analyses required for establishing colonies, especially in quarantine situations.

Biological control programs will benefit from molecular techniques that are now implemented for model organisms. Large DNA sequence datasets derived from genomic resources will soon be realized for the typical non-model organisms found in biological control projects (Thomson et al., 2010), eventually followed by affordable full genome sequencing of organisms involved in biological control. Comparing whole genomes of model organisms and biological control agents and weeds, along with determining gene functions and how they interact with other genes, will help in understanding traits that make a weed so successfully invasive or an agent host-specific and effective (Stewart et al., 2009, and e.g. Broz et al., 2007, Foley et al., 2010). Full genome sequencing will soon be affordable and accessible through Single Molecule Real Time Sequencing (SMRT). SMRT is a process in which the synthesis of a single DNA molecule is viewed and nucleotide base calls are made according to the corresponding fluorescence of the dyes corresponding to G, C, A or T. Tens of nucleotides can be read per second, and reads of up to 1000 or more base-pairs can be combined to obtain the whole genome of an organism. This rapid method will also allow processing numerous environmental samples of a single or a few genes, which could vastly speed up biological control population studies. Costs of SMRT may be uneconomical for biological control projects at first.

Other recently developed molecular tools, such as real-time qPCR, microarrays and pyrosequencing, described in Section 1, are not yet routinely used in biological control of weeds programs, mostly due to higher costs and specific technical knowledge required. In the future we expect to see increases in use of these tools in biological control programs. Real-time qPCR can be used to quickly diagnose the presence and quantify levels of pathogens in plant tissue or endosymbionts in an agent. Microarrays can be used to determine which genes are being expressed in an organism or populations of organisms, and can give insight into how plants, or which parts of a plant, are increasing chemical defenses in response to herbivory. Pyrosequencing can be used to quickly sequence large portions of a genome, which could help us understand the genetic basis for resistance in plant/herbivore relationships. As more of the tools recently developed for model species are brought to biological control programs, questions we never considered possible to answer in the past, or even considered to ask, will launch our science onto a new plane of discovery and applications.

5.3. Revisiting biological control projects

It is likely that molecular methods will be used to revisit some old weed biological control projects, not only to understand the results achieved (e.g. the toadflax program, Section 4.2.3), but also to identify new lines of research for targets for which biological control solutions have yet to be found. The *Lantana camara* sensu lato complex of interbreeding taxa is a good candidate. The hugely variable, polyploid status of the species complex has made biological control very difficult, since most of the weedy genetic forms do not occur naturally in the native range. Indeed, although around 40 species of natural enemies have been released to date in the invasive range, those agents that have become established have been only partially successful. A component of this limited impact is because each agent can usually only develop on a few of the weedy lantana forms. Historically, the forms have been separated on morphological differences, particularly flower color, but earlier molecular work had shown this not to be meaningful from the perspective of biological control agents (Scott et al., 1997). Molecular and cytological analyses need to be fully utilized to improve the understanding of the relationships of the different ploidy levels and hybrids within the *L. camara* complex, so that natural enemies can be collected and tested that will address the huge amount of genetic variation within the weedy forms.

Another project where molecular tools would have helped years ago, and will still be needed to complete our understanding, is that against *Chromolaena odorata* (Zachariades et al., 2009). There are more than 165 species in the Neotropical genus *Chromolaena*, but only *C. odorata* has become invasive in the Old World. It is now understood that there are two main invasive forms of *C. odorata*: one occurring in Asia and West Africa and the other in southern Africa. They differ from one another in morphology, biology, and ecology, but with little variation within each form. Thus, they are functionally distinct entities, and have been characterized as biotypes. Further work is needed to understand the relation of the two invasive biotypes to the situation in the indigenous range, and this will provide better indications on where co-evolved natural enemies for each 'biotype' might be found.

5.4. Conclusion

In the last two decades approaches in biological control of weeds have changed substantially by incorporating molecular techniques at each step of the process; from the identification of target weeds and related natural enemies (potential agents) to genetically comparing released agents (Table 3). Some of the novel molecular results presented in this review were determined following field observations of unexpected insect behaviour or unusual plant population structure. This combination of "ancestral" and "emergent" techniques is the key for increasing the global knowledge in biological control of weeds. Worldwide, there is a diminishing pool of taxonomic experts for both plants and arthropods (Goolsby et al., 2006), and even when an expert exists, their research interests may not overlap with the needs of biological control researchers. New molecular and statistical methods can answer some previously intractable questions about biological control targets and agents, but the role of traditional taxonomists cannot be underestimated (Wheeler et al., 2004), and they should always be included to examine results based on molecular data. Many taxonomic experts may not have been trained in nor have time to perform molecular methods, thus, biological control researchers need to at least be familiar with the tools available or build collaborations that involve molecular researchers and taxonomic experts. In many cases, biological control researchers have found themselves filling these roles when no suitable collaborations can be found.

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