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Reevaluating establishment and potential hybridization of different biotypes of the biological control agent *Longitarsus jacobaeae* using molecular tools

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ABSTRACT

Evaluation of past and current biological control programs using molecular tools can clarify establishment success of agent biotypes, and can contribute to our understanding of best practice for natural enemy importations. The flea beetle, *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae) has successfully controlled the weed tansy ragwort, *Jacobaea vulgaris* Gaertn., in Pacific coastal areas of the USA. A *L. jacobaeae* biotype introduced in 1969 from Italy is assumed to provide this control. A cold-adapted biotype from Switzerland was also released in 1969 to California, but its establishment was never confirmed. Recent infestations of tansy ragwort into parts of Montana with continental, winter-cold climates prompted introduction of the Swiss biotype in 2002. The Italian and Swiss biotypes cannot be separated morphologically and are able to hybridize in the laboratory. We used amplified fragment length polymorphisms to assess which biotypes established in California, Oregon, and Montana at sites with varying climatic conditions, and whether the biotypes have hybridized in nature. The analysis was based on 216 *L. jacobaeae* individuals collected from 13 populations in the introduced and native ranges in 2006 and 2007. Clustering and assignment tests showed that the Italian biotype successfully established at all study sites, including those characterized by continental, winter-cold climates. We also found hybrids of the two parental biotypes, which in one study location constituted 47% of the population. Future studies are needed to evaluate whether to release either biotype alone or in combination on new tansy ragwort infestations in winter-cold climates in North America.

1. Introduction

Early classical biological control programs followed differing exploration principles for the importation and release of natural enemies. Some focused on explorations for biological control agents in climatic regions within the native range of the target pest that closely matched the areas of introduction to increase the probability of establishment (Bartlett and van den Bosch, 1964; Wapshere, 1985). Others emphasized the genetic diversity of the source populations and the collection of different strains or biotypes of a control agent from a range of climates throughout the range of a species to facilitate adaptation to new environments post-release (Bartlett and van den Bosch, 1964; DeBach and Rosen, 1991). Since experimental evidence supporting either strategy was not available at the time (Sands and Harley, 1980), choosing a region from which to select potential agents has typically been based on the availability of agents, resources and other practical considerations (Myers and Sabath, 1980).

Advances in ecological research and retrospective analyses of past biological control programs have shown that there are limitations and risks associated with both approaches. While climatic matching is considered a useful tool to focus research efforts to certain areas for exploration of new biological control agents (Hoelmer and Kirk, 2005; Hufbauer and Roderick, 2005; Sands and Harley, 1980), its importance is also being questioned as an increasing number of cases has been documented in which climate did not reliably predict the establishment and performance of agents in the introduced range (e.g., Harris, 1984; McFadyen, 1984; van Klinken et al., 2003). Importation and release of additional biotypes of biological control agents have led to improved control and establishment in several cases (e.g., DeBach and Rosen, 1991; Room et al., 1981; Unruh and Messing, 1993). For example, introduction of a Persian biotype of the parasite *Trioxys pallidus* Haliday has led to successful control of the walnut aphid (*Chromaphis juglandicola* Kaltenbach) in California, where a French biotype previously failed (DeBach and Rosen, 1991). However, recent studies have raised concerns about the safety and efficacy of this practice since biological control agent biotypes can differ in host ranges

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and other traits including developmental time, fecundity, longevity, egg size, and sex ratio (DeBach and Rosen, 1991; Doutt and DeBach, 1964; Messing and Aliniazee, 1988; Goldson et al., 2005; Hoffmann et al., 2002; Mathenge et al., 2009; Sands and Harley, 1980; reviewed in Hopper et al., 1993). Moreover, mixing and/or hybridization between biotypes can lead to unexpected consequences that could either increase or decrease the fitness of future generations or may result in changes in host-specificity (Bartlett and Lagace, 1961; DeBach and Rosen, 1991; Hoffmann et al., 2002). Hoffmann et al. (2002) have shown that two biotypes of *Dactylopius opuntiae* Cockerell, a biocontrol agent of invasive *Opuntia* species in South Africa, lost specificity for their respective *Opuntia* species hosts when hybridizing. While the ability of F1 hybrids to attack different invasive *Opuntia* species may be advantageous, the F2 generation included both host-specific and non-host specific genotypes, which could be detrimental when host-specific genotypes hatch on a non-host. The authors concluded that only pure strains should be released in different areas to avoid hybridization (Hoffmann et al., 2002).

Until recently, biological control efforts have been hindered by the fact that identification of closely related species, species complexes, cryptic species or species biotypes, both pre- and post-release, was often inconclusive using traditional identification methods (González and Gilstrap, 1992; Hoelmer and Kirk, 2005; Schauff, 1992; Unruh and Wooley, 1999). The unintended release of cryptic species and the inability to identify biotypes prevented establishment or successful control in a number of cases (examples in Barratt et al., 2010; Clarke and Walter, 1995; DeBach and Rosen, 1991; Gordh and Beardsley, 1999; Hoelmer and Kirk, 2005). These problems can nowadays be resolved with molecular methods, which are reliable for identifying cryptic species and species biotypes (Hoelmer and Kirk, 2005; Unruh and Wooley, 1999).

The highly successful tansy ragwort biocontrol program in the western USA (Coombs et al., 2004; McEvoy et al., 1991) provides a good example to study the outcome of past introductions since two biotypes of the most effective agent, *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae), have been introduced on multiple occasions from Italy and from Switzerland (Frick, 1970b; Frick and Johnson, 1973; Littlefield et al., 2008; Turner and McEvoy, 1995). Evaluation of the current distribution of these two biotypes and their potential hybrids in the Pacific Northwest using genetic tools would clarify the nature of these populations and contribute to our understanding of the consequences of multiple biotype release as an approach in biological control. Identification of which biotypes established in different environments can give an indication of the adaptive potential or phenotypic plasticity of *L. jacobaeae* and provide a basis for recommendations concerning the source populations for future releases of this agent to control additional tansy ragwort infestations.

Our study focuses on the introduced biotypes of the ragwort flea beetle, *L. jacobaeae*: one from a Mediterranean climate near Rome in Italy, and the other from a more continental, winter-cold climate from the northeast Swiss Jura Mountains (Frick, 1970b; Frick and Johnson, 1973; Littlefield et al., 2008). The two biotypes exhibit distinct phenologies that assumedly make them more suitable for certain climates, and are able to hybridize under laboratory conditions (Frick, 1971; Frick and Johnson, 1973). It is assumed that the Italian biotype released in northern California between 1968 and 1970 has established, but not the single small (40 females) release of the Swiss biotype in northern California in 1969 (Frick, 1970b; Frick and Johnson, 1973). Italian beetles have been distributed and provide control of tansy ragwort along the Pacific coast where the mild climate is similar to the Mediterranean region (Hawkes, 1980; Turner and McEvoy, 1995). More recently tansy ragwort invaded higher elevation areas (>1000 m) in the inland Pacific Northwest, in Idaho and northwestern Montana, where continental, winter-cold climates prevail (Littlefield et al., 2008). *L. jacobaeae* collected from low elevation (<100 m) coastal areas in Oregon failed to establish in Montana, while a population from a site from Mt. Hood Oregon (1100 m) established in Montana by 2002. Nevertheless, the Swiss biotype has been re-introduced in 2002, often to the same areas where Italian beetles already established (Littlefield et al., 2008).

Establishment of presumably Italian beetles at the high elevation sites in Oregon and Montana may have been facilitated by genetic introgression from Swiss beetles. Accordingly, our first objective was to assess whether Swiss *L. jacobaeae* established from the 1969 release in California. Our second objective was to determine the ancestry of *L. jacobaeae* established at high elevation locations in Oregon and Montana. Finally, we sought to ascertain if the two biotypes have hybridized at these and other sites in nature.

2. Materials and methods

2.1. Study organism

L. jacobaeae is a univoltine herbivore. Its native range encompasses a wide range of climates, extending from the British Isles through Europe and North Africa to Siberia, Tibet and Turkestan (Shute, 1975). During the foreign exploration for biocontrol agents against tansy ragwort, *Jacobaea vulgaris* (syn *Senecio jacobaea* L.) in the 1960s, two *L. jacobaeae* biotypes were studied, one collected near Rome, Italy and one from Delémont, Switzerland (Frick, 1970a, 1971; Frick and Johnson, 1973). Annual, winter and summer mean temperatures are 14.6, 7.5 and 22.4 °C for the region around Rome, and 8.7, 0.5 and 16.7 °C at Delémont, respectively (MeteoSwiss, 1961–1990; NCDC, 1955–1980). Host-specificity tests were conducted for both biotypes, and both were found to be environmentally safe for release in North America (Frick, 1970a). As a consequence of changes to the taxonomic status of tansy ragwort, which is now placed in the genus *Jacobaea* (Pelser et al., 2007), additional plants were tested prior to the release of the Swiss biotype in Montana and as part of a new petition to release Swiss beetles in Canada (Puliafico, 2003, unpublished; U. Schaffner, personal communication).

The Italian and Swiss biotypes have different phenologies (Frick, 1971; Frick and Johnson, 1973). Italian beetles emerge late May to early June, and feed briefly before entering aestivation throughout the hot and dry summer. When temperatures decrease and rainfall recommences in fall, the beetles become active again. After 2–3 weeks of adult feeding on the foliage of tansy ragwort, beetles mate and females start to lay eggs around rosettes. Most larvae hatch within 3 weeks. Larvae mine in the roots, petioles and lower leaves throughout the winter until spring, when they leave the roots for pupation in the soil (Frick and Johnson, 1973). By contrast, adults of the Swiss biotype emerge from late June to mid July. Oviposition starts 2 weeks after emergence and eggs are laid throughout the cooler and moister summer, compared to Italy, until late fall. Eggs exhibit a facultative diapause and hatch the following spring. Larvae initially mine in the leaf petioles and later in the root crowns. Pupation takes place between late spring and early summer (Puliafico et al., 2008). The biotypes hybridize under laboratory conditions, and the resulting hybrid beetles show intermediate values for most life history traits compared to the parental biotypes (Frick and Johnson, 1972, 1973; M. Szűcs unpublished).

2.2. Introduction history of L. jacobaeae biotypes

A low number (25 females and 15 males) of Italian beetles was introduced in 1968 near Fort Bragg, California (Frick, 1970b) (Fig. 1). This first release attempt did not result in establishment

and was followed by larger releases (54 females and 75 males in 1969, and 419 females and 369 males in 1970), which led to successful establishment of the beetle (Frick and Johnson, 1973). Three hundred Italian biotype beetles were also released in southern Washington in 1970 (Frick and Johnson, 1973). There was a single release of beetles from Switzerland (40 females and 46 males) at the beginning of the biocontrol program in 1969 near Smith River, California, but its establishment was never confirmed (Frick, 1970b; Frick and Johnson, 1972). Based on the better climatic match between Italy and the targeted northern California and coastal Oregon, chances for establishment of the Italian biotype were generally assumed to be higher, and no further attempts were made at that time to establish Swiss beetles. The number of Italian beetles recovered in surveys sharply increased and by 1973 showed the first signs of control (Hawkes and Johnson, 1978).

Redistribution of Italian beetles from the Fort Bragg area began in 1971 (Isaacson, 1978). More than 20 beetle shipments from this area were sent to and released in Oregon between 1971 and 1975 (Isaacson, 1978). By 1975, beetle populations at the 1971 release sites in Oregon reached densities so high that it was the insectary used for within state redistribution (Isaacson, 1978). Releases of beetles at infestations at higher elevations (>1000 m) in the Cascade Mountains began in 1978 (Hawkes, 1980). Beetles collected in the Salem, Oregon area were used for repeated releases on Mt. Hood, OR (E. Coombs personal communication). During the 1980s, several releases were made east of the Cascade Mountain range where climatic conditions are more continental, all of which failed to establish (Coombs et al., 1996).

A major tansy ragwort infestation, spreading at high elevation locations in Lincoln and Flathead Counties, Montana, was discovered following the Little Wolf fire in 1994 (Littlefield et al., 2008). *L. jacobaeae* collected from lowland populations in Oregon (<100 m elevation) were released in 1997 and 1998 with little success (Littlefield et al., 2008). Few adults were recovered for three years following the releases but none thereafter (Littlefield et al., 2008). Subsequently, flea beetles originating from high elevation populations at Mt. Hood (1100 m elevation) were released between 1999 and 2001 (Littlefield et al., 2008). These beetles established in Montana but their distribution was limited to moister environments (Littlefield et al., 2008), thus the known cold-adapted biotype was re-introduced from Switzerland from 2002 to 2006 (Littlefield et al., 2008). The Swiss biotype was identified as *L. jacobaeae* using mitochondrial DNA sequences (Puliafico, 2003, unpublished; Puliafico et al., 2004) to avoid importation of the cryptic species *L. flavicornis* Stephens, which in the past was accidentally introduced into Australia and Canada (McLaren et al., 2000; Shute, 1975). Currently, there are several sites in Montana where both Italian and Swiss beetles have established. This provides a situation under which hybridization between the two biotypes could occur in a natural environment.

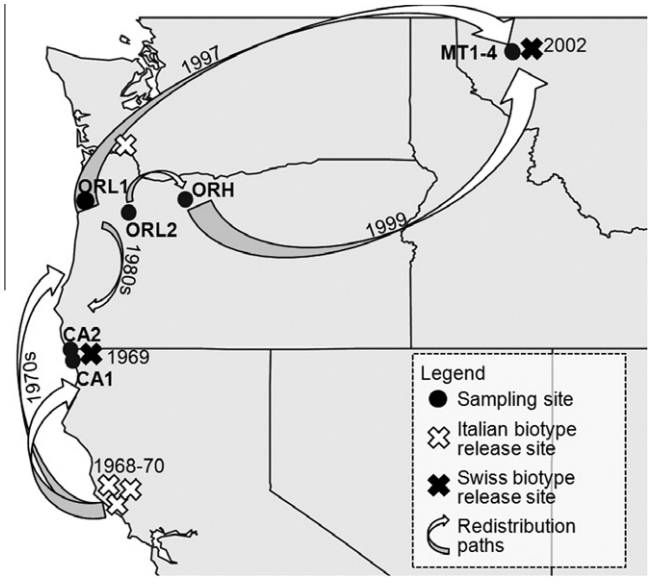

Fig. 1. Western USA field study population locations, release sites of Italian and Swiss biotypes and some of the redistribution paths of *L. jacobaeae* relevant for the study. See text for details.

2.3. L. jacobaeae sampling

Individuals from 12 *L. jacobaeae* populations were sampled in the native range in Switzerland and Italy, and the introduced range in the western USA in 2006 and 2007 (Table 1, Fig. 1). Beetles were collected in Italy close to Nazzano, 40 km north of Rome next to a gravel road running along the Tiber River, and in Switzerland in the southeast Jura Mountains at L 'Himelette from a grazed pasture (Table 1). Individuals from a second Swiss population were collected from a laboratory and common garden rearing at the University of Idaho, in Moscow, Idaho. These beetles were originally collected at St. Imier and Mettembert in Switzerland, introduced to Montana in 2002, and provided to the University of Idaho in 2004 (Table 1). Beetles were also collected from two populations (Smith River and Crescent City) in northern California from abandoned pastures, close to the original release site of the Swiss biotype beetles in 1969 (Table 1). In Oregon, beetles were collected at two low (Scherzinger and Salem; <100 m elevation) and one high elevation populations (Mt. Hood; 1000 m elevation) from grazed pastures and open woodland, respectively (Table 1). Finally, beetles were sampled from four high elevation sites (>1000 m) in Montana from previously burned timberland, where both Italian and Swiss beetles have been released (J. Littlefield personal communication) (Table 1). Adult beetles from populations in the introduced range were collected during the summer except for one of the California (Smith River) and all Montana populations, which were sampled in the fall (Table 1). Since late fall sampling may fail to capture Swiss individuals, which are near the end of their lives by that time, a second population 25 km distant from Smith River, (Crescent City) was also sampled during the summer (Table 1). Unfortunately, beetles from earlier collection dates from Montana were not available. Known hybrid individuals were also included to serve as reference samples for the identification of potential hybrids from field collections. These individuals represented reciprocal F1 and F2 crosses (without backcrossing) between *L. jacobaeae* from Salem (Italian ancestry) and our Swiss laboratory rearing at the University of Idaho. All collected beetles were either stored in 100% ethanol or at −80 °C until DNA extraction.

2.4. AFLP analyses

DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol using the entire individual. Egan et al. (2008), who also used entire larvae and adults, argued that the use of animal-specific DNA extraction kit and DNA degradation in the gut would make contamination by host plant material unlikely. Moreover, chances for the small amount of DNA that might be extracted from the gut to outcompete the high concentration beetle DNA during PCR are very low (Egan et al., 2008). The AFLP method followed Vos et al. (1995) with these modifications: restriction and ligation were performed during a single step in an 11 μl reaction containing 500 ng genomic DNA, 2 U *Mse*I (New England Biolabs [NEB], Ipswich, MA, USA), 1 U *Eco*RI (NEB), 1× T4 DNA ligase buffer (NEB), 0.45 U T4

Table 1
Overview of study populations, sample sizes, information of release histories of the two biotypes of *L. jacobaeae* at the sampled locations, and the results of assignment test as determined by STRUCTURE v. 2.3.3. Site codes correspond with those presented in legends of Assignment Tests and PCOA in Figs. 3 and 4.

Code	N	Location	Coordinates	Altitude	History	Collection date	Assignment test results		
		Europe		(m)			Italian	Swiss	Hybrid
CHI	20	L'Himelette, Switzerland	N 047°08'00.0" E 007°01'00.0"	1173	Native	2006 July		✓	
IT	7	Nazzano, Italy	N 042°14'11.0" E 012°37'47.0"	29	Native	2006 Oct	✓		
		United States							
CH2	20	St. Imier and Mettembert, Switzerland (reared at Moscow, ID)	N 047°09′00.0″ E 006°59′00.0″	933	Released in Montana 2002–2006, and Idaho in 2005[1]	2006 July		✓	
CA1	21	Crescent City, Del Norte Co., CA	N 041°44′01.5″ W 124°08′57.1″	21	Both Swiss and Italian beetles introduced in this county in late 1960s[2]	2006 July	✓		
CA2	14	Smith River, Del Norte Co., CA	N 041°57′58.8″ W 124°12′03.4″	23	Both Swiss and Italian beetles introduced in this county in late 1960s[2]	2006 Nov	✓		
ORL1	18	Scherzinger road, Tillamok Co., OR	N 045°07′48.0″ W 123°57′50.3″	28	Beetles released from California collections in 1970s[3]	2006 July	✓		
ORL2	20	Salem, Marion Co., OR	N 044°52′50.1″ W 122°57′56.3″	84	Beetles released from California collections in 1970s[3]	2006 May*	✓		
ORH	22	Mt. Hood, Clackamas Co., OR	N 045°09′20.6″ W 121°46′15.4″	1003	Beetles from Salem area introduced first in 1978[4,5]	2007 May*	✓		
MT1	7	Site #1, Lincoln Co., MT	N 048°16′31.2″ W 114°51′00.7″	1312	Italian beetles released in 2000[6]	2007 Oct	✓		
MT2	20	Site #2, Lincoln Co., MT	N 048°16′50.5″ W 114°51′59.2″	1229	Both Swiss and Italian beetles released 2001–2004[1,6]	2007 Oct	✓		✓
MT3	19	Site #3, Lincoln Co., MT	N 048°17′05.8″ W 114°52′17.8″	1201	Both Swiss and Italian beetles released 2001–2005[1,6]	2007 Oct	✓		✓
MT4	8	Site #4, Lincoln Co., MT	N 048°17′05.8″ W 114°52′33.7″	1187	Italian beetles released in 2001[1,6]	2007 Oct	✓		
HYB	20	Moscow, ID rearing	N 046°44′17.3″ W 117°01′42.4″	792	F1 and F2 reciprocal crosses between Salem (Italian) and Swiss beetles	2007/2008 July			✓

[1] Littlefield et al., 2008;
[2] Frick, 1970b; Frick and Johnson, 1972;
[3] Isaacson, 1978;
[4] Hawkes, 1980;
[5] E. Coombs, personal communication;
[6] J. Littlefield, personal communication.
* The Salem and Mt. Hood populations were sampled by digging up infested tansy ragwort plants in May and rearing out adults in a greenhouse in Moscow, Idaho.

DNA ligase (NEB), 0.05 M NaCl, 0.5× BSA, 4.5 μM *Mse*I adaptor, 0.45 μM *Eco*RI adaptor, and H_2O. The restriction–ligation was incubated at room temperature overnight, after which 5.5 μl of the product was diluted to 100 μl in TE (15 mM Tris and 0.1 mM EDTA). A pre-selective Polymerase Chain Reaction (PCR) was performed in a 20 μl reaction containing 4 μl of the diluted, restricted-ligated product, 1× PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM of each pre-selective amplification primer (*Mse*I + C and *Eco*RI + A), 0.5 U goTaq polymerase (Promega) and H_2O. The pre-selective PCR consisted of 20 cycles of: 30 s at 94 °C, 60 s at 56 °C, and 60 s at 72 °C. Ten microlitres of the pre-selective amplification product was diluted to 200 μl in TE (15 mM Tris and 0.1 mM EDTA). The selective amplification was performed in a 20 μl reaction containing 3 μl of the diluted pre-selective amplification product, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 μM *Mse*I selective primer, 0.05 μM *Eco*RI selective primer dye-tagged with 6-FAM (Integrated DNA Technologies, Coralville, IA, USA), 0.5 U of goTaq polymerase and H_2O. The selective PCR was 120 s at 94 °C; 10 cycles of: 20 s at 94 °C, 30 s at 66 °C (decreasing by 1 °C each cycle), 120 s of 72 °C; 25 cycles of: 20 s at 94 °C, 30 s at 56 °C, 120 s at 72 °C. Each selective PCR product (0.5 μl) was combined with 0.25 μl of 600 base pair (bp) size standard (GeneScan, Applied Biosystems, Foster City, CA, USA) and 9.25 μl of de-ionized formamide and loaded into an Applied Biosystems 3130 Genetic Analyzer. Loci were initially scored by the fragment analyzer software GeneMapper v. 3.7 (Applied Biosystems). These bins were then double checked and edited manually, making this a semi-automatic scoring method, as suggested by Papa et al. (2005). Thirty-nine primer combinations were used for initial screening and the three most polymorphic primer pairs (*Mse*I + TG – *Eco*RI + ACC, *Mse*I + TA – *Eco*RI + ACC and *Mse*I + CT – *Eco*RI + ACC) were used in this analysis. Repeat runs, starting from restriction/ligation, were run for 28 individuals (13% of samples) and scored blindly and compared to original runs to estimate AFLP error rate.

2.5. Data analyses

The Bayesian clustering and assignment software STRUCTURE v. 2.3.3 (Falush et al., 2003, 2007; Pritchard et al., 2000) was used to infer the number of genetic groups (K) and to assess the ancestry proportion of individuals (q) within those groups. STRUCTURE uses allele frequencies across loci to infer population structure and to calculate a membership coefficient ($q = 0–1$) for each individual for each inferred genetic group. Individuals can be probabilistically assigned to one or more populations if they are admixed, based on their q-values.

The recessive alleles model was used for all analyses, which is designed to handle dominant genetic data, such as AFLPs (Falush et al., 2007). In the first analysis the admixture ancestry model was used with default settings, without giving any *a priori* population information to determine the number of genetic groups (K) the data represented. In these analyses only native populations with known ancestry were used (one Swiss, one Italian; $n = 27$) and those USA populations (one from Idaho, two from California, and three from Oregon; $n = 115$) that consisted of individuals of most likely either pure Italian or Swiss ancestry based on published literature data ($n = 142$). An initial burn-in period of 10,000 was followed by 100,000 iterations, and repeated five times for $K = 1–10$. The ANCESDIST function was used to calculate 95% probability

intervals around the estimated q values for each individual. The criteria implemented by Blair and Hufbauer (2010) were used to distinguish truly hybrid individuals from those whose q value may have indicated hybridization (<0.9 membership coefficient in a given group) but the probability intervals included values of 1.00, and thus should not be considered as true hybrids. The most probable K value was chosen by calculating Ln P (D) and ΔK values (Evanno et al., 2005). Both estimates use the log probability of data from the STRUCTURE output by either taking the mean from independent runs for each K value (Ln P [D]) or by using a second order rate of change of the likelihood function with respect to K (ΔK) (Evanno et al., 2005). The least negative probability values of Ln P (D) with the least variance and the mode in the distribution of ΔK indicate the correct number of genetic groups (K) (Evanno et al., 2005).

In a second analysis, the POPINFO option was turned on, which allows users to give prior population information to a few or to all samples in order to help with classification of samples with unknown origin (Pritchard et al., 2010, unpublished), especially if those likely have mixed ancestry (i.e. our Montana individuals). Population information was given to the 142 individuals, as inferred by the first analysis (Swiss or Italian), and no information to the Montana individuals with uncertain ancestry ($n = 54$) and the reference hybrid samples ($n = 20$). The latter setting was used to test if the program would correctly identify the known hybrids. Allele frequencies were updated using only individuals with POPFLAG = 1, and according to the admixture model for samples with POPFLAG = 0. This setting creates so-called "learning samples" from the predefined populations, which are used to cluster the remaining individuals (Pritchard et al., 2010, unpublished). Our dataset is particularly suitable to take advantage of this option in STRUCTURE since the two populations used to create the known hybrids (ORL2 and CH2, Table 1), and the populations that were the sources of releases in Montana (ORH and CH2, Table 1) were all included in the predefined "learning samples". Following the recommendation of Falush et al. (2007), who also studied two genetically distinct groups (ecotypes) and hybrids between those, we set GENSBACK = 3, and MIGRPRIOR = 0.001. The first value allows reclassification of individuals for which population information was given, in case they do not have pure ancestry in the assigned group. The second value ensures strong statistical support for inference of hybrid ancestry. The burn-in period was set to 100,000 followed by a run length of 1000,000 at $K = 2$.

Principal Coordinates Analysis (PCOA) was performed using NTSYS-pc ver. 2.21 h software, which allows visual comparison of the genetic clusters (Rohlf, 1994). The Dice (1945) similarity coefficient was calculated (identical to the Nei and Li (1979) coefficient): $2a/(2a + b + c)$ where a = number of bands present in both samples, b and c = number of bands present in one or the other sample, then the DCENTER (to transform a symmetric similarity or dissimilarity matrix to scalar product form) and EIGEN (computes eigenvalues and eigenvectors for real symmetric matrices) modules of NTSYS. The first PCOA analysis was performed on the same 142 individuals that were used for the first STRUCTURE analysis to visualize the number of genetic clusters found in Italian and Swiss beetles. The same settings were used in the second analysis, where all known hybrids and the Montana individuals with unknown ancestry were included, similar to the second STRUCTURE run described above.

3. Results

The three primer pairs yielded 261 polymorphic loci for the 216 individuals scored. The error rate was calculated as 1.1% for the 28 repeated individuals. One allele was biotype-specific.

According to the Ln P (D) values (Fig. 2a) of the first STRUCTURE run, the data could have represented two or three genetic groups (least negative values). However, the variability was higher at $K = 3$ (S.D. = 11.15) than at $K = 2$ (S.D. = 2.9), therefore $K = 2$ is more likely to be the correct estimate. The ΔK values confirmed that, indicating $K = 2$ as the most likely number of genetic groups (Fig. 2b). The assignment of individuals to the two identified groups (Italian and Swiss) corresponded always with known ancestry information, and with the release and establishment history of the beetles in the USA. All individuals, except for three, had ancestry values (q) >0.9 and were assigned to either the Swiss or Italian genetic groups (Fig. 3a). The 95% probability intervals for the three individuals, whose q values were lower than 0.9, included 1.00, therefore it is very unlikely that they had mixed ancestry (Blair and Hufbauer, 2010). This is also corroborated by the fact that one of those individuals was collected in the native range in Switzerland. The first STRUCTURE analysis not only identified Italian and Swiss beetles as distinct genetic groups, but confirmed that Swiss beetles from the original 1969 release in California most likely did not establish, and that *L. jacobaeae* redistributed within California and Oregon have a pure Italian ancestry (Fig. 3a).

The information above was used in the second analysis to help the assignment of the unknown Montana individuals. The known hybrids from the laboratory rearing served as reference samples, and were all correctly identified as having mixed ancestry (with $q < 0.9$, and 95% probability intervals excluding 1.00). The status of individuals in the predefined clusters (Fig. 3a) did not change following this analysis. Most Montana individuals were found to have Italian ancestry with q values >0.89 and probability intervals overlapping 1.00; two hybrid individuals were identified from site MT2 and nine from site MT3 (Table 1, Fig. 3b).

The results of the Principle Coordinates Analysis (Fig. 4) were in agreement with those obtained by the Bayesian assignment tests conducted in STRUCTURE, but this method does not allow for the unambiguous identification of hybrid individuals. The two separate clusters in Fig. 4a correspond to the Italian and Swiss genetic groups. Both the laboratory reared hybrids and those individuals identified as hybrids by STRUCTURE tend to fall between the Italian and Swiss clusters, with some overlap (Fig. 4b). The remainder of the Montana individuals fell within the Italian cluster, in agreement with the results of STRUCTURE.

4. Discussion

All *L. jacobaeae* specimens from California and Oregon were determined to have pure Italian ancestry (Fig. 3a, 4a). This is in agreement with the generally held assumption that the Swiss biotype of *L. jacobaeae* did not establish from the original release in northern California in 1969, and that only the Italian biotype beetles are the source for the distribution program along coastal areas of the western USA (Turner and McEvoy, 1995). Even if Swiss beetles originally did establish in the 1970s, the small release size (<100 individual) and the continuous releases of Italian beetles within the same vicinity could have led to either the extinction of the Swiss biotype altogether or at least to significant introgression of the Swiss biotype to an extent that renders its contribution to the current gene pool undetectable at this point.

We found that beetles with pure Italian ancestry established at all of the studied high elevation populations at Mt. Hood, Oregon and Lincoln County, Montana. The mean annual temperatures for these areas are 5.6 and 4.5 °C, with mean winter temperatures of −0.76 and −5.13 °C, respectively (WRCC, 1971–2000). These sites represent different climatic conditions with their cool winters and shorter growing seasons than either the western coastal areas (mean annual temperature of Fort Bragg, California and Salem,

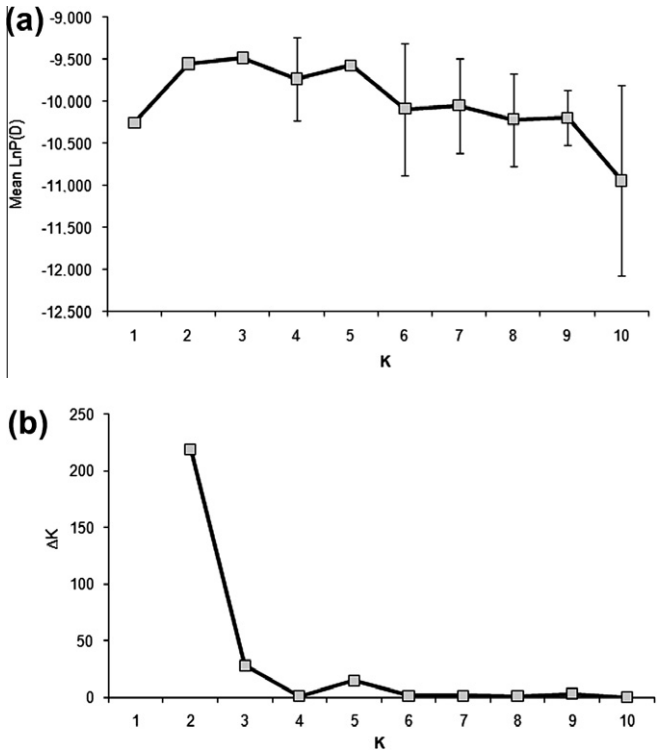

Fig. 2. (a) Mean posterior probabilities (±SD) for five independent runs for each *K* (number of clusters) value as estimated using the admixture model in STRUCTURE v. 2.3.3 (Pritchard et al., 2000; Falush et al., 2007) for 142 *L. jacobaeae* individuals. Note that SD values for $K = 1, 2, 3$, and 5 are relatively low and thus are not visible. (b) ΔK values for the same runs determined using the method developed by Evanno et al. (2005). The highest (least negative) values with the least variability for LnP (D) and ΔK, respectively indicate the most likely value for *K*, the number of clusters.

Oregon are 11.8 and 11.4 °C, with mean winter temperatures of 9.1 and 5.1 °C, respectively) or the region around Rome, Italy (mean annual and winter temperature is 14.6 and 7.5 °C, respectively) where the Italian beetles were originally collected (WRCC, 1971–2000; NCDC, 1955–1980).

There are several cases for the successful establishment of biological control agents in presumably unsuitable climates. For example, *Chrysolina quadrigemina* Suffrian successfully established in British Columbia, Ontario and Nova Scotia, Canada from source populations collected in California (Harris, 1984; Harris et al., 1969; Julien and Griffiths, 1998). Mediterranean areas of France, closely matching the climate of collections in California, were the original sources of these beetles (Julien and Griffiths, 1998). *Rhopalomyia californica* Felt, a gall midge from California where winter rains prevail also performed well in southern Queensland in Australia, where rains fall in the summer (McFadyen, 1984). In contrast, a number of biocontrol agents fail to establish in areas where climatic or environmental conditions substantially differ from their area of collection. As an example, *Diorhabda elongata* Brullé *deserticola* Chen, the leaf beetle introduced to control saltcedar from Fukang, China and Chilik, Kazakhstan was only able to establish in latitudes north of the 38th parallel in the USA (DeLoach et al., 2004).

The underlying question of whether plasticity, post-release adaptation, or other unknown factors enable some biological control agents to establish and thrive in new environments while others fail to do so, is rarely tested in biocontrol programs (Hufbauer and Roderick, 2005; Roderick, 1992). These mechanisms, however, have important consequences for the effectiveness of agents in suboptimal environments. McFadyen (1991) argued that climate modeling should be used to predict where an already established agent will be most effective. However, van Klinken et al. (2003) found that neither the establishment nor the performance of a leaf-tier *Evippe* sp. (Lepidoptera: Gelechiidae) introduced against *Prosopis* spp. were correctly predicted in Australia using climate matching. Similarly, *C. quadrigemina* has been successful for control of *Hypericum perforatum* L. in Canada despite the climatic mismatch with California (Harris, 1984). In our case, the effectiveness of Italian *L. jacobaeae* in high elevation environments has not been directly measured. However, a study by McEvoy et al. (1991) suggests that the beetles are similarly effective in controlling tansy ragwort at low and high elevation infestations. In that study, tansy ragwort densities were surveyed across Oregon at 42 infestations for 2–13 years. One of the criteria used to summarize and analyze the data was the time since release of the ragwort flea beetle at each site. Unfortunately, five of the six high elevation sites (800–1200 m) were only surveyed 2–4 years following the beetle release, which was not sufficient to judge the effectiveness of biological control. At one of those high elevation sites, Mt. Hood, a 33%

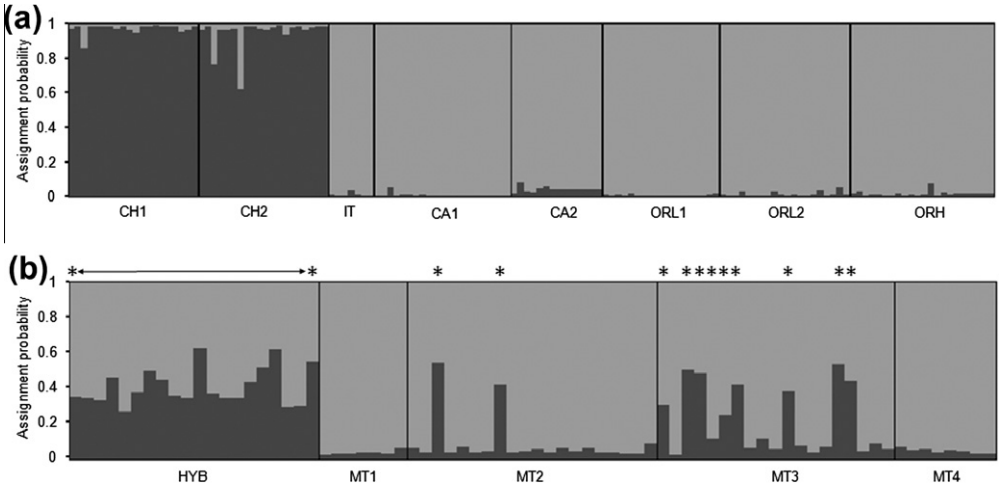

Fig. 3. Proportion of Swiss (dark portion of bar) and Italian (lighter colored portion of bar) ancestry for *L. jacobaeae* individuals, based on AFLP allele frequencies. (a) The assignment probabilities of those eight populations of *L. jacobaeae* that have either pure Italian or Swiss ancestry according to sample origin or published literature. Membership proportions (*q*-values) are based on admixture analysis by the Bayesian clustering software STRUCTURE v. 2.3.3 and shown at the most likely number of genetic clusters ($K = 2$). (b) Partial results of the second STRUCTURE run with POPINFO function turned on (see text for additional information). Although the eight populations shown in (a) were included in the analysis, data not plotted here since their assignment results did not change during the second analysis. $*$ indicates individuals with $q < 0.9$ and probability intervals not overlapping 1.00 (i.e., hybrids). Codes for the study populations are as in Table. 1. The figures were prepared using the program *Distruct* (Rosenberg, 2004).

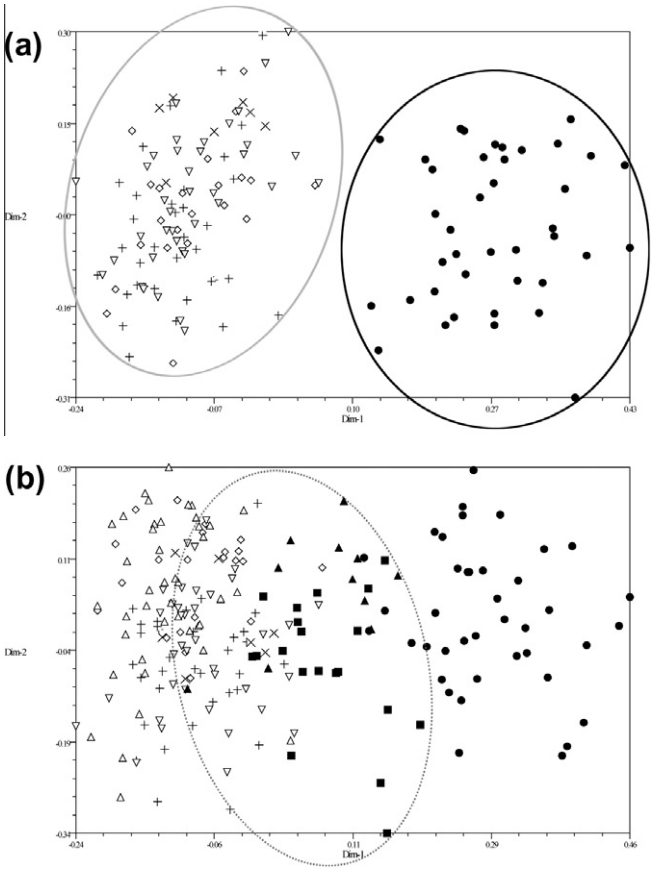

Fig. 4. Results of Principle Coordinates Analysis based on AFLP data. (a) 142 *Longitarsus jacobaeae* individuals, with known Italian (enclosed by the light grey ellipse) or Swiss ancestry (enclosed by the black ellipse). (b) 212 *L. jacobaeae* individuals, including those shown in (a) and additional known hybrids and Montana specimens of unknown ancestry. The dotted line encircles laboratory reared hybrids and hybrids identified by STRUCTURE v. 2.3.3 from Montana populations. Symbols represent the following study populations: In (a), ●, CH1 and CH2 (native and introduced Swiss populations); +, CA1 and CA2 (CA populations); ▽, ORL1 and ORL2 (OR, low elevation populations); ◇, ORH (OR, high elevation population); ×, IT (native Italian population); additional populations included in (b), ■, HYB (laboratory reared hybrids); △, MT1–4 (not hybrid from Montana populations); ▲, hybrids from MT1–4 study populations as assigned by STRUCTURE v. 2.3.3.

decline in tansy ragwort density was reported four years post-release of *L. jacobaeae*. In addition, the sixth high elevation site (1200 m) was included in the final analysis, which involved 30 sites throughout western Oregon representing a variety of land uses, spanning a range of elevations (sea level to 1200 m) and annual precipitation (1000–2500 mm) (McEvoy et al., 1991). Successful control of tansy ragwort was not correlated with either elevation or precipitation, and did not depend on land-use types (McEvoy et al., 1991). These results indicate that Italian beetles can be effective in high elevation environments. Successful control may take longer when biotypes are used that are climatically not pre-adapted but there is a trade-off between the time needed for success and the cost of introducing novel biotypes or agents, as suggested by Harris (1984).

Eleven intraspecific hybrid individuals were identified at two of the sites in Montana where both Italian and Swiss *L. jacobaeae* have been released. This is the first record of hybridization of *L. jacobaeae* biotypes in nature, which has previously only been demonstrated under laboratory conditions (Frick and Johnson, 1972, 1973). In accordance with release and establishment records, the remainder of the beetles collected in Montana was assigned an Italian ancestry. Swiss beetles were not found in the Montana study populations, which may be explained by the late collection date in the season (October 2007). By that time, Swiss beetles are close to the end of their life span and are rare in the field (M. Szűcs personal observation).

A surprisingly large proportion of hybrid individuals were found (47% of individuals at the MT3 site) given that the differing phenologies of the biotypes leave only a short window for inter-mating. Swiss beetles lay the majority of their eggs during the summer when Italian beetles are aestivating. By the time the Italian beetles mate in September, most of the Swiss female's egg load has been depleted and most Swiss males die during September and October. The large proportion of hybrid individuals found at least at one study site may indicate that the novel genotypes have a greater fitness or that their phenology may be better adapted to the given environment than that of their parents'. Alternatively, the relatively large proportion of hybrids may be due to the seasonal timing of the sampling (October) and only reflect a time of greater activity and availability relative to pure biotypes. The results of STRUCTURE and the release history of the two biotypes at these locations indicate that hybridization is in its early stage. In the assignment test, ancestry values of the hybrids from Montana were close to the known laboratory reared hybrids (Fig. 3b), which were not allowed to backcross and thus represented 50% of each Italian and Swiss ancestry. Considering release and establishment records from Montana (Littlefield et al., 2008) and the sampling date of the populations (October 2007), it is probable that F1 hybrids were collected. Both biotypes have a very narrow host range (Frick, 1970a; Puliafico, 2003, unpublished), limited to the new genus *Jacobaea* and a few other species of the old genus *Senecio* s.l. (Pelser et al., 2007; U. Schaffner, personal communication). We are not aware of any biological control agents where the host-specificity of hybrids of two biotypes, both with very similar and narrow host-ranges would change upon hybridization.

As tansy ragwort is spreading to new environments east of the Cascades, further redistributions of *L. jacobaeae* will be necessary. Our results provide evidence that the Italian beetles, which are readily available for collections in coastal Oregon areas, can potentially establish and successfully control tansy ragwort in a wide range of environments, including those with a winter-cold climate such as Mt. Hood. While the phenology of the Swiss biotype may be more suitable to colder climates, it may also reduce the beetle's control potential since the larval feeding is concentrated to the milder spring months. Tansy ragwort may be able to compensate more for the damage at this time of the year than during winter. The severity of infestations, applicability of alternative control means, and availability of resources may decide on a *L. jacobaeae* biocontrol strategy: Introduce the climatically best pre-adapted population in the hope to accomplish control as quickly as possible; or collect flea beetles where they are readily available and accept a lag-time period for adaptation of that population. Alternatively, the Swiss and Italian biotypes may be released combined. Windig (1991) suggested that releasing both *L. flavicornis* and *L. jacobaeae* combined could increase biocontrol efficacy since *L. flavicornis* larvae feed throughout the winter and *L. jacobaeae* larvae from The Netherlands hatch in the spring and feed until early summer. Hence, both species combined should inflict more damage by stressing tansy ragwort plants continuously from fall to early summer. A similar strategy could be achieved with Italian and Swiss *L. jacobaeae*, whose larvae either feed from fall until late spring or from early spring to summer, respectively. However, our results indicate that this release method will likely result in hybridization of the biotypes, which can be beneficial by increasing the genetic variation available for selection to act on, and thus facilitating adaptation to new environments. On the other hand, traits (e.g., life history, cold/warm tolerance) of hybrids may be altered in such ways that they become detrimental in certain environments.

Monitoring the demography and impact of the existing pure and mixed-biotype populations in Oregon, Montana and Idaho over several generations would be a good approach to assess the most suitable strategy for future releases of *L. jacobaeae* at sites with winter-cold climates in North America.

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