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REVIEW

The importance of cryptic species and subspecific populations in classic biological control of weeds: a North American perspective

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Abstract Classical biological control of weeds depends on finding agents that are highly host-specific. This requires not only correctly understanding the identity of the target plant, sometimes to subspecific levels, in order to find suitable agents, but also identifying agents that are sufficiently specific to be safe and effective. Behavioral experiments and molecular genetic tools have revealed that some arthropod species previously thought to be polyphagous really consist of multiple cryptic species, host races or biotypes, some of which are more host-specific than others. Whereas true species are reproductively isolated, individuals from subspecific populations may potentially interbreed with those of other populations if they should encounter them. Furthermore, biotypes may consist of individuals sharing a genotype that is not fixed within a monophyletic group, and thus may not be evolutionarily stable. This raises the question of how such populations should be classified, and how to confirm the identity of live arthropods before releasing them as classical biological control agents. The existence of host races or cryptic species may greatly increase the number of prospective biological control agents available. However, it may also create new challenges for governmental regulation. These issues are discussed using pertinent examples, mainly from North America.

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Introduction

The safety and efficacy of classical biological control depend on correctly understanding the taxonomy of both the target plant and prospective biological control agents. It is important to determine if there is one or more distinct races, varieties or species in the invaded region to properly define the weed(s) being targeted: the taxonomy must be compatible with that in the region of origin to avoid searching for agents on the wrong target plant. Lastly, prospective agents need to be tested on appropriately representative accessions of the target weed to determine their potential efficacy. The advent of molecular genetic taxonomic tools and integrative taxonomy has greatly facilitated our ability to verify traditional taxonomic concepts as well as to understand the genetic structure of populations, including detection of cryptic species, subspecies or races (Drès and Mallet 2002; Bickford et al. 2007; Gaskin et al. 2011). These tools are also helping us to discover new prospective biological control agents by revealing host-specific populations that are hidden within species that were thought to be polyphagous. Cryptic species appear to be much more common than was previously thought (Bickford et al. 2007), which may greatly improve our ability to find safe and effective biological control agents.

A clear understanding of taxonomy depends on the application of appropriate concepts of taxonomic categories and their evolutionary stability. Despite its limitations regarding applicability to all types of organisms (Noor 2002), the ‘biological species concept’ (Mayr 1942), based on interbreeding reproductively-isolated populations, is generally used today to delineate living species of plants and arthropods (Coyne and Orr 2004), but morphological characters are used for describing species (Cook et al. 2010). Thus, integrative taxonomy, which combines the analysis of genetic, morphological and other characters, is recognized as a way to improve our ability to define taxa (Padial et al. 2010). However, species are not immutable in time and space, and the process of speciation is expected to produce a continuum or spectrum of entities ranging from polymorphisms to races to species (Powell et al. 2013). Furthermore, of greatest importance to practitioners of classical biological control is an accurate understanding of the behavioral, ecological and physiological characteristics of the population(s) of interest, and differences in such phenotypes may occur between groups that are currently recognized as belonging to one species (Mopper and Strauss 2013). The term ‘cryptic species’ designates populations that are reproductively isolated, but that have not been (or cannot be) reliably identified by morphological characters (Darlington 1940). Populations that are not completely reproductively isolated may be categorized as subspecies or races (Fox and Morrow 1981; Drès and Mallet 2002; Hufbauer and Roderick 2005). The terms ‘biotype’, ‘race’ and ‘strain’ have often been used to describe populations that have a distinctive biological characteristic of interest (Downie 2010). However, much confusion has been caused when such classifications have been used without a clear description of the genetic structure of the group being studied (Downie 2010). For example, if a group of insects shows a particular characteristic, e.g. an ability to attack a different plant variety or resistance to an insecticide, this could be caused either by a change in gene frequencies within the insect population (an intra-specific change) or by replacement of one taxonomic group by another (an inter-specific change). This distinction is critical because the former reflects evolutionary plasticity of a group, whereas the latter indicates replacement of one evolutionarily stable taxon by another. With regard to host plant specificity of a classical biological control agent, the former case is undesirable because it suggests the potential for host plant specificity to change, whereas the latter is desirable because host plant specificity of a population should be relatively fixed.

The purpose of this paper is to describe some examples, primarily from North America, in which cryptic species or subspecific groups were involved in the identity of either the target weed and/or the biological control agent and were critical to achieving successful biological control. We also discuss issues related to future research and regulatory control.

Identification of weed targets

Correct identification of the target weed is critical for determining its geographic region of origin and for directing the search for prospective biological control agents. Misunderstanding the identity of the target weed can lead to looking for agents in the wrong region, or of selecting prospective agents that are not well-adapted to the target. Here we present several samples of invasive plants in North America that posed a challenge to biological control because of misidentification or because of the discovery of biotypes, cryptic species and/or hybrids. Confusion due to historical synonymies and incorrect species concepts have affected projects on many targets, including leafy spurge (*Euphorbia* spp., Malpighiales: Euphorbiaceae; Crompton et al. 1990), toadflaxes (*Linaria* spp., Lamiales: Plantaginaceae; De Clerck-Floate and McClay 2013; De Clerck-Floate and Turner 2013) and Russian thistle (common tumbleweed, *Salsola* spp., Caryophyllales: Amaranthaceae; Hrusa and Gaskin 2008). Furthermore, hybridization of invasive species with other invasive species or with native species has proved a challenge for projects on French broom (*Genista monspessulana* [L.] LAS Johnson, Fabales: Fabaceae; Kleist et al. 2014), purple loosestrife (*Lythrum salicaria* L., Myrtales: Lythraceae; Houghton-Thompson et al. 2005), Russian thistle (*Salsola* spp.; Hrusa and Gaskin 2008), saltcedar (*Tamarix* spp., Caryophyllales: Tamaricaceae; Gaskin and Shafroth 2005; Gaskin and Kazmer 2009), toadflax (*Linaria* spp.; Ward et al. 2009; Boswell et al. 2016), and water milfoil (*Myriophyllum* spp., Saxifragales: Haloragaceae; Moody and Les 2002). In the case of Russian thistle, genetic and morphological studies distinguished *Salsola tragus* L. from *S. australis* R. Brown, which had previously been considered a synonym (Hrusa and Gaskin 2008). The latter species is now thought to originate from Australia, which was very surprising because all the other species closely related to *S. tragus* are believed to originate from Eurasia (Rilke 1999; Borger et al. 2008). Both a fungal pathogen (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz.; Deuteromycotina: Coelomycetes) and a gall-forming midge (*Desertovellum stackelbergi* Mamaev; Diptera: Cecidomyiidae) from Eurasia are far more damaging to *S. tragus* than they are to *S. australis* (Sobhian et al. 2003; Bruckart et al. 2004). On the other hand, in an analysis of perennial pepperweed (*Lepidium latifolium* L., Brassicales: Brassicaceae), morphological and molecular data, based on AFLPs (amplified fragment length polymorphisms) from populations in Eurasia and North America, indicated the existence of three genetic clusters, but that these did not correspond to morphological characters, and that there was no support for differentiating between *L. latifolium*, *L. affine* Ledeb. and *L. obtusum* Basiner (Gaskin et al. 2013b).

Subspecific variation in target weeds that affects suitability to attack by prospective biological control agents has interfered with the progress of many projects. This has been especially true for highly specific agents such as rust fungi, mealybugs, gall-forming insects and eriophyid mites. For example, rush skeletonweed (*Chondrilla juncea* L., Asterales: Asteraceae) is now known to have three genotypes in Australia and at least three other genotypes in North America (Gaskin et al. 2013a). This helps explain why the rust fungus, *Puccinia chondrillina* Bubák and Sydow (Uredinales: Pucciniaceae), introduced from Italy in 1976 suppressed the weed in California but much less so in the Pacific Northwest, where different genotypes occur (Supkoff et al. 1988; Gaskin et al. 2013a; Pitcairn et al. 2014). Ultimately five strains of the rust were released in Australia to control three morphological ‘forms’ of rush skeletonweed (Cullen 2012). Plant ‘forms’ also varied in resistance to the eriophyid mite, *Aceria chondrillae* (Canestrini) (Acari: Eriophyidae). A population of the mite from Greece was introduced to Australia that was effective on one form of the weed, and a population of the mite from Italy was introduced to the USA that attacked a different form of the plant (Sobhian and Andres 1978; Smith et al. 2010). Analysis of plant AFLPs has pointed to Bulgaria as the best place to look for agents that would be adapted to genotypes #1 and #2, which are still uncontrolled in Idaho and Montana (Gaskin et al. 2013a).

The availability of molecular genetic tools for identifying subspecific groups, has encouraged the search for agents that are specific enough to attack an invasive alien subspecies of a native North American species, common reed (*Phragmites australis* (Cav.) Trin. ex Steud., Poales: Poaceae; Tewksbury et al. 2002; Casagrande et al. submitted). Although hybrids between the alien and native genotypes generally do not occur at sites inhabited by both types, a hybrid population has been found in New York (Saltonstall et al. 2014). However, an adventive midge, *Lasioptera hungarica* Möhn (Diptera: Cecidomyiidae), which is known to attack the European subspecies, but not the American subspecies, was observed attacking hybrid plants in the field. Thus, there appears to be at least one insect natural enemy that is specific to the invasive subspecies and its F1 hybrid.

Determining the geographic origin of the target weed

The desire to find highly specific agents that are well-adapted to attacking the target weed makes it important to determine the geographic origin of the target weed. Plants exhibiting relatively little genetic heterogeneity in the invasive region and that have a clear phylogeographic structure in their native range are relatively easy to match with potential geographic origins. Some examples include the various genotypes of perennial pepperweed (*L. latifolium*; Gaskin et al. 2013b) and rush skeletonweed (*C. juncea*; Gaskin et al. 2013a), discussed above. Species that are outcrossing and that are the consequence of many introductions may present a more difficult challenge, such as yellow starthistle (*Centaurea solstitialis* L., Asterales: Asteraceae; Barker et al. 2017), French broom (*Genista monspessulana*; Kleist et al. 2014), and Russian thistle (*Salsola* spp.; Hrusa and Gaskin 2008). Nevertheless, molecular genetic tools are now commonly used to help characterize the origin of target weeds (Gaskin et al. 2011).

The right agent for the right target

Coevolutionary adaptation between isolated populations of plants and their natural enemies can result in intra-specific variation that can be crucial for the success of a biological control project (Kniskern and Rausher 2001; Hufbauer and Roderick 2005). Plant populations can exhibit resistance to some populations of host-specific herbivores or pathogens, and some populations of the latter are adapted to tolerate such resistance mechanisms. Thus, some introduced biological control agents have failed to establish because they were not adapted to the host plant genotypes in the invaded range. An example is the gall-forming tephritid fly *Urophora jaculata* Rondani (Diptera: Tephritidae) from Italy which failed to attack yellow starthistle (*Centaurea solstitialis*) in California because that genotype was resistant to the fly (Clement 1994). However, proper characterization of the target weed has helped to direct exploration for agents capable of attacking the targeted weed genotype. For example, proper identification of the floating aquatic plant, *Salvinia molesta* D.S. Mitchell (Salviniales: Salviniaceae) led to use of the appropriate population of a stem-mining weevil, *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae), which was later described as a different species, *C. salviniae* Calder and Sands (Madeira et al. 2006).

Eriophyid mites are often highly host-specific and may even be limited to attacking only certain genotypes of the host plant (Skoracka et al. 2010). The example of *Aceria chondrillae* on rush skeletonweed was described above. However, anticipating a similar situation helped to direct the search for a leaf-curling mite (*Floracarus perrepae* Knihinicki and Boczek, Acari: Eriophyidae) that could attack the genotype of climbing fern, *Lygodium microphyllum* (Cav.) R. Br. (Schizaeales: Lygodiaceae), which is invasive in Florida (Goolsby et al. 2006). Testing of mites collected from various sites during exploration in the South Pacific led to the discovery of an appropriate population to introduce, which became successfully established (Lake et al. 2014). In another example, molecular genetic analysis of perennial pepperweed (*Lepidium latifolium*) indicated that plants in North America are most similar to those in Kazakhstan and China (Gaskin et al. 2013b). The eriophyid mite *Metaculus lepidifolii* Monfreda and De Lillo (Acari: Eriophyidae) had been found on *L. latifolium* in Turkey (Monfreda and De Lillo 2012). However, this population of the mite produced much higher numbers on *L. latifolium* plants from Turkey than from the USA in a field garden experiment (M. Cristofaro unpubl. data). Thus, the search for a well-adapted mite population is focusing on Kazakhstan where mites differ by about 2% (difference in *COI* sequence) from those in Turkey, which suggests a possible cryptic species (T. Cvrković unpubl. data).

Brazilian pepper, *Schinus terebinthifolius* Raddi (Sapindales: Anacardiaceae), has two distinct chloroplast haplotypes and their hybrids in Florida, where it is invasive (Williams et al. 2005, 2007). A thrips initially thought to be *Pseudophilothrips ichini* Hood

(Thysanoptera: Phlaeothripidae) was collected in Brazil and tested on Florida Brazilian pepper genotypes. Molecular genetic and morphological analyses distinguished two species of thrips: *P. ichini*, which was found on haplotypes A, K, N, and M in the native range and is well adapted to both Florida haplotypes (A and B), and a new species, *P. gandolfoi* Mound, which is only associated with haplotypes C and D from southern Brazil (Manrique et al. 2008; Mound et al. 2010). Further study of two Brazilian populations of *P. ichini*, one from haplotype K from Salvador (13°S lat.) and one from haplotype A from Ouro Preto (20°S), indicated a difference in their adaptation to temperature (Manrique et al. 2014). The Ouro Preto population could survive freezing temperatures longer and developed equally well on the A and B haplotypes, whereas the Salvador population developed better on A than on B. Climate matching analysis based on known distributions in Brazil predicted that only the Ouro Preto population would be able to establish in Florida, thus identifying this as the best population to introduce to Florida.

In the case of toadflaxes, three species were targeted for control in North America: Dalmatian toadflax (*Linaria dalmatica* (L.)) Miller, broomleaf toadflax (*L. genistifolia* (L.) Miller) and yellow toadflax (*L. vulgaris* Miller) (Lamiales: Plantaginaceae) by the stem-boring weevil, *Mecinus janthinus* Germar (Coleoptera: Curculionidae), which was thought to attack all of them (Wilson et al. 2009; De Clerck-Floate and McClay 2013; De Clerck-Floate and Turner 2013). However, it was later discovered that there are at least two cryptic species of *Mecinus*: *M. janthinus* Germar, which is better adapted to *L. vulgaris*, and *M. janthiniformis* Toševski and Caldara, which is better adapted to *L. genistifolia* and *L. dalmatica* (Toševski et al. 2011, 2013).

Specialists hidden within polyphagous taxa

In the search for prospective biological control agents, taxa that are known to have a broad range of host plants, based on museum specimen records, or reports in the literature, are typically discarded from further consideration. However, there are several cases in which a host-specific population has been discovered to exist within such a species. The weevil *Ceutorhynchus assimilis* Paykull (Coleoptera: Curculionidae) has been reported to be a pest of more than 13 species in the mustard family, Brassicaceae, including several crops (Hoffmann 1954). However, preliminary host-specificity tests showed that *C. assimilis* populations reared from hoary cress (*Lepidium draba* L., Brassicales: Brassicaceae) occurring in southern France were only able to complete their larval development on *L. draba* (Fumanal et al. 2004). Preliminary crossing experiments between this host race and the other genetic entities developing on *L. draba* showed an absence of prezygotic barriers but the existence of partial postzygotic barriers (M.C. Bon, unpubl. data).

In another example, the weevil *Trichosirocalus horridus* (Panzer) (Coleoptera: Curculionidae) was considered to be a single species when it was introduced in 1974 to control thistles (*Cirsium* and *Carduus* spp., Asterales: Asteraceae) in North America, Australia and New Zealand (Kok and Trumble 1979; Harris 1984; Jessep 1989; Woodburn 1997). Subsequent exploration for agents of Scotch thistle (*Onopordum* spp., Asterales: Asteraceae) led to the discovery of a population in Spain that did not attack *Carduus* or *Cirsium* spp. (Briese et al. 2002). This entity was described as a new species, *T. briesei*, based on morphological and molecular genetic analysis (Alonso-Zarazaga and Sánchez-Ruiz 2002; De Biase et al. 2016), and was successfully released in Australia (Briese 2012). A similar situation was discovered for the flea beetle, *Psylliodes chalcomera* (Illiger) (Coleoptera: Chrysomelidae), which had been unsuccessfully introduced to North America in 1997 to control musk thistle (*Carduus nutans* L., Asterales: Asteraceae; Andres and Rees 1995). Subsequent studies on molecular genetics of adults and larvae extracted from host plants in Eurasia showed that this species comprised at least three clusters based on haplotypes: one associated with yellow starthistle (*Centaurea solstitialis*), which is a new host plant record, one associated with Scotch thistle (*Onopordum acanthium* L., Asterales: Asteraceae), and one that is apparently more polyphagous that is associated with musk thistle, Scotch thistle and yellow starthistle (De Biase, unpubl. data). The genetic divergence between specimens from the yellow starthistle cluster and the two other clusters is small (0.007 and 0.024, respectively), suggesting that these may be host races rather than cryptic species.

Evolutionary stability of agents

Coevolution of insects and host plants is a never-ending process, but the speed at which evolution proceeds is variable (Singer et al. 1994). Some taxa may be more heterogeneous and dynamic than others. For example, the oligophagous weevil *Ceratapion basicorne* (Illiger) (Coleoptera: Brentidae) showed little *COI* variation across its geographic range from France to Turkey (Antonini et al. 2009), whereas the weevil *T. horridus* sensu lato, and the flea beetle *P. chalcomera* showed much higher variation, including host races and/or cryptic species. The combination of geographic and/or phenological isolation and relative abundance of different host plants may facilitate the development of subpopulations that differ in host plant specificity (Diehl and Bush 1984; Mopper and Strauss 2013). For example, a widely distributed haplotype clade of *P. chalcomera* occurs on several species of Cardueae plants (*C. nutans*, *O. acanthium*) but an isolated population in southern Russia is apparently adapted to *C. solstitialis*. Similarly, populations of the root weevil *C. assimilis* from central Europe appear to be more polyphagous than populations from southern France that are associated with *L. draba* (Fumanal et al. 2004). The widespread European weevil *T. horridus* sensu lato develops on many Cardueae plants, but a sister species, *T. briesei*, found only in northern Spain, is adapted to *Onopordum* (Briese et al. 2002). Thus, we may find more host-specific populations of prospective agents by examining the genetic isolation of populations that are associated with isolated patches of the target plant.

Conclusions

Improved molecular genetic tools for identifying target weeds and distinguishing them from close relatives (whether true species or subspecific populations) should improve our ability to find prospective biological control agents that are likely to establish and multiply, thus improving the likelihood of significantly suppressing the target weed. Correct systematic identification helps direct exploration for agents to the appropriate geographic region of origin and enables us to test prospective agents on the appropriate genotypes of the weed that are targeted for biological control. However, some target weeds have evolved during the process of introduction such that they present novel genotypes that cannot be found in their region of origin. Although this complicates the decision of where to look for well-adapted agents, it is nevertheless important to understand the genetic variation of the target weed populations to enable us to test the potential of prospective agents to attack the various genotypes of the target weed.

The fact that populations of prospective biological control agents that differ in important physiological or behavioral characters may be difficult or impossible to distinguish morphologically raises the question of how to avoid releasing the wrong individuals. Use of ‘standard barcodes’, such as *COI* or other mitochondrial markers may be sufficient. However, in cases where hybridization may occur, nuclear markers would be needed (Gaskin et al. 2011). Furthermore, barcoding might not correspond with morphological characters attributed to known species (Stepanović et al. 2016). While agents originating from one location are likely to be more genetically similar than those coming from multiple locations, this is not sufficient to guarantee that only one biotype has been collected. Polyphagous genotypes of *T. horridus* have been collected at *T. briesei* sites in Spain and polyphagous *P. chalcomera* has been collected at a yellow starthistle-genotype site in Russia (Cristofaro et al. 2004; De Biase et al. 2016). Thus, in cases where multiple biotypes are suspected, it is important to screen all members of a colony to ensure that test results pertain to the appropriate biotype and that only their descendants are used for eventual release. This may be done by nondestructive analyses, such as by extracting DNA from frass (Fumanal et al. 2005) or by isolating progeny from individual females and adding them to a colony only after the mothers have been genotyped.

The use of phylogenies based on statistical analysis of large numbers of characters, whether molecular genetic or morphological, has begun to erode traditional taxonomic hierarchies (Peccoud et al. 2009). This also applies to the subspecific populations. There are no standard thresholds for determining when a genetic distance is sufficient to consider different populations to be species, subspecies or biotypes (Cognato 2006). However, if only one population is being considered for introduction as a biological control agent, then these distinctions may be less relevant because the population would be geographically isolated from its close relatives in the region of introduction, and thus could not interbreed with them. Furthermore, rarely has the ability of cryptic species to hybridize been tested experimentally (Paterson et al. 2016). In any case, it is important to define how to identify a member of the population that we want to study and release. Such information should be publicly available, such as by posting on a genetic database (e.g., GenBank). Regarding the possible impact on regulation of biological control, the guidelines used in the USA (USDA-APHIS 1998) already request reporting if DNA analysis was used to identify the agent and what the geographic origin of the agent is. They also request reporting the methods used to ensure that colonies are pure and to verify the correct identification of the agent to be released. Although identification is currently required only to the species level for insects, strain, race and type are requested for pathogens, so it would seem reasonable that regulation could easily move in the direction of identifying a particular strain of insect or mite to release.

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