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Assessing the invasion of *Ambrosia artemisiifolia* in Italy through the analysis of genetic variability and herbarium data

Ciappetta S.[1], Ghiani A.[1], Gilardelli F.[1], Bonini M.[2], Citterio S.[1], Gentili R.[1*]

[1]Department of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, I-20126 Milan, Italy.

[2] Department of Medical Prevention, ASL (Local Health Authority) Milan 1, Public Health Service, via Spagliardi 19, Parabiago I-20015 Milan, Italy.

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***Correspondence: Rodolfo GENTILI,** Department of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, I-20126 Milan, Italy.

Email: rodolfo.gentili@unimib.it

Fax: +39 02 64482996; tel.: +39 02 64482700

Summary

Investigations of the genetic pattern and colonisation sources and the routes of invasion by alien species populations are crucial for identifying invasion mechanisms and the reasons for the bio-ecological success of invasive species. The aim of our work was to study the genetic pattern of Italian populations of *Ambrosia artemisiifolia* in comparison with that of some French and Canadian populations and to use herbarium records to characterise the colonisation areas of *A. artemisiifolia* across Italy.

Molecular investigations were based on a set of nuclear SSR marker loci, which we used to analyse a number of Italian, Canadian and France populations. The time-spatial spread of *A. artemisiifolia* was reconstructed through the distributional pathway of 194 herbarium specimens.

Ambrosia artemisiifolia Italian populations (H_E = 0.687) had higher values of genetic diversity when compared to Canadian (H_E = 0.639) and French (H_E = 0.643) populations. Nevertheless, the time of residence of the Italian populations was positively correlated with the observed and expected heterozygosity (H_O, H_E). Genetic clustering inferred in STRUCTURE suggested admixture of populations with different ancestry. Historical-distributional data highlighted that *A. artemisiifolia* first colonised the Po plain and different phased localities of the Mediterranean region.

This study synthesises genetic and historical-distributional data, highlighting that several invasion events have occurred across the Italian peninsula in different spatio-temporal steps, establishing high levels of historic gene flow between populations with mixed ancestry.

Key words: annual weed**,** dispersal, gene flow, invasive species, mapping, spatial dynamics and distribution

1. Introduction

The increasing incidence of biological invasion phenomena is a consequence of globalisation (global trade, transport and tourism) and climate change (Clements and Ditommaso, 2011; Kissling et al., 2015). One species, *Ambrosia artemisiifolia* L. (common ragweed; Asteraceae), has become a major problem in European countries due to its invasiveness (Chauvel et al., 2006; Galzina et al., 2010; Gladieux et al., 2011). It also represents a health risk as it is a source of highly allergenic pollen (Ghiani et al., 2012; Smith et al., 2013). This plant, an annual weed native to North America, was introduced to Europe at the end of the 18th century, when the species was cultivated in botanical gardens (Allioni, 1770-73). Nevertheless, its invasion into and across Europe probably started later, during the 19th century in France and in East Europe, due to accidental introduction events (Chauvel et al., 2006; Pinke et al., 2011).

Since then, *A. artemisiifolia* has become a widespread alien species. To date, populations of the species are expanding toward Central and Northern Europe and they are expected to further expand due to this plant's great dispersal ability (Storkey et al., 2014) and favoured by climate change (Cunze et al., 2013; Wasowicz et al., 2013.). *Ambrosia artemisiifolia* preferentially colonises anthropogenic habitats such as ruderal areas and disturbed bare soils (construction sites, gravel pits and quarry areas), as well as cultivated and abandoned fields, and it spreads along roadsides, railways and river corridors (Chauvel et al., 2006).

The investigation of invasion sources and routes of populations is a crucial for identifying the invasion mechanisms and the causes for success of a non-native species and then to implement action plans for controlling its spread (Lawson Handley et al., 2011). The study of population genetic processes involved in species spreading is a key step toward understanding the evolutionary implications of the invasion and for defining future scenarios of potential distribution. On the other hand, although the expansion of invasive species is a discontinuous process in space and time, it can be documented through the study of herbarium specimens and field observations.

On this basis, both genetic and distributional studies have been conducted in recent years to understand the origin of European *A. artemisiifolia* populations, to monitor the extent of their spread, to promote management activities (Brandes and Nitzsche, 2006; Buttenschøn et al., 2009; Gentili et al., 2015) and to investigate their short-term evolutionary potential in terms of adaptation and fitness with respect to changing environments in new colonisation areas (Chun et al., 2011). Gaudeul *et al*. (2011) analysed the genetic variability of populations of *A. artemisiifolia* at the global level and found evidence for multiple introductions of the species in most parts of its invasive range. That study supported the hypothesis that introductions into Europe were probably derived from two different regions of the native area: a) a population from eastern North America first established in Central Europe and b) a population from western North America that first colonised Eastern Europe. This pattern could reflect the distinct rules for trade and exchange from America to Western and Eastern Europe during most of the twentieth century (Gladieux et al., 2011). At a more regional level, *A. artemisiifolia* populations across France have also been suggested to include plants from a mixture of sources (Genton et al., 2005).

The distributional studies of Chauvel et al. (2006) documented the invasion history (introduction and spread) of *A. artemisiifolia* in France by collecting information from herbarium specimens. According to their study, prior to 1890, *A. artemisiifolia* was mostly found in cultivated fields (about 80% of all specimens) and was later found along roads and in waste areas. Galzina *et al*. (2013) collected detailed field data on the current distribution of the species in Croatia and reported its presence in crop fields (particularly in sunflower, *Helianthus annuus* L.) and non-agricultural plots in urban and industrial areas. Tokarsta-Guzik et al. (2011) reconstructed the spread of *A. artemisiifolia* in Poland using herbarium data and field observation over three consecutive periods starting from 1850; they found that the distribution has been increasing for the last fifty years.

In Italy, the species occurs at the southern boundary of the European distribution. It was first reported in the wild at the beginning of the 19th century in north-western Italy (Vignolo-Lutati, 1934; Bouvet, 2013); after 1950, its occurrences across the Po valley and toward Central (and

South) Italy drastically increased (Celesti-Grapow et al., 2009). Gladieux et al. (2011) reported that some Italian populations of *A. artemisiifolia* seem to have originated in the eastern part of Northern America. However, exhaustive studies on both genetic patterns and colonisation/distribution of Italian *A. artemisiifolia* populations are still lacking.

The aim of the present study was to evaluate the genetic pattern of Italian populations in comparison with French (located in early introduction sites of Europe) and Canadian (from the native range of the species) populations and to use herbarium records in order to map the colonisation areas and routes of *A. artemisiifolia* across Italy. Specific aims of our study were: a) to focus on *A. artemisiifolia* habitat preferences during its invasion into Italy and b) to highlight the relationships between spatio-temporal (i.e. time of colonisation and distribution) and genetic diversity patterns of *A. artemisiifolia* Italian populations.

2. Material and Methods

2.1 Population sampling

Seeds from 18 different *A. artemisiifolia* populations were collected from (Table 1): a) Canada, in the native range of the species; b) France, in the Rhone region, highly infested by ragweed and located in early introduction sites; and c) Italy. Seeds were then used to obtain plantlets. Germination was promoted by subjecting the collected seeds to a cold stratification (4°C) for 30 days; after this period, seeds from each population were placed in a growth chamber and left to germinate in controlled conditions (20°C; relative humidity 50–80%, 10 h dark/14 h light; light intensity 150 µmol m^{-2} sec^{-1}). Leaf tissues were collected from germinated plantlets of each of the 18 populations (Table 1) and stored at -20°C.

2.2 Microsatellite analysis

The Simple Sequence Repeat (SSR) technique was applied to assess population genetic variability. The DNA was extracted for SSR analysis by disrupting leaf material with a TissueLyser (Qiagen)

and using the EUROCLONE plant DNA extraction kit. Extracted DNA was frozen at - 20°C until use. We used six microsatellite markers (Ambart 04, 06, 09, 18, 24, 27; GenBank accessions: FJ595149, FJ595150, FJ595151, FJ595153, FJ595155 and FJ595156; Chun et al., 2010). PCR was performed in an Eppendorf Mastercycler Gradient thermal cycler in a 10 µL final reaction volume containing 10 ng genomic DNA, 0.04 µM forward primer 0.16 µM reverse primer, 0.16 µM M13 primer, 0.5 U TopTaq DNA Polymerase and 1X TopTaq PCR Buffer (Qiagen). Amplification cycles included an initial denaturing of 94°C for 4 min, 30 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 45 s, 8 cycles at 94°C for 30 s, 53°C for 45 s, 72°C for 45 s and the final extension step of 72 °C for 5 min (Schuelke, 2000)

Amplified products were genotyped using a 3730XL DNA Analyser sequencer (Applied Biosystems) and allele sizes were analysed using GENESCANVIEW 1.1. The software MICRO-CHECKER version 2.2.3 was used to identify genotyping errors such as large allele dropout or stutter peaks (van Oosterhout, 2004).

2.3 Genetic data analysis

The proportion of polymorphic loci (P), the number of observed alleles (Na), the mean number of rare allele per locus (Ra), the observed heterozygosity (H_O), the expected heterozygosity (H_E) across the populations were determined using POPGENE v. 1.31 (Table 2). Exact tests for population differentiation and tests for Hardy-Weinberg equilibrium (HW) at each locus were calculated using the TFPGA version 1.3 software (Miller, 1998). F-statistics (F_{ST}) and inbreeding coefficients ($F_{IS} = (H_E - H_O)/ H_E$) were calculated for each population using the FSTAT software (Goudet, 1995). Differences in F_{ST} values across countries (Canada, France and Italy) were assessed by ANOVA analysis.

Within each population, linkage disequilibrium was tested between loci using exact tests performed with the FSTAT software. Statistical significance (p = 0.05) was evaluated based on 1000

permutations, and then corrected for multiple tests using the sequential Bonferroni method (Rice, 1989).

2.4 Population structure

The genetic distance matrix according to Nei (1972) was subjected to Principal Coordinates Analyses (PCoA) using the PAST 2.1 software (Hammer *et al.* 2001). A Neighbour-joining (NJ) analysis, based on the same matrix, was conducted with TREECON 1.3b (Van de Peer and De Wachter, 1994). The tree was graphically edited using the program SplitsTree 4.13 software (Huson and Bryant 2006); support of nodes was assessed with 1000 bootstrap replicates.

The ancestry of *A. artemisiifolia* samples was estimated performing a Bayesian cluster analyses to model population structure, using SSR markers, in STRUCTURE v. 2.3.4 (Pritchard et al., 2000; Falush et al., 2007). The allele frequencies of the different *A. artemisiifolia* populations were assumed to be correlated, which is a realistic model for populations that are likely to be similar due to common migration events and/or shared ancestry. The best number of clusters was determined by performing 20 independent runs of K (K = 1 to 18) with an admixture model at 1,000,000 Markov chain Monte Carlo (MCMC) iterations and a 100,000 burn-in period (LOCPRIOR option; estimate λ). We used ΔK, the second-order rate of change in ln P ($X|K$), for successive values of K to determine the number of clusters (Evanno *et al*., 2005). These calculations were carried out by processing the STRUCTURE result files in Structure Harvester v. 0.6.1 (Earl and vonHoldt, 2011), a software program that implements the method of Evanno *et al*. (2005) (Supplementary File SF1). Sampled populations of *A. artemisiifolia* where then mapped in representative pie charts with the percentage of the K genetic pools from each population (Fig. 1A,B,C).

Analysis of molecular variance (AMOVA) was performed using the Genalex software (Peakall and Smouse, 2006) to estimate genetic structure and degree of genetic differentiation within populations, among populations and among geographic provenance (Canada, France and Italy; Table 3). The significance of the estimates was obtained through 999 data replications.

We assessed the relationships between the residence time of the investigated Italian populations and the genetic parameters at the population level by performing Kendal tau correlations (Table 4). We estimated the residence time of populations by verifying the age of the oldest herbarium specimen within 30 kilometres from the sampled population. This distance takes into account the long distance dispersal ability of *Ambrosia* pollen (Sikoparija et al., 2013). We then assigned a residence category following Fenesi and Botta-Dukát (2012): 1: populations established less than 30 years ago; 2: established more than 30 years ago; 3: established more than 40 years ago; 4: established more than 60 years ago; and 5: stablished more than 80 years ago (Table 1).

2.5 Herbarium data collection and analysis

We planned to identify the time-spatial spread of *A. artemisiifolia* L. in Italy through the study of herbarium specimens using the approach of Chauvel et al. (2006). We therefore examined specimens present in 56 institutions holding herbarium collections: museums, universities, botanical gardens, high schools, local associations and libraries (see Supplementary Table ST1). We visited herbaria directly or requested high resolution images of scanned specimens of the *Ambrosia* genus. We collected the following information for each specimen: the sampling location, date of sampling, collector (legit, determinavit) and type of habitat.

We georeferenced specimens following the description of the sampling locality (municipality, address, altitude, habitat, description of the locality and sometime coordinates) and according to the UTM ED1950 system. Data were used to build a GIS database (software ArcMap 10.1) of the species occurrence; we then created a point-shapefile with the findings and distribution maps for the studied species, representing its spread in time (Fig. 2A,B). In this analysis, we did not consider specimens collected by the same population on the same date by the same collectors; we then excluded samples with uncertain taxonomic designation (after specimen examination), those without the mention of the locality of collection and those collected/cultivated in botanical gardens.

We verified possible changes in habitat preference of the species over time by individuating five a priori temporal patterns corresponding to about the 20% of the *A. artemisiifolia* specimens collected over time, as shown in the cumulative distribution of the number of herbarium specimens over time. We then applied a chi-square test to different temporal patterns of *A. artemisiifolia* distribution in natural habitats in comparison to the whole period of observation (i.e. sample collections Fig. 3A,B).

We investigated the species spatio-temporal invasion of *A. artemisiifolia* across the main distribution range of the herbarium specimens in the Northern Italy by calculating the median of specimens age coming from western (Po1), central (Po2) and central-eastern (Po3) Po plain areas, and from the eastern Po plain in Friuli Venezia Giulia (Ts=Trieste), Liguria (Gen=Genoa), Marche (Pes=Pesaro) and Lazio (Rm=Rome). We emphasise that the last four areas fall within the Mediterranean bioclimatic region: They are separated from the Po plain by the Apennines chain (Liguria, Marche and Lazio regions) or are far from the Po plain area. The unique sample from Tuscany (Fi) was not considered for additional analyses (Fig. 2B). We then displayed jitter and box plots (Fig. 3C). Differences in the mean age of specimens between geographical areas were tested with the Kruskal-Wallis test.

3. Results

3.1 SSR genetic variability across populations

All loci were polymorphic in all populations of *A. artemisiifolia*; the percentage of polymorphic loci was 100% in almost all populations. The software MICRO-CHECKER did not evidence for large-allele dropout for the analysed locus, across populations. As a general rule, the Italian populations (IT) showed higher values for genetic diversity parameters than did the Canadian (CAN) and French (FRA) populations. Genetic variation, measured as number of alleles, number of rare alleles or expected heterozygosity, tended to increase slightly from the native range (CAN) to France and Italy (Table 2).

Considering all populations, the number of alleles (N_a) per population varied from 4.667 in CAN4 and IT_P and to 8.333 in IT_TO (Table 2). The Ra value was the highest in IT_TO. The mean values for observed heterozygosity showed the highest value for the IT_TV population (H_O = 0.972) and the lowest value for the IT_P population (H_O = 0.411); the mean values for expected heterozygosity showed the highest value for the IT_TO population (H_E = 0.804) and the lowest value for the CAN4 population (H_E = 0.505). Populations with a surplus or a deficiency of heterozygosity (indicating a deviation from Hardy-Weinberg equilibrium) were practically equivalent in number, so that the overall F_{IS} value displayed only a moderate value of surplus heterozygosity (mean F_{IS} = -0.042). Population differentiation using F-Statistics (F_{ST}) accounted for an overall value of 0.120. Considering the CAN, FRA and IT populations, the among-population differentiation measured as F_{ST} did not differ significantly between Canada (F_{ST} = 0.096), France (F_{ST} = 0.072) and Italy (F_{ST} = 0.123), according to ANOVA analysis; however IT populations accounted for the highest value. Results of exact Hardy-Weinberg tests are presented in Table 2.

Considering only the Italian range, the IT_TO, IT_TV and IT_PU populations exhibited the highest level of genetic diversity values (for instance H_E = 0.804, 0.782 and 0.727, respectively) while the IT_P population exhibited the lowest value (H_E = 0.556; Table 2). None of the tests for linkage disequilibria showed statistical significance.

3.2 Population structure

The relationships among the analysed populations were investigated with a PCoA; the results are shown in Fig. 1D. The first three principal components explained 10.45, 8.13 and 6.07% of the variation, respectively. The PCoA analysis showed no evidence of a subdivision between the Canadian, French and Italian populations (see also Neighbour-joining analysis, Supplementary Fig. S1).

STRUCTURE analysis estimated the highest mean log likelihood at K = 3 [lnP(D) (–5202.5)], indicating that populations of *A. artemisiifolia* are subdivided into three distinct genetic clusters

(Supplementary File SF1). The results depicted in Fig. 1A,B,C are based on an admixture model, where individuals may have mixed ancestry from the different populations. The results indicated that individuals have mixed ancestry from the different populations. Indeed, Fig. 1A,B,C shows a low degree of population structure in *A. artemisiifolia*. However, some trends can be observed in the genetic structure of populations: a) as a general rule, the Italian populations exhibit a non-homogeneous allelic pattern; b) Canadian and French populations have a similar genetic composition, with the "red" cluster being the less frequent, whereas, by contrast, most Italian populations have a high proportion of this red cluster.

AMOVA analyses performed for SSR markers based on the geographic subdivision of *A. artemisiifolia* populations (Canada, France, Italy; Table 3), revealed that most of the total genetic variation can be attributed to individuals within populations (92.0%), while the genetic variation attributable to difference among populations and region was low (7.9%) or scarce (1.1%), respectively (Table 3).

3.3 Temporal invasion and habitat preference of A. artemisiifolia

We collected 248 specimens assigned to *A. artemisiifolia* (or synonyms) from 56 Italian herbaria (out of a total of 131), mostly from northern and central Italy. However, after examination of specimens, only 194 were considered (Supplementary Table ST2) as we excluded samples with uncertain designation, those without the mention of the locality of collection and those collected/cultivated in botanical gardens.

During the 20th century and until the present time, the collection of new herbarium specimens has increased (Fig. 3A). The ancient specimen of *A. artemisiifolia* accidentally introduced into Italy dates back to 1902 and was located in the viticulture garden school in Alba. The primary range invasion of *A. artemisiifolia* in Italy was mainly in the Piedmont, Liguria and Lombardy regions (Fig. 2A). The plant then spread toward the east, down the Po Valley (Fig. 2B). However, in both the most eastern and the southern ranges of the plant's Italian distribution, distinct events of

284 colonisation probably occurred close to a) Trieste (Friuli Venezia-Giulia region), b) Pesaro (Marche
285 region) and c) Rome (Lazio region). More recent colonisation events have occurred in Tuscany
286 (close to Florence).
287 In Fig. 3C, the jitter plot and box plots show the different temporal patterns of *A. artemisiifolia*
288 specimens over the main geographic areas of species colonisation. The median age of specimens
289 collected in the different geographic areas was statistically different according to the Kruskal-Wallis
290 test (H-chi^2 = 47.69; $p < 0.001$; see also Supplementary Table ST3 for pairwise comparisons). In
291 particular, along the Po plain, from west to east, the specimens were progressively more recent.
292 The finding of new specimens of this considered invasive species was infrequent until the '70s; the
293 frequency of specimen collection then greatly increased during the '80s and '90s and continues at
294 present. The sampling location of *A. artemisiifolia* specimens is related to the presence of roads and
295 water networks, as well field areas (Fig. 3B). In particular, *A. artemisiifolia* seems to use both
296 indiscriminately, even if over time, based on the a priori temporal patterns (each comprising about
297 20% of the specimens), a closer relation to a certain habitat has occurred.
298 For the whole period of observations (1902–2012), the majority of specimens have been collected
299 along roads/railways (29.8), rivers/lakes (29.2%) and fields (25.2%). Habitat frequency
300 comparisons between the whole period of observations (1902–2012) and temporal steps of invasion,
301 individuated through the cumulative distribution of the number of herbarium specimens over time
302 (Fig. 3A,B), revealed the following significant differences: a) in the 1902–1977 period, Other
303 habitats frequency is higher and Field habitat frequency is lower than during the 1902–2012 period
304 (Df=4; Chi-square=17.83; $p<0.01$); b) in the 2000–2005 period, river and lake habitats exhibited
305 lower habitat frequency than in the 1902–2012 period (Df=4; Chi-square=11.03; p=0.026).
306
307 ***3.4 Genetic variability and invasion time***
308 The correlations according to the Kendall tau coefficient between time of residence of the
309 investigated Italian populations and parameters of genetic diversity at the population level are

shown in Table 4. Time of residence was positively correlated with the observed and expected heterozygosity (H_O and H_E), both without considering the IT_TV population or attributing a high residence time to that population.

4. Discussion

Understanding source populations and invasion paths is a crucial phase in deciphering biological invasion phenomena, with obvious practical implications for implementing effective and appropriate control strategies (Roderick and Navajas, 2003). The results of this study, focusing on population genetics and spread history of *A. artemisiifolia* in Italy, indicated high levels of genetic diversity within, and low levels among, its populations. Despite a fairly moderate sample sizes, that may have partially influenced the results, these are clearly in accordance with the general trend of previously published papers on the population genetics of *A. artemisiifolia* for both Western and Eastern Europe (Genton et al., 2005; Gladieux et al., 2011; Kočiš Tubić et al., 2015). Previous studies that have investigated the genetic diversity of *A. artemisiifolia* populations with microsatellites have observed positive F_{IS} values, that is, an overall deficit in heterozygotes (Genton et al., 2005; Kočiš Tubić et al., 2015). In our study populations with a surplus or a deficiency of heterozygosity were practically equivalent in number; in all probability, those populations that exhibited a surplus of heterozygosity have been subjected to a high gene flow in environments favouring high out-crossing rate (i.e. exchange of seeds and pollen).

Our study also suggested several spatio-temporal introductions of the species across its Italian range, starting from the beginning of the 20th century in north-eastern Italy (herbarium record collected in 1902).

No strong evidence was obtained for possible ancestry relationships between Italian, Canadian and France populations based on our genetic clustering inferred in STRUCTURE. The analysis highlights that such a pattern may be the consequence of the species ecological characters: *A. artemisiifolia* is awide-ranging habitat generalist exhibiting a high potential for gene flow (Coltman,

2008; Kočiš Tubić et al., 2015). On the other hand, a degree of differentiation between French and Italian populations can be observed (prevalence of green colour in the French populations; prevalence of red in the Italian populations). The different STRUCTURE pattern between the French and Italian populations suggests that the Italian populations did not directly originate from the French populations previously established in Europe (although pollen or propagule exchanges cannot be excluded), but they were probably founded through different colonisation events from North America and other European countries. This type of genetic configuration has already been hypothesised for *A. artemisiifolia* populations of Eastern Europe (Gladieux et al., 2011). For instance, our results confirm the findings of Gaudeul et al. (2011) wherein the most eastern Italian population has a different genetic pattern compared to the western ones and they are probably more strongly linked to populations growing in Eastern Europe.

Our findings, in all probability, reflect a combination of historical, biological and ecological factors. First, numerous successful invader species, like *Ambrosia*, have been documented to show high within-population genetic diversity (Groves and Burdon, 1986; Bossdorf et al., 2005). Some part of the high genetic diversity levels of *A. artemisiifolia* can be ascribed to its monoecious, wind-pollinated, out-crossing (predominantly) breeding system, due to a self-incompatibility mechanism (Li et al., 2012). In contrast, the scarce genetic differentiation across populations and regions that we report here probably reflects its high natural or human-mediated dispersal ability followed by gene flow: a) pollen can be transported over large distances by the wind (Prank et al., 2013); b) seed production is extremely high, with a single individual capable of producing as many as 60,000 seeds (Brandes and Nitzsche, 2006); c) factors that have contributed to the high levels of propagule pressure (e.g. contaminated crop fields and bird seed, agricultural machines, transport of soil; Essl et al., 2009). If genetic variability is recognised to determine a population's capacity to adapt to new or changing environmental conditions (Sakai *et al*., 2001), almost certainly, from an evolutionary point of view, these bio-ecological features have contributed to the generalist character and to the phenotypic plasticity of the species that is able to colonise a variety of habitats (Fumanal *et al*.,

362 2008). Our results confirm a strong propensity of *A. artemisiifolia* to be a generalist species, based
363 on the analysis of its habitat spectrum through herbarium data.
364 In keeping with the genetic observations, the results from herbarium data suggest distinct
365 introduction events, which have occurred in at least five different geographical areas: a) first in the
366 western Po plain (specimen dated in 1902; Piedmont region) with a very likely expansion toward
367 the east; b) then in the Rome (Lazio region), Genoa (Liguria region) and Trieste (Friuli-Venezia
368 Giulia region) areas, with specimens from 1934 to 1939; more recently at Pesaro (Marche region),
369 with specimens in 1963. Interestingly, these areas correspond to the most developed regions of Italy
370 from a *commercial and industrial point of view:* Turin (and its surrounding) was one of the first
371 industrial cities in Italy while Genoa, Pesaro and Trieste are important seaports. These areas have
372 probably functioned as important introduction pathways for contaminated seeds of cereals and other
373 agricultural crops from Canada and the USA into Europe (Buttenschøn et al., 2009).
374 The occurrence of *A. artemisiifolia* is sporadic and/or ephemeral toward the central and southern
375 regions of Italy in the Mediterranean region (e.g. the city of Rome). Cunze *et al*. (2013), in their
376 ecological niche modelling of *A. artemisiifolia* in Europe, hypothesised that "a possible reason for
377 *Ambrosia'*s absence in the Mediterranean region, despite of predicted habitat suitability, may be
378 that North American populations adapted to Mediterranean climatic conditions did not reach the
379 adventive range (Europe) yet". In our opinion, the explanation lies in the differences at the
380 ecosystem level, in the competition of better adapted species (Kueffer et al., 2013) and in
381 bioclimatic filters (Haider et al., 2010). On the other hand, the general trend of increased "genetic
382 potential" (in terms of H_O and H_E) that we found in populations with a higher residence time
383 suggests a future increase in the ability of a population to adapt to new environmental
384 characteristics.
385 Our findings, both from genetic analyses and herbarium data, are consistent with those of Genton et
386 al. (2005); Gaudeul et al. (2011) and Gladieux et al. (2011) in that their genetic studies of *A.*
387 *artemisiifolia* populations across France, the world, and Eastern Europe, respectively, highlighted a

high genetic diversity within populations of the species that may reflect multiple introduction events.

5. Conclusions

Our study, synthesising both genetic and historical-distributional data, highlighted that several invasion events have occurred throughout the Italian peninsula in different spatio-temporal steps: a) first in the Po plain (continental areas), from west to east, where a population with a higher residence time exhibits an increased genetic diversity (and then a higher invasion potential); followed by b) in certain areas in the Mediterranean regions (Genoa and Pesaro). The difficulty in finding herbarium specimens from the Mediterranean range of the species seems to reflect the inhibition of expansion of the species toward southern regions, likely due to bioclimatic filters.

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FIGURE LEGENDS

Fig. 1. A,B) Spatial genetic structure and population clusters (K=3) of *A. artemisiifolia* are inferred by the Bayesian cluster method implemented in STRUCTURE. At each location, pie charts in the maps indicate the mean proportion of membership of individuals at each location for the K = 3 genetic groups. C) In the bar diagram different colors represent the proportion of ancestry in each of the K populations. D) Principal coordinate analysis of the (PCoA) 3D plot based on the genetic distance matrix of 216 samples of *A. artemisiifolia* from Canada (square), France (triangle) and Italy (circle).

Fig. 2. A) Italian distributions of the *A. artemisiifolia* herbarium specimens that were collected before 1950, between 1951 and 2001, between 1902 and 2012. In the first illustration of Italy the names of the cited Italian regions are reported. B) Main colonization areas and likely invasion trend of *A. artemisiifolia* in Italy and likely invasion trend in the Po plan area. Specimens were subdivided in subset corresponding to different colonisation regions: western (Po1), central (Po2) and central-eastern (Po3) Po plain areas, eastern Po plain-Friuli Venezia Giulia (Ts), Liguria (Gen), Marche (Pes) and Lazio (Rm).

Fig. 3. A) Cumulative distribution of the number of herbarium specimens during time. B) Spectrum of habitats indicated on the label of the herbarium specimens of *A. artemisiifolia* for the whole observation period (1902-2012) and for the temporal steps individuated through the cumulative distribution of the number of herbarium specimens during time. C) On the left, Jitter plot showing the frequency and temporal range of herbarium specimens by the most likely A. *artemisiifolia* colonization areas; on the right, box-plots of the age of the herbarium specimens by colonization areas. Legend= western (Po1), central (Po2) and central-eastern (Po3) Po plain areas; eastern Po plain-Friuli Venezia Giulia (Ts=Trieste), Liguria (Gen=Genua), Marche (Pes=Pesaro) and Lazio (Rm=Rome).

TABLES

Table 1. Sampled populations of *A. artemisiifolia* across Canada, France and Italy.

Pop. name	Pop code	Locality	State	N	E	n. samples	Residence time category*
CAN 1	LOT 6	L'Acadie	Canada	45°18'52"	73°21'19"	12	/
CAN 2	LOT 18	Mirabel	Canada	45°39'45"	73°00'10"	12	/
CAN 3	LOT 800	Ste Clotilde de Chateauguay	Canada	45°09'49"	73°40'17"	12	/
CAN 4	LOT 878	Ste Clotilde de Chateauguay	Canada	45°11'24"	73°38'59"	12	/
CAN 5	LOT 990	Ste Clotilde de Chateauguay	Canada	45°09'17"	73°41'02"	12	/
FRA 1	01P01	Ambronay	France	45°59'35"	5°19'37"	12	/
FRA 2	26P18	Allex	France	45°44'52"	4°55'04"	12	/
FRA 3	26P19	Grane	France	44°44'58"	4°52'56"	12	/
FRA 4	26P21	Livron sur Drôme	France	44°46'02"	4°50'45"	12	/
FRA 5	39P04	Saint Germain les Arlay	France	46°45'56"	5°34'25"	12	/
IT_P	P	Pavia	Italy	45°11'43"	9°10'05"	12	1
IT_MM	MM	Magenta	Italy	45°27'15"	8°53'46"	12	4
IT_BR	BR	Brescia	Italy	45°29'23"	10°11'47"	12	2
IT_MI	G	Greco	Italy	45°30'26"	9°12'39"	12	3
IT_L	L	Lodi	Italy	45°18'52"	9°31'05"	12	2
IT_TO	TO	Torino	Italy	45°09’08”	7°44’55”	12	5
IT_TV	TV	Treviso	Italy	45°46’01”	12°20’03”	12	?
IT_PU	PU	Pesaro	Italy	43°41'16"	12°48’12”	12	1

*Only for Italian populations

Table 2. Genetic diversity parameters at SSR loci. For each population the following parameters are reported: %P= percentage of polymorphic loci; N_a = Number of alleles; Ra = Mean number of rare allele per locus. H_O= observed heterozygosity; H_E= expected heterozygosity; F_{IS} = inbreeding coefficient; HW = results of exact Hardy-Weinberg tests; n_{ds}= number of loci that deviate significantly from HWE ($P < 0.05$); F_{ST} = genetic differentiation among populations.

Pop		**%P**	**N_a**	**Ra**	**H_O**	**H_E**	**F_{IS}**	**HW**	**n_{ds}**	**F_{ST}**
CAN1		100.00	6.333	2.932	0.833	0.662	-0.261	*	2	
CAN2		100.00	6.667	3.196	0.611	0.704	0.211	**	4	
CAN3		100.00	5.667	3.005	0.667	0.713	0.053	*	4	
CAN4		66.67	4.667	2.195	0.444	0.505	0.113	**	4	
CAN5		100.00	6.000	2.801	0.667	0.611	-0.093	*	4	
	Mean									
	CAN	*93.33*	*5.867*	*2.826*	*0.644*	*0.639*	*0.029*			
	Tot									0.096
	CAN									
FRA1		100.00	7.667	2.901	0.667	0.706	0.007	***	4	
FRA2		100.00	7.667	3.183	0.556	0.698	0.167	**	6	
FRA3		100.00	6.667	3.005	0.611	0.616	-0.022	**	4	
FRA4		100.00	5.667	2.195	0.556	0.630	0.060	***	4	
FRA5		100.00	5.000	2.801	0.722	0.568	-0.223	***	2	
	Mean									
	FRA	100.00	6.533	*2.817*	0.622	0.643	-0.002			
	Tot									0.072
	FRA									
IT_P		100.00	4.667	2.938	0.411	0.556	0.154	*	4	
IT_MM		100.00	5.000	2.797	0.767	0.693	-0.097	*	4	
IT_BR		100.00	6.000	3.001	0.778	0.699	-0.148	**	4	
IT_MI		100.00	5.333	2.667	0.589	0.626	0.018	*	4	
IT_L		100.00	6.000	2.801	0.556	0.611	0.042	**	4	
IT_TO		100.00	8.333	3.750	0.861	0.804	-0.089	***	6	
IT_TV		100.00	7.000	3.535	0.972	0.782	-0.278	***	5	

IT_PU		100.00	5.667	3.015	0.944	0.727	-0.315	**	4	
	Mean IT	100.00	6.000	3.073	0.735	0.687	-0.089			
	Tot IT									*0.123*
Total	**Mean**	98.15	6.111		0.678	0.662	-0.042			0.120
	SE	1.85	0.262		0.027	0.024	0.022			0.003

Table 3. Results from analysis of molecular variance (AMOVA) from SSR markers for *A. artemisiifolia* populations of Canada, France and Italy.

Source	df	SS	MS	Est. Var.	%	Pvalue
Among Regions	2	16.831	8.415	0.014	1.1%	0.001
Among Pops	15	95.850	6.390	0.180	7.9%	0.001
Within Pops	414	861.000	2.080	2.080	92.0%	0.001
Total	431	973.681		2.274	100%	

Table 4. Kendal tau rank correlation tables between time of residence category (see Table 1) of investigated *A. artemisiifolia* Italian populations and parameters of genetic diversity of the same populations. As the uncertain residence time category attribution of TV population, correlations were repeated three times: a) considering TV population as missing data; b) considering TV population in class 4 of residence time; c) considering TV in class 1 of residence time. In bold significant values are reported.

	Without TV population		With TV population class 4		With TV population class 1	
	Kendall tau	p	Kendall tau	p	Kendall tau	P
Ho	0.65081	**0.040**	0.617	**0.032**	0.264	0.359
He	0.75094	**0.017**	0.771	**0.007**	0.415	0.149
Fis	-0.45056	0.155	-0.462	0.108	-0.188	0.512

644 FIG. 1

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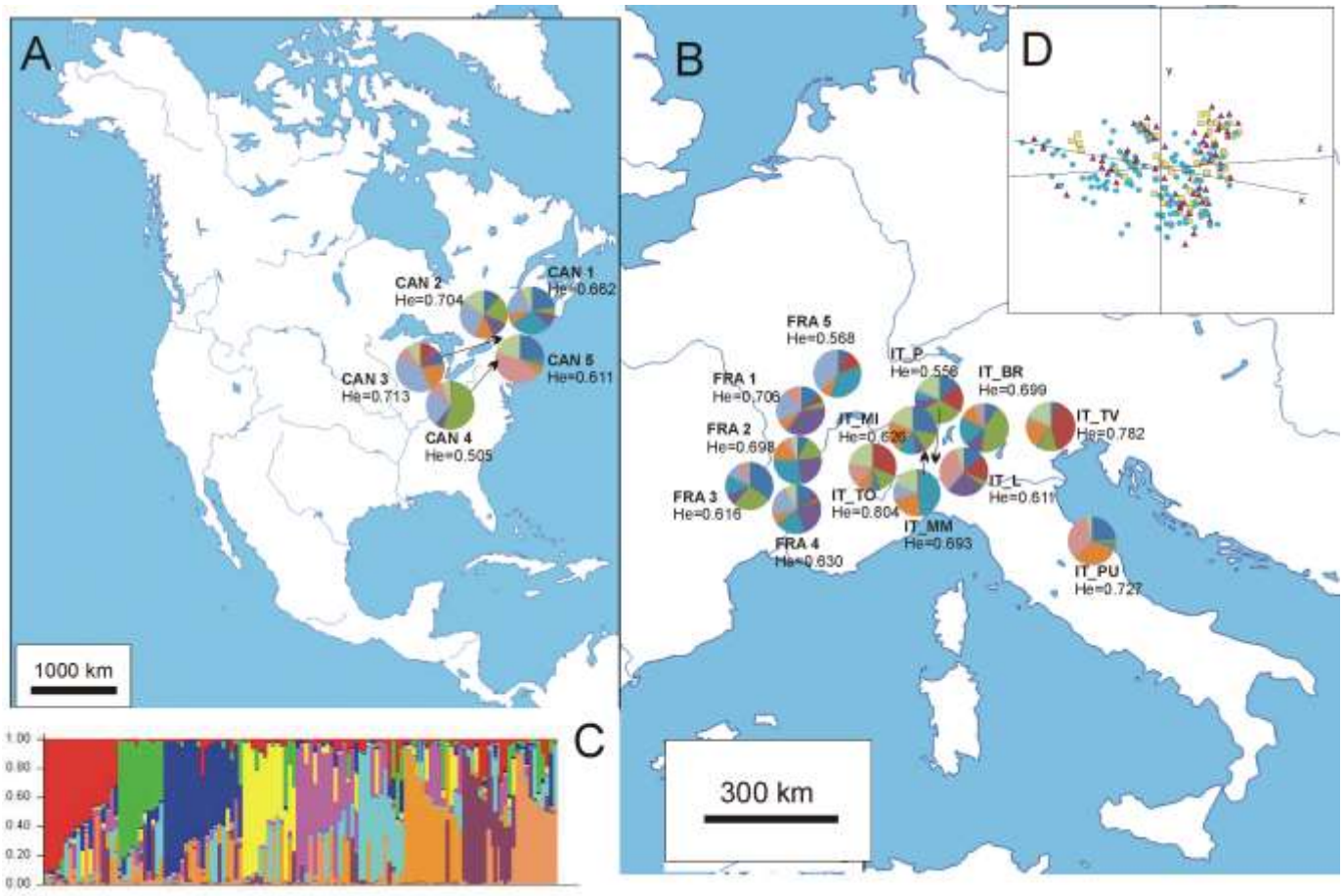

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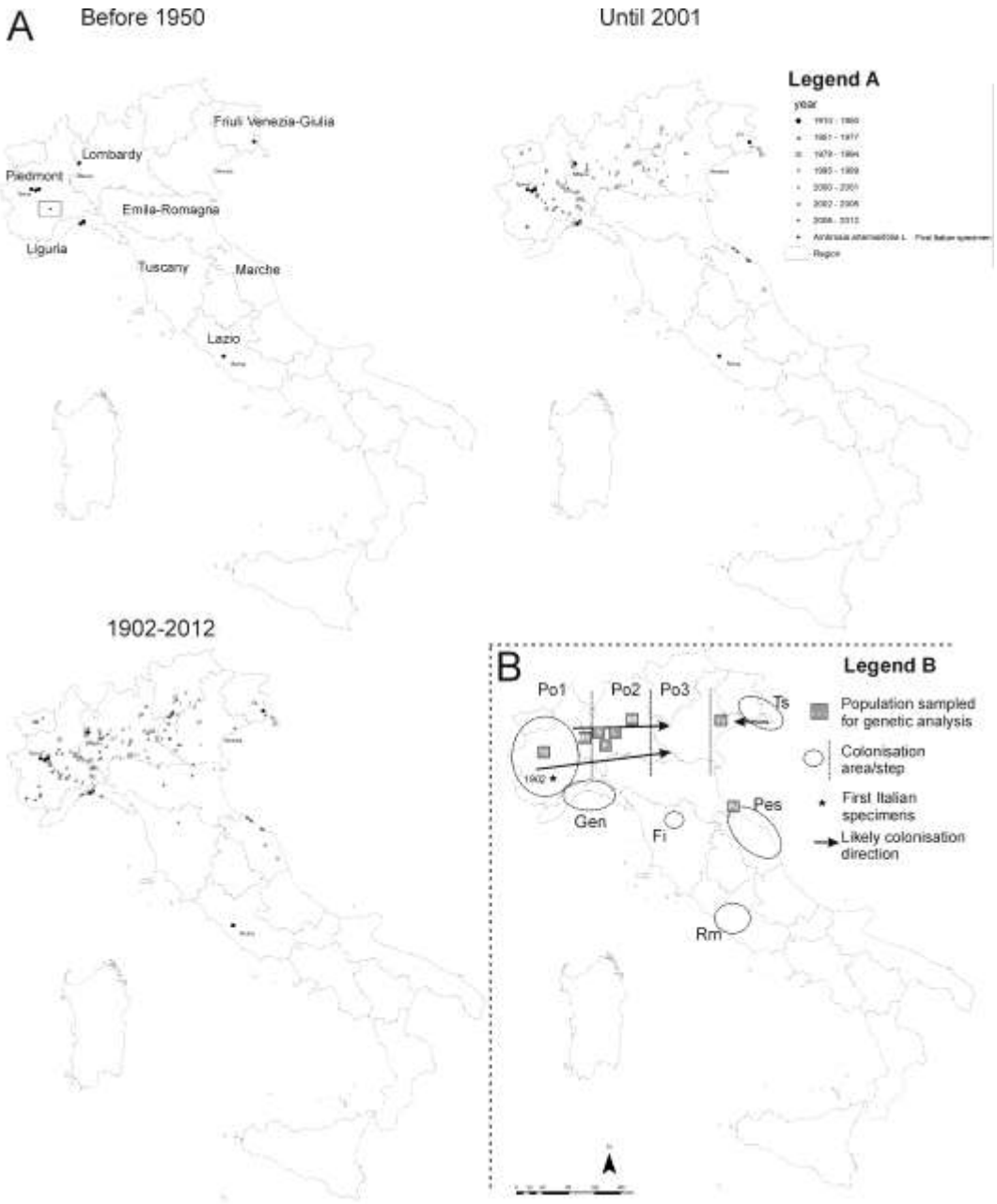
FIG. 2



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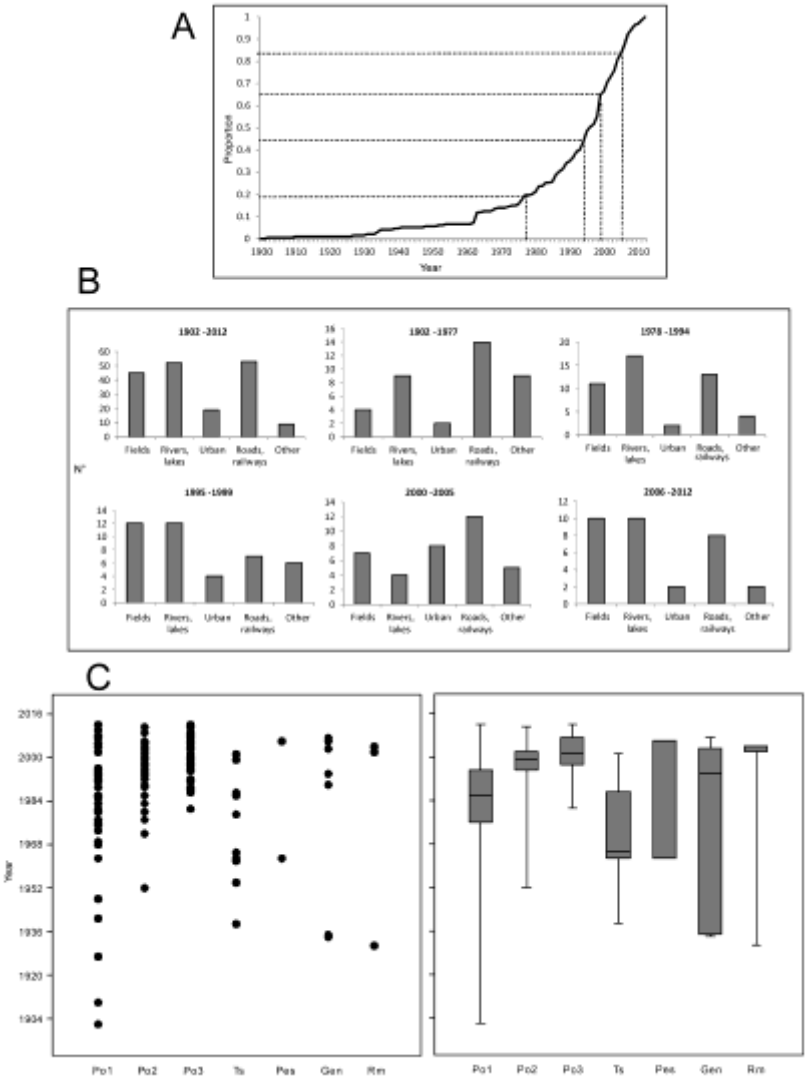
667 FIG. 3



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