UNIVERSITY OF MILANO - BICOCCA

Department of Earth and Environmental Sciences

Ph.D. Program in Biology (XXVIII cycle)

Study of intra and inter population variability of common ragweed (*Ambrosia artemisiifolia* L.) in relation to Amb a 1 isoforms and their allergenicity.

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1. Allergies: the 21st century epidemic

Allergy is a chronic disease that is expected to affect more than 50% of all Europeans in 10 years' time (EAACI, 2011).

While at the beginning of the 20th century allergy was seen as a rare disease, in the last few decades we have witnessed a dramatic increase in disease burden (EAACI, 2015).

Over 150 million people have allergies in Europe, the most common chronic disease (EAACI, 2014) and it is estimated that 10 million adults suffer from more than one allergy (Mintel, 2010). Further, these data are considered to be underestimated, as many patients do not report their symptoms or are not correctly diagnosed, with an estimated 45% of patients having never received an allergy diagnosis.

The prevalence of allergic diseases is growing rapidly in parallel to allergy triggers which include urbanisation, industrialisation, pollution and climate change, factors that are not expected to reduce in the foreseeable future (EAACI, 2015).

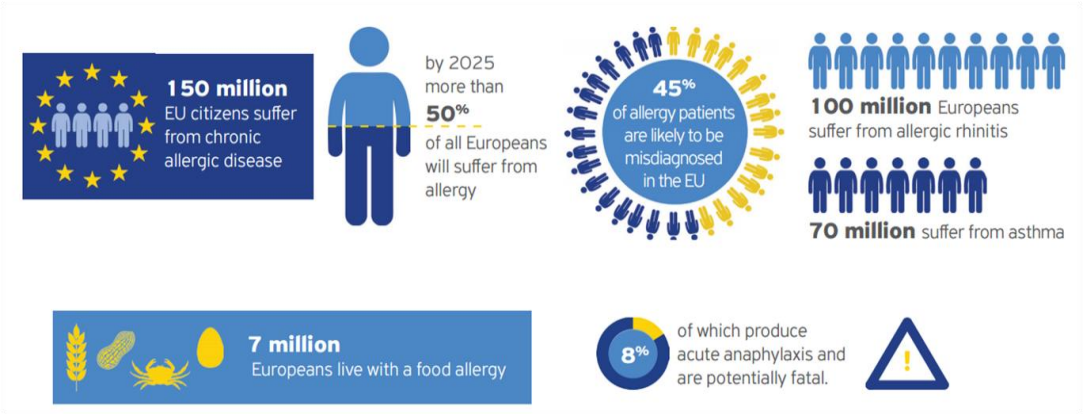

Fig. 1 - Summary figure from EAACI site.

1.1 The immune system

During their evolution, living organisms have developed many defensive mechanisms against potentially harmful agents like micro-organisms and chemical substances. Humans, in particular, have a complex immune system, composed by the innate immune system and by the adaptive immune system. With regard to the former, Elie Matchnikoff discovered that many microorganisms could be engulfed and digested by phagocytic cells called macrophages (Tan *et al*., 2009). These cells are immediately available to combat a wide range of pathogens without requiring prior exposure to them and they represent the key component of the innate immune system.

Antibodies, on the contrary, are specific of the adaptive immune system; they are produced only after infection in response to the infecting pathogen.

Indeed, antibodies can be induced against a vast range of substances. Such substances are known as antigens because they can stimulate the generation of antibodies. However, not all adaptive immune responses entail the production of antibodies, and the term antigen is now used in a broader sense to describe any substance that can be recognized by the adaptive immune system.

The innate immune and the adaptive immune systems together provide a remarkably effective defense system (Janeway *et al*., 2001).

Both innate immunity and adaptive immune responses depend upon the activities of **white blood cells or leukocytes**.

Leukocytes are classified in the following classes:

(1) Granulocytes,

Also called polymorphonuclear leukocytes, they are a collection of white blood cells whose prominent granules give them their characteristic staining patterns; they include three types of granulocytes, all of which are relatively short lived and are produced in increased numbers during immune responses, when they leave the blood to migrate to the sites of infection or

inflammation. These particles have different affinity with neutral, acid or basic dyes. This interaction changes the color of the cytoplasm, allowing the classification of granulocytes in neutrophils, acidophilus (or eosinophils) and basophils.

Neutrophil granulocytes

They are the most numerous and important cellular component of the innate immune response: hereditary deficiencies in neutrophil function lead to overwhelming bacterial infection, which is fatal if untreated.

Basophil granulocytes

Their main function is to secrete immunoglobulins, which are essential to mediate hypersensitivity reactions. Basophils are also characterized by the presence of specific receptors for IgE immunoglobulins on their surface.

Eosinophils granulocytes

They are thought to be important chiefly in defense against parasitic infections, because their number increases during this type of infections.

(2) Monocytes

Macrophages are the mature form of monocytes, wich circulate in tha blood and continually migrate into tissue, where they differentiate. They are relatively long-lived cells and perform several different functions throughout the innate immune response and the subsequent adaptive immune response. Both monocytes and macrophages are phagocytic, but as most infections occur in the tissues, primarily macrophages perform this important protective function. An additional and crucial role of macrophages is to orchestrate immune responses: they help to induce inflammation, which is a prerequisite to a successful immune response, and they secrete signaling proteins that activate other immune-sistem cells (Murphy *et al*., 2008).

(3) Lymphocytes

A common lymphoid progenitor gives rise to **lymphocytes**. The lymphocytes represent 20-40% of all leukocytes and most of them are in a resting state. Some lymphocytes remain in the bone marrow as "memory" and they enter the bloodstream only if necessary.

There are two major types of lymphocyte: B and T lymphocytes, distinguished by their sites of differentiation. They both originate in the bone marrow but only B lymphocytes mature there; T lymphocytes, on the contrary, migrate to the thymus to undergo their maturation. Once they have completed their maturation, both types of lymphocyte enter the blood-stream, from which they migrate to the peripheral lymphoid organs. B cells are the only cells capable of producing antibodies. They recognize both extracellular soluble and cell surface antigens, and they differentiate into antibody-secreting plasma cells, functioning as mediators of the humoral immunity. T cells are instead responsible for cellular immunity and they are divided into three categories: Tc (cytotoxic), Th (helpers), Ts (suppressors). T lymphocytes recognize the antigens of intracellular microbes and they also help phagocytes to destroy them or to kill the infected cells. T cells do not produce antibody molecules. Their antigen receptors are membrane molecules distinct but structurally related to antibodies. T lymphocytes have a restricted specificity for antigens; they recognize peptides derived from foreign proteins that are bound to host proteins called major histocompatibility complex (MHC) molecules, expressed on the surfaces of other cells. As a result, these T cells recognize and respond to cell surface-associated antigens but not soluble ones.

Unfortunately, the immune system not always works properly: sometimes it can generate an altered response against harmless substances or environmental factors, triggering intense and prolonged inflammatory reactions that can damage surrounding tissues and create harmful effects and even death. Such a

malfunctioning is commonly defined “allergy” and the substances responsible for it are called “allergens”.

1.2 The allergy mechanism

The term allergy was originally defined by Clemens Von Pirquet as “an altered capacity of the body to react to a foreign substance” (Hamelmann *et al.*, 2002). Nowadays this definition is considered extremely broad as it included all immunological reactions and allergies are defined in a much more restricted manner as “diseases following a response by the immune system to an otherwise innocuous antigen” (Murphy *et al*., 2008) .

Immunoglobulins or antibodies (Fig. 2) are globular proteins of the serum and have an important defensive function: they are able to specifically bind antigens. Biochemists have identified five distinct classes of immunoglobulins: IgG, IgA, IgD, IgM and IgE.

The IgE, commonly known as "reagins", were discovered and described by Ishizaka and co-workers in 1966. The IgE are large glycoproteins (about 190 kDa) characterized, like all immunoglobulins’ classes, by a typical quaternary structure consisting of two light chains (L) and two heavy chains (ε) of peptides, each constituted by two regions, a variable region (V), in correspondence of the aminoterminal end, and a constant region (C) at the carboxyl end.

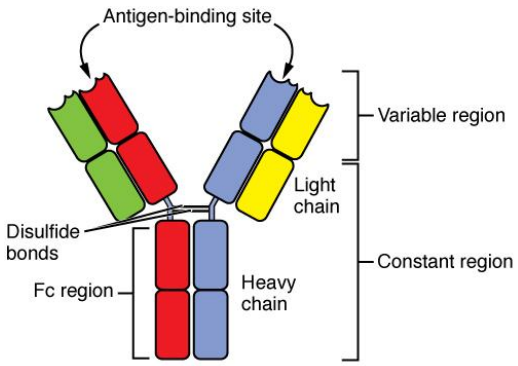

Fig. 2 - The typical four-chain structure of a generic antibody.
From: Human Anatomy and Physiology (http://philschatz.com/anatomy-book/contents/m46844.html).

All allergic reactions share common features, although they differ greatly in the types of antigens that elicit these reactions and their clinical and pathologic manifestations.

- The hallmark of allergic diseases is the production of IgE antibody, which is dependent on the activation of IL-4-producing helper T cells. Whereas healthy individuals either do not respond or have harmless T cell and antibody responses to common environmental antigens, atopic individuals develop strong IL-4-producing helper T cell responses and produce IgE on exposure to these allergenic substances.
- The typical sequence of events in immediate hypersensitivity consists of exposure to an antigen, activation of lymphocytes specific for the antigen, production of IgE antibody, binding of the antibody to Fc receptors of mast cells, and triggering of the mast cells by re-exposure to the antigen, resulting in the release of mediators from the mast cells and the subsequent pathologic reaction (Fig. 3). Binding of IgE to mast cells is also called sensitization because IgE-coated mast cells are ready to be activated on antigen encounter.
- Allergy is the prototypic T_H2-mediated disease. Many of the early events and pathologic features of the reaction are trigged by T_H2 cytokines, which way be produced by T_{FH} cells in lymphoid organs and by classical T_H2 cells in tissue. This contrasts with delayed type hypersensitivity, which is largely a T_H1-mediated immune reaction.
- The clinical and pathologic manifestations of allergy consist of the vascular and smooth muscle reaction that develops rapidly after repeated exposure to the allergen (immediate hypersensivity) and a delayed late phase inflammatory reaction. These reactions may be initiated by IgE-mediated mast cell activation, but different mediators are responsible for the immediate versus late-phase reactions. Because mast cells are present in connective tissue and under epithelia, these tissue are the most common

sites of immediate hypersensitivity reactions. Some immediate hypersensitivity reactions may be triggered by non-immunologic stimuli, such as exercise and exposure to cold. Such stimuli induce mast cell degranulation and the release of mediators without antigen exposure or IgE production. Such reactions are said to be non-atopic.

- Allergic reactions are manifested in different ways, depending on the tissues affected, including skin rashes, sinus congestion, bronchial constriction, abdominal pain, diarrhea, and systemic shock. In the most extreme systemic form, called anaphylaxis, mast cell-derived mediators can restrict airways to the point of asphyxiation and produce cardiovascular collapse leading to death.
- The development of allergies is the result of complex and poorly understood gene-environment interactions. There is a genetic predisposition for the development of allergies, and relatives of allergic individuals are more likely to also have allergies than unrelated people, even when they do not share environments. Various environmental factors, especially in industrialized societies, including the presence of allergens and exposure to microbes, have a profound influence on the propensity to develop allergies (Abbas *et al*., 2015).

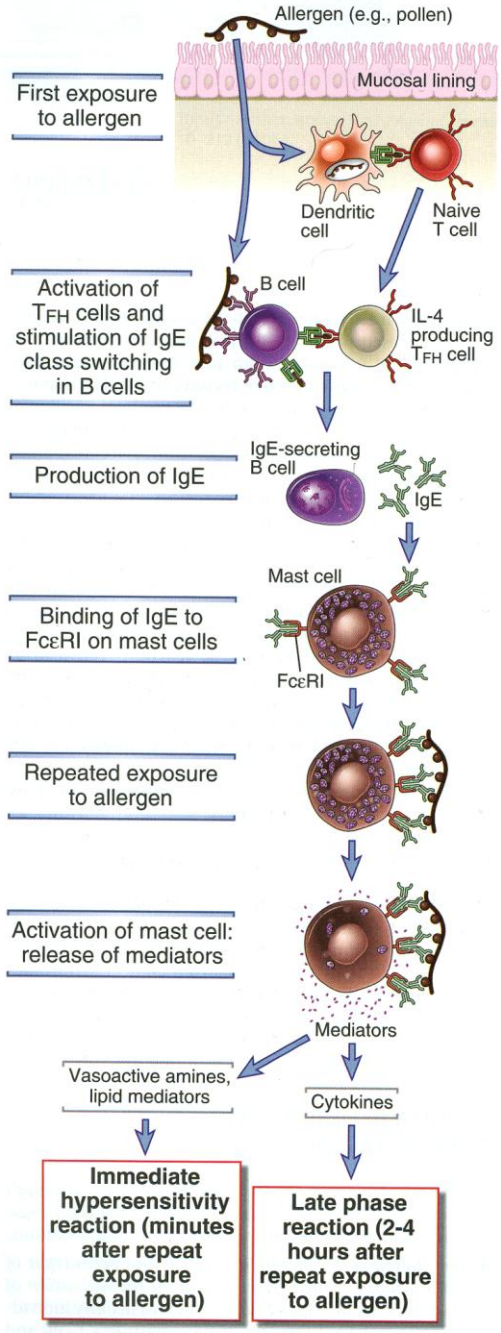

Fig. 3 - Sequence of events in immediate hypersensitivity reactions. immediate hypersensitivity diseases are initiated by the introduction of an allergen, which stimulates IL-4 producing helper T cell responses and IgE production. IgE sensitizes mast cells by binding to FceRI, and subsequent exposure to the allergen activates the mast cells to secrete the mediators that are responsible for the pathologic reactions of immediate hypersensitivity (Abbas *et al.*, 2015).

2. Plant allergy

Plants may trigger allergic reactions in some people after a direct contact, inhalation of plant pollens or consumption of foods derived from plant materials. Inhalation of pollens can cause pollinosis, resulting in symptoms such as rhinitis, sneezing, and itching (see below). After eating certain plant foods, sensitive people may suffer diarrhea, emesis, and anaphylaxis, which is a severe systematic allergic reaction (Nakamura and Teshima, 2013). Two forms of food allergy, class 1 and class 2, can be distinguished, according to the clinical appearance, the pattern of allergens, and the underlying immunologic mechanisms (Breiteneder *et al*., 2000). Class 1, it is one of the first manifestations of the atopic syndrome and affects young children. This kind of food allergy is rare in adults. The sensitization process occurs in the gastrointestinal tract. One special feature of the allergens eliciting this manifestation is their particular resistance to gastric digestion. The most important allergens are cow's milk, hen's egg, and legumes. It can be expected in the majority of the cases that these manifestations disappear later during childhood and are replaced by other manifestations of the atopic syndrome. Class 2 allergy, typically affects older children, adolescents and adults and is the consequence of primary sensitization due to inhaled allergens. The immune basis involves a cross-reactivity phenomenon that may be clinically relevant or irrelevant (Ebner *et al*., 1991). Food will induce symptoms in those patients previously sensitized to homologous allergens present in the inhaled aeroallergens (Bartra *et al*., 2009).

2.1 Plant allergens

Approximately 41% of identified allergens have a plant origin (pollen, food, latex). With the implementation of molecular biological techniques in the field of allergen characterization, the sequence, nature, and three-dimensional structure of several important allergens have been revealed. Over 25 years ago, the gene encoding the

major birch pollen allergen Bet v 1 was the first such gene to be cloned and its product characterized. Since that time, 53 tree pollen allergens have been identified and acknowledged by the WHO/IUIS allergen nomenclature subcommittee (Asam *et al*., 2015).

Plant allergens have various physiological roles; some are storage proteins, some are enzymatic, and others are structural proteins. Plant allergens are classified into families according to their functions and structures, as shown in Table 1. The binding sites of allergen to patients' IgE, epitopes, are eather linear stretches of 6–20 amino acids or a conformational structure; IgEs that recognize linear epitopes can cross-react with proteins from different plants that have similar sequences as well as IgEs that recognize conformational epitopes can cross-react with proteins showing similar structures. Therefore, members of allergen families may show cross-reactivity to specific IgEs. For example, subjects who are sensitized to pathogenesis related class 10 (PR-10) proteins from pollens can also react to fruit proteins with similar structures; it is the so called "pollen-food syndrome".

Here below the main characteristics of five of the major groups of plant allergens are described.

1) Calcium-binding proteins (Ca-Bp).

   In a 1999 study, Rozwadowski and colleagues characterized a Ca-Bp in pollen of *Brassica* and *Arabidopsis*. This type of allergenic proteins, called polcalcins constitute the majority of allergenic Ca-Bps, and their expression seems to be restricted to pollen, both from monocots and dicots (Hauser *et al*., 2010). The biological function of polcalcins is still unclear. However, due to their pollen-specific localization and their ability to bind calcium, it has been proposed that polcalcins function in the control of intracellular calcium levels during pollen germination (Wopfner *et al*., 2007). Two conformational states of CBPs can be distinguished, i.e. the closed calcium-free "apo", and the open calcium-associated "holo" forms. Several studies demonstrated that the apo-forms are less stable to thermal denaturation and

display decreased IgE-reactivity when compared to their calcium-bound counterparts (Hauser *et al*., 2010).

2) Expansins (EXP).

   The EXP proteins are involved in the plant cell wall remodeling (Cosgrove, 2000). When the first expansin was sequenced, a database research found a similarity with a group of allergens of herbaceous monocots known as Group 1. In fact, Group 1 allergens are structurally and functionally similar to expansins and they are the most important allergens of the herbaceous monocots' pollen. These are glycoproteins with molecular weight of approximately 31-35 kDa and with slightly acid pI; localization studies have shown the presence of these proteins in the cytoplasm, and also in the exine of pollen grain (Taylor *et al*., 1994). In recent years the knowledge relating to expansins has gotten more complex through bioinformatic analysis of expansin distribution and evolution, as well as through expression analysis, dissection of the upstream transcription factors regulating expression, and identification of additional classes of expansin by sequence and structural similarities.

   Today we know that transgenic modulation of expansin expression modifies growth and stress physiology of plants, but not always in predictable or even understandable ways (Cosgrove, 2015).

3) Pathogenesis-related proteins (PRPs).

   The PRPs are an important group of plant allergens, whose expression can be regulated in plants in response to environmental stresses (pathogenic, temperature variations, pollutants) (Hanninen *et al*., 1999). Some of them are constitutively expressed in those parts of plant, as pollen and fruits, which are more easily attacked by pathogens or more exposed to environmental stress. In fact an important common function of most PRPs is their antifungal effects and some PRPs also exhibit antibacterial, insecticidal or antiviral action.

One of the most important family belongin to the PRPs is the Bet v 1 superfamily (Indirli *et al.*, 2012). It was cloned for the first time by Breiteneder and associates in 1989 and has been one of the most studied allergens. These allergens are heat-sensitive and unstable and therefore they usually only affect the oral cavity.

4) The prolamine superfamily.

   The prolamine superfamily comprises three major groups of allergens: lipid transfer proteins (LTPs), albumins 2S and the trypsin and α-amylase inhibitors. All are rich in cysteine and are stable in response to thermal processing and enzyme proteolysis.

   They have been described for the first time by Pastorello and collaborators in 1999 and since that time they have been the focus of many studies for their clinical relevance.

   Plant lipid transfer proteins (LTPs) are cationic peptides whose name is related to their ability to reversibly bind and transport hydrophobic molecules in vitro.

   These proteins have been described in many plant foods and are able to sensitize the host through the digestive tract and probably via the inhalatory route too.

5) Profilin.

   The first allergenic profilin was described in birch pollen by Valenta and collaborators in 1991 and was designated Bet v 2. Profilins represent a family of small (12 to 15 kDa), highly conserved molecules sharing sequence identities of more then 75% even between members of distantly related organisms (Hauser *et al.*, 2010). Profilins are found in all eukaryotic cells and the principal function of this protein in plant is the fertilization process (Valenta *et al*., 1992).

   It is a molecule present in many foods and pollens; specifically allergenic profilins were identified in pollen of trees, grasses, and weeds, in plant

derived foods, as well as in latex. This type of proteins are little resistant to digestion; thus we would expect to have answers only in the mouth but nevertheless there is evidence of systemic response (Bartra *et al*., 2009).

Tab. 1 - Allergen protein families of plants (from: Nakamura and Teshima, 2013; Ferreira, 2004).

Allergen	Function	Pollens			Fruits and vegetables	Legumes, nuts and seeds	Latex
		Tree	Grasses	Weeds			
Fagales, group 1	Plant steroid hormone transporter, PR-10	Aln g 1* Bet v 1* Car b 1* Cas s 1* Cor a 1* Fag s 1 Que a 1*			Api g 1* Dau c 1* Fra a 1 Mal d 1* Pru ar 1* Pru av 1* Pru p 1 Pyr c 1* Vig r 1	Cor a 1.04* Gly m 4*	
Profilins	Actin-binding protein	Bet v 2* Car b 2 Cor a 2* Fra e 2 Ole e 1 Ole e 2* Pho d 2* Pla a ?	Cyn d 12* Lol p 12 Ory s 12 Phl p 1 Phl p 11 Phl p 12* Poa p 12 Zea m 12	Amb a ? Art v 4* Che a ? Hel a 2* Mer a 1* Par j 3* Zyg f ?	Ana c 1* Api g 4* Aspa o ? Cap a 2* Cit l ? Cuc m ? Cuc p ? Cuc s ? Dau c 4* Fra a 4 Gly m 3* Lit c 1* Lyc e 1* Man I 3 Mal d 4* Mus xp 1* Pru av 4* Pru p 4* Pyr c 4*	Ara h 5* Bra n ? Cor a 2*	Hev b 8*

Allergen	Function	Pollens			Fruits and vegetables	Legumes, nuts and seeds	Latex
		Tree	Grasses	Weeds			
Polcalcins	Calcium-binding protein	Aln g 4 Bet v 3* Bet v 4* Car b ? Jun o 4* Fra e 3 Ole e 3* Ole e 8* Syr v 3	Cyn d 7 Phl p 7	Amb a ? Art v ? Bra n ? Bra r ? Che a ? Par j ?			
Oleaceae, group 1	Trypsin inhibitor	Fra e 1* Lig v 1* Ole e 1* Syr v 1*	Lol p 11* Phl p 11*	Che a 1* Pla l 1* Sal k ?			
Thaumatins	PR-5	Act d 2 Cry j 3 Cup s 3 Jun a 3* Pru p 2			Act c 2* Cap a 1* Mal d 2* Ole e 13 Pru av 2* Tri a TLP Vit v ?		
Grasses, group 1	B-Expansin		Agr a 1 Ant o 1 Ave s 1 Cyn d 1* Dac g 1* Fes e 1 Fes p 1 Hol l 1* Hor v 1 Lol p 1* Ory s 1* Pha a 1* Phl p 1* Phr a 1 Poa p 1* Sec c 1 Sor h 1* Tri a 1 Zea m 1*				

Allergen	Function	Pollens			Fruits and vegetables	Legumes, nuts and seeds	Latex
		Tree	Grasses	Weeds			
Grasses, group 5	Unknown		Ant o 5 Ave s 5 Cyn d 5 Dac g 5* Fes e 5 Fes p 5 Fes r 5 Hol l 5 Hor v 5 Imp c 5 Lol p 5* Pha a 5 Phl p 5* Phr a 5 Poa p 5* Sec c 5				
Ragweed, group 1	Pectate lyase	Cha o 1 Cry j 1* Cup a 1* Cup s 1* Jun a 1*		Amb a 1* Art v ?			
Compositae, group 1	PR-12, defensin domain			Art v 1* Hel a ? (SF18) Par h 1			
Enolases	Glycolytic enzyme						Hev b 9*
SOD	Manganese super oxide dismutase						Hev b 10*
Glucanase **Chitinase**	PR-2 PR-3				Mus xp ? Act c ? Bra r 2 Cas s 5* Mus xp ? Pers a 1* Vit v ?		Hev b 2* Hev b 6.02* Hev b 11*

Allergen	Function	Pollens			Fruits and vegetables	Legumes, nuts and seeds	Latex
		Tree	Grasses	Weeds			
nsLTP	Non-specific Lipid Transfer Protein, PR-14	Ole e 7		Amb a 6	Aspa o 1*	Ara h 7	Hev b 12*
				Art v 3*	Dau c ?	Bra o ?	
				Par j 1* Par j 2* Par o 1*	Hor v ? Lac s 1* Mal d 3* Pru ar 3* Pru av 3* Pru d 3* Pru p 3* Pru du ? Pyr c 3 Tri a 14 Tri s ? Vit v 1* Zea m 14*	Bra r ? Cas s 8* Cor a 8* Jug r 3*	
Plant proteases	Cystein protease				Act c 1* Ana c ? (Bromelain) Car p ? (Papain) Fic c ? (Ficin)	Gly m 1	
Plant albumins Storage protein	2S albumin					Ana o ? Ara h 2* Ara h 6* Ara h 7* Ber e 1* Bra j 1* Bra n 1* Jug n 1* Jug r 1* Pru du ? Ric c 1* Ric c 3 Ses i 1* Ses i 2* Sin a 1*	

Allergen	Function	Pollens			Fruits and vegetables	Legumes, nuts and seeds	Latex
		Tree	Grasses	Weeds			
Plant globulins (1)	7S globulin Cupin (Vicilin) superfamily					Ana o 1* Ara h 1* Cor a 11* Gly md 28 K Jug n 2* Jug r 2* Len c 1* Pis s 1 Ses i 3*	
Plant globulins (2)	11S – 13S globulin			Ber e 2* Cor a 9* Fag e ? Gly m ? Sin a 1 Pis v 2 Car i 4 Jug r 4 Fag e 1		Ana o 2* Ara h 3* Ara h 4*	
Alpha-amylase/ trypsin inhibitor	Protease inhibitor					Tri a 15 RAG1/ RAG2/RA5	

*Allergenic molecules listed in the Official International Union of Immunological Societies Allergen Nomenclature website (http://www.allergen.org).
In bolditalic are reported crossreactive molecules. In bold molecules with function and sequence homologies but lacking demonstrated crossreactivity.

2.2 Pollinosis

Among allergic diseases, the respiratory allergies are certainly the most common forms. The respiratory allergens are carried by airborne particles of different origin: animal (droppings of dust mites, epithelial fragments of mammals), vegetal (pollen) and chemical (smoke, resins).

Depending on the characteristics of exposure to respiratory allergens, it is possible to distinguish between seasonal and perennial allergies.

The allergic reaction to pollen, causing hay fever, is generally seasonal and called pollinosis. It is the most common and widespread manifestation, especially in industrialized countries.

This kind of allergy has been identified and described for the first time in the XIX century by Dr. Charles Blackley, who first interrelated the allergic symptoms with the seasons of the year and then with the inhalation of pollen.

The symptoms caused by the inhalation of this category of allergens affect eyes, the upper respiratory tract (nose, pharynx and larynx) and the lower one (trachea, bronchi and lungs). A common disorder associated with pollinosis is allergic rhinitis.

It is hard to determine the exact number of people suffering by allergic rhinitis, but it has been estimated that about 20% of the Italian population will suffer sooner or later (Clough, 2008).

Not all pollens can induce allergy. For a pollen to be considered allergenic, it has to follow the following set of requirements originally described by August Thommen (1931) and called Thommen's Postulates:

1. The pollen must be allergenic.
2. The pollen must be wind-borne.
3. The pollen must be produced in large quantities.
4. The pollen must light and small enough to be carried by the wind for considerable distances.

5. The plant must be abundantly distributed in a region, or habitually grown.

However there are exceptions. For instance, willow pollen breaks these rules but it is allergenic; althougt it is a generally insect-pollinated tree, it has been shown to cause allergies in human.

2.2.1 Pollen

Pollen is the gametophyte responsible for the production and transport of the male gamete in the reproductive system of spermatophytes (Gymnosperms and Angiosperms).

The word "pollen", from the Latin *pollen*, "fine flour", was coined by the German physician and botanist Valerius Cordus (1515-1544) after observing the presence of a powder in the anthers of flowers. Later, the invention of the optical microscope in the XVII century and the first publications and descriptions of pollen grains officially gave start to the scientific study of pollen.

At maturity stage, the pollen surface can be divided into three principal layers (Fig. 4), that differ in dimension between species: (1) an outer exine wall, itself multilayered, composed of the chemically resistant polymer sporopollenin and interrupted by openings called “apertures”; (2) an inner intine, also sometimes multilayered, made primarily of cellulose; and (3) a pollen coat, composed of lipids, proteins, pigments, and aromatic compounds, that fills the sculptured cavities of the pollen exine (Edlund *et al*., 2004).

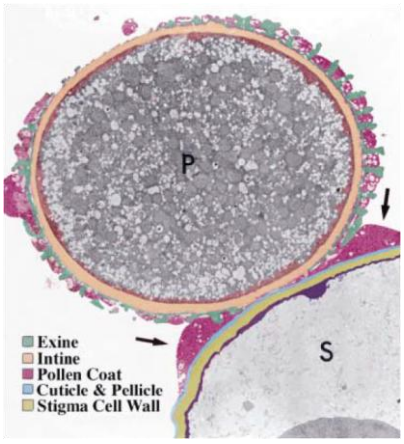

Fig. 4 - Transmission electron micrograph showing the point of contact between a pollen grain (P) and a stigma papillus (S), colorized to highlight the pollen coat (pink), intine (peach), exine (green), stigma cell wall (yellow), and stigma cuticle (blue). A foot of lipid-rich material (arrows) collects between the two surfaces (Edlund *et al*., 2004).

The pollen development is completed only when the pollen has reached the female structure with the formation of the pollen tube which acts as a carrier for the male gametes.

In Spermatophytes, pollination can be achieved in three different ways:

1- Pollination by wind: passive transport of pollen grains in the atmosphere occurs by wind, until they don't meet a female structure.
2- Pollination by water: water is the dispersion medium of the pollen grains (system limited to a few plant species).
3- Pollination by animals: some species of animals, called pollinators (insects, birds, bats, molluscs) actively attend the pollen dispersion.

The anemophilous transport is not very efficient and consequently the anemophilous plants produce large amounts of pollen grains in the atmosphere to ensure a successful fertilization thus assuming an important role in the development of pollinosis.

At first it was believed that the allergens pollen were mostly located on the exine, later it was ascertained that instead most of the allergens were contained within the granule and that they had the property to escape from the pores very quickly, once a contact with a moist surface.

However, immunochemical studies suggested typical sites for different pollen allergens; experiments have localized allergens in the cell wall (exine and intin) or in the cytoplasm. A moderate amount of allergens was also localized near the apertures.

Humans exposition to allergens occurs in two way:

(1) through inhaling pollen which diffuse proteins during rehydration at mucosa level and
(2) directly through the inhalation of free aeroallergens, released from pollen under high humidity condition or undergoing osmotic breaking before mucosa contact.

There are numerous studies reporting a correlation between hospital admissions for asthma attacks and heavy storms. This can be explained by the sudden increase in the atmosphere concentration of allergens released from pollen grains undergoing osmotic shock during severe thunderstorms.

2.2.2 Plant inducing pollinosis in Europe

The concentration of allergenic pollen in the atmosphere varies according to geography, climate and vegetation. The more allergenic plant in Europe are listed below (D'Amato *et al*., 2007).

Herbaceous:

- *Gramineae.*

  The grass family comprises more than 600 genera and over 10 000 species, of which more than 400 herbaceous, wind-pollinated plants are found in Europe and among these the most important are*: Phleum pratense, Dactylis glomerata, Alopecurus pratensis, Secale cereale*.

- *Urticaceae.*

  Parietaria is the main allergenic genus and the most important species are *Parietaria judaica* and *Parietaria officinalis*.

- *Asteraceae.*

  The *Compositae* is one of the largest plant families with almost 20000 species. Ragweed (*Ambrosia*) and mugwort (*Artemisia*) are the most involved in pollinosis. The most common species of *Artemisia* are *A. vulgaris* (mugwort), which grows throughout Europe, and *A. Annua* and *A. verlotorum*, which grow mainly in southern Europe. The genus *Ambrosia* (*A.*), which includes both *A. artemisiifolia* (short or common ragweed) and *A. trifida* (giant ragweed), has long been recognized as a significant cause of allergic rhinitis.

Arboreal:

- *Fagales.*

  This order comprises three families: *Betulaceae*, including the genera *Betula* (birch, the major pollen-allergen-producing tree in northern Europe) and *Alnus* (alder); Corylaceae, including the genera *Corylus* (hazel), *Carpinus* (hornbeam), and *Ostrya* (hopbeam); *Fagaceae*, including the genera *Quercus* (oak), *Fagus* (beech), and *Castanea* (sweet chestnut).

- *Oleaceae.*

  Olive pollen is considered as one of the most important causes of respiratory allergic disease in the Mediterranean region. *Olea europaea* pollinosis is clinically characterized by rhinoconjunctival symptomatology and bronchial asthma.

- *Cupressaceae.*

  The genus *Cupressus* is widely spread in Mediterranean area, where the most common species are *C. sempervirens*, *C. arizonica*, *C. macrocarpa* and *C. lusitanica*. Cypress is responsible for a large part of total annual amount of airborne pollen in several Mediterranean areas.

The vegetational areas and prevalent distribution of allergenic plants in Europe are listed below.

Arctic: birch.

Central: deciduous forest, birch, grasses.

Eastern: grasses, mugwort, ragweed.

Mountains: grasses (with a pollination season delayed by three-four weeks in comparison with areas at sea level).

Mediterranean: *Parietaria*, olive trees, grasses and also cypress.

2.3 Pollinosis and climate change

According to the Fourth Assessment Report by the IPCC released in 2007, warming of the global climate system is unequivocal, and there is a >95% certainty that the cause is extrinsic. As stated in the Working Group I Report of the Intergovernmental Panel on Climate Change (IPCC) "most of the observed increase in globally averaged temperatures since the mid-20th century (Fig. 5) is very likely due to the observed increase in anthropogenic greenhouse gas concentrations". Human activities have a net warming effect (>90% confidence) that is dominated by greenhouse gas (GHG) emissions. The most important GHG is carbon dioxide released by the burning of fossil fuels and to a lesser extent land use practices, followed by nitrous oxide and methane. Over the past 100 years, CO_2 emissions have accelerated globally, driving faster temperature rise.

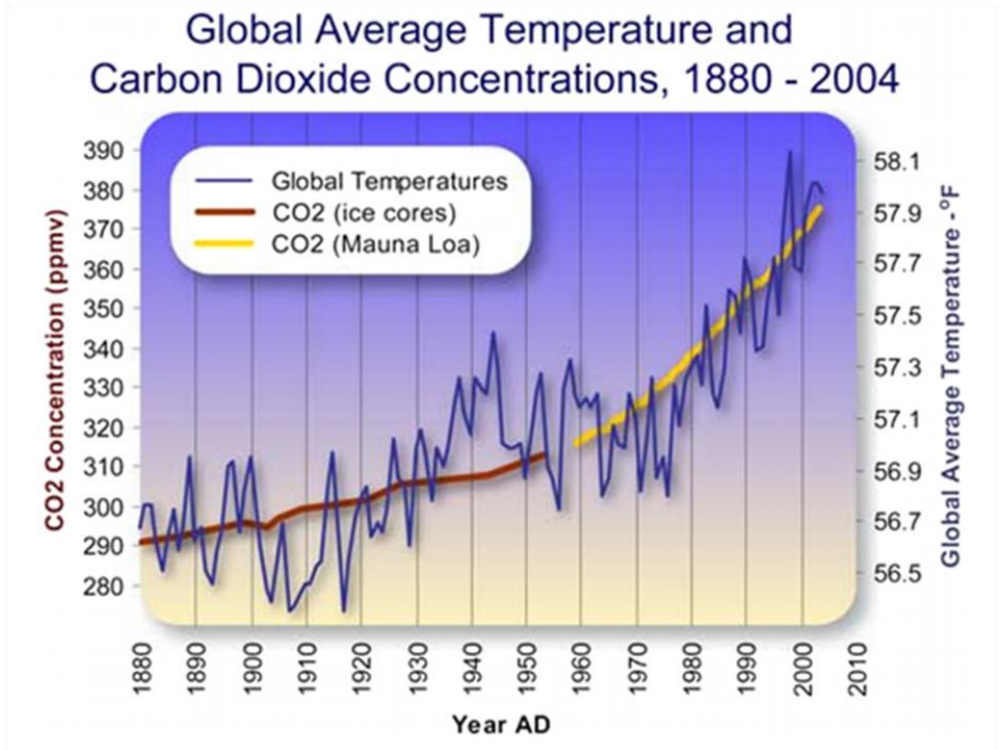

Fig. 5 - Global average temperature and carbon dioxide concentrations, 1980–2004 (D'Amato *et al.*, 2013).

Because of the inertia in the climate system and the long residence time of CO_2 in the atmosphere, even if emissions were abruptly reduced to zero, global warming would continue throughout the 21st century and likely persist for hundreds of years (Shea *et al.*, 2008). In turn, these changes are expected to have a wide range of current and projected effects on human systems; indeed, the Lancet acknowledged that climate change represents the greatest threat to human health in the 21st century (Ziska, 2013). Concerning pollinosis, to date, the data indicates that plant-based aeroallergens are likely to be significantly altered with earlier pollen initiation, greater pollen loads, greater allergenicity and longer exposure times. In addition data suggests that asthma and allergic disease will also likely be worsened because of interaction between heavier pollen loads and increased air pollution, thunderstorms and extreme precipitation events, worsening heat-related ground-level ozone pollution and increased ambient air pollution from natural and anthropogenic sources (Shea *et al*., 2008).

Specifically the correlation between climate changes, allergenic plants and pollen distribution can be summarized in the following points:

1. <u>Increase in plant biomass and pollen production</u>

   increased CO_2 and temperature affect plant growth. Generally plants grown in higher concentrations of CO_2 develop faster, are larger at maturity and produce more pollen. For instance it has been reported an increased short ragweed biomass and an increase in pollen production of 61% to 90% with increased ambient CO_2 (Shea *et al.*, 2008; Wayne *et al*., 2002).

   Different studies in Denmark (Rasmussen, 2002), Switzerland (Frei, 1998) and North America (Levitin, 2001) reported an increase of cumulative pollen of a number of taxa over the latter decades of 1900s that were related with climate change.

   In another study Ziska *et al*. (2003) showed that ragweed produced significantly greater above-ground biomass and pollen at urban locations

than at rural locations were the concentration of greenhouse gases were lower.

2. Increase in pollen allergenicity: the amount of allergenic proteins contained in pollen was detected in many species grown at different climatic conditions. For instance studies on mountain birch (*Betula pubescens*), grown at two different temperatures, showed a higher level of the major pollen allergen Betv1 in pollen collected from trees grown at the higher temperature (Ahlholm *et al*., 1998). Moreover, Hjelmroos (1995) examined the heterogeneity of antigenic proteins and allergens within individual white birch (*B. pendula*). He proved that the allergen level was greatest in pollen developed on south-facing branches and suggested that this was at least partly due to higher temperature. In addiction an experiment of Singer and collaborators in 2005 demonstrated that the quantity of Amb a 1, a major allergen of *Ambrosia artemisiifolia*, increased with the increase of the concentration of CO_2. Finally Kelish *et al*. (2014) showed that the expression of most Amb a 1 mRNA increased in pollen of plants exposed to high concentrations of CO_2 and under drought stress.
3. Prolonged pollen seasons: there is high confidence about a trend in many regions towards earlier greening of vegetation in the spring linked to longer thermal growing seasons due to recent warming (Smith *et al*., 2014). Both higher temperatures and higher ambient CO_2 levels, speeds flower development, resulting in earlier blooming. A 2002 study of 385 British plant species found that the average first flowering had advanced by 4.5 days during the previous decade. One sixth of species had a marked advancement of 15 days, whereas 10 species (3%) had delayed flowering. Spring-flowering plants were the most affected and were sensitive to temperature in the preceding month. Entomophilous plants were more affected than anemophilous ones, although many anemophilous aeroallergenic pollens have demonstrated altered seasons (Shea *et al.*,

2008). For instance spring flowering species such as birch (Emberlin *et al*., 2002, Van Vliet *et al*., 2002), mugworth (Stach *et al*., 2007), grass (Emberlin *et al*., 1999; Burr, 1999), showed an earlier start of flowering and an extent of pollen season. The duration of season was also shown to be extended in some summer and late flowering species (Beggs, 2004). However warming has been slightly greater in the winter hemisphere and changes in spring phenophases are more pronounced than those that occur in summer and autumn (Aasa *et al*., 2004; Ahas *et al*., 2002; Bertin, 2008; Fitter and Fitter, 2002; Solomon *et al*., 2007; Walther *et al*., 2002).

4. Changing plants and pollen distribution. A wide literature is available for this topic. For instance it has been reported that many plants such as *Betula pubescens* (Troung *et al*., 2006) and *Brachypodium pinnatum* (Buckland *et al*., 2001) expanded to different regions (i.e. higher altitudes) in response to recent warming of the climate. Similar extension could be expected for *Olea, Parietaria, Ambrosia*, *Poaceae* (Emberlin, 1994).

   A study of Weber (2002) has suggested that one of the implications of increased pollen production associated with increased CO_2 concentration, could be more efficient wind pollination, and ultimately, greater propagation of the plant species.

In conclusion an increase of allergenic plant biomass along with a longer duration of their flowering period and with an increased production of pollen raise the period of exposure to allergens and may therefore increase the duration of the symptoms and allergic reactions.

However, although now there is the certainty that climate change will affect the reproduction and distribution of allergenic plants both woody and herbaceous, the effect of these changes on the prevalence of allergies remains speculative (Cecchi *et al*., 2010).

3. *Ambrosia artemisiifolia:* one of the most allergenic alien species in Italy

Ambrosia artemisiifolia is an herbaceous annual plant, native from North America, which in recent times has rapidly spread in the European continent. This plant has a very allergenic pollen and is greatly responsible for the late onset of summer oculorhinitis and bronchial asthma in susceptible subjects. The oculorhinitis involves nasal mucosa and ocular conjunctiva, and is characterized by the onset of disorders such as ocular and nasal itching, photophobia, intense lacrimation, redness and watery rhinorrhea.

In North America ragweed is the main cause of allergy, affecting over 20% of the population (Katz and Carey, 2014).

In Europe, the nation with the higher incidence of pollinosis to ragweed is Hungary (>50% of the population, Bullock *et al*., 2010).

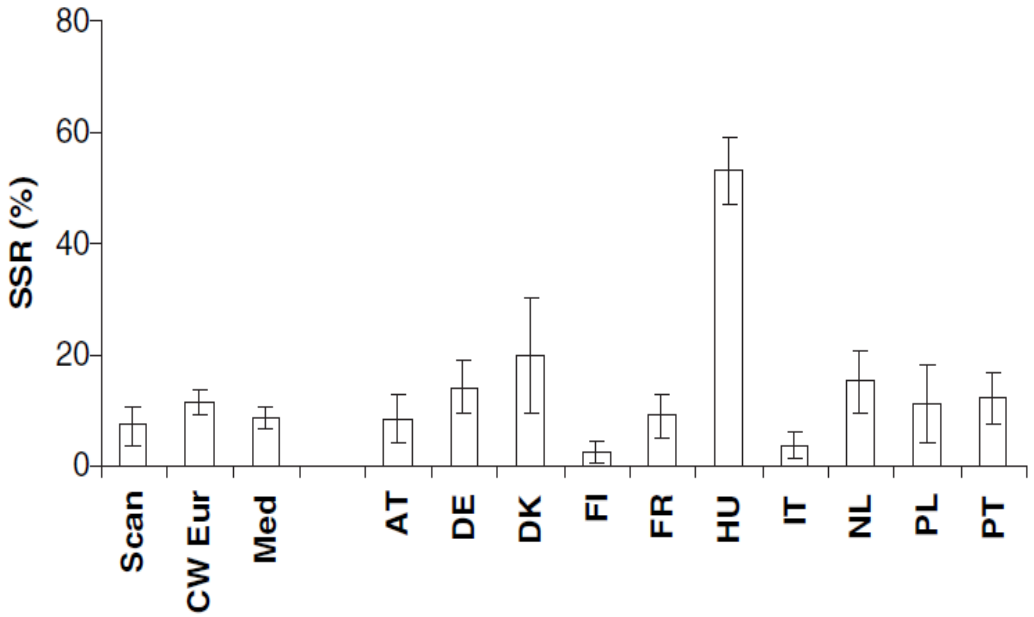

Fig. 6 - Standardized sensitization rates for Ambrosia (SSR, n = 2026) by region [Scandinavia (Scan), Central/Western Europe (CW Eur), Mediterranean (Med)] and country. The following countries were subsumed in regions: Denmark (DK) and Finland (FI): Scandinavia; Austria (AT), Belgium, Germany (DE), Netherlands (NL) and Switzerland: Central and Western Europe; France

(FR), Greece (GR), Italy (IT), Portugal (PT): Mediterranean Countries. The Eastern European countries Poland (PL) and Hungary (HU) were analyzed separately. A total of 95% confidence intervals are depicted as error bars (Burbach *et al*., 2009).

This plant, 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.).

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*. (2010) 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 (Fig. 7).

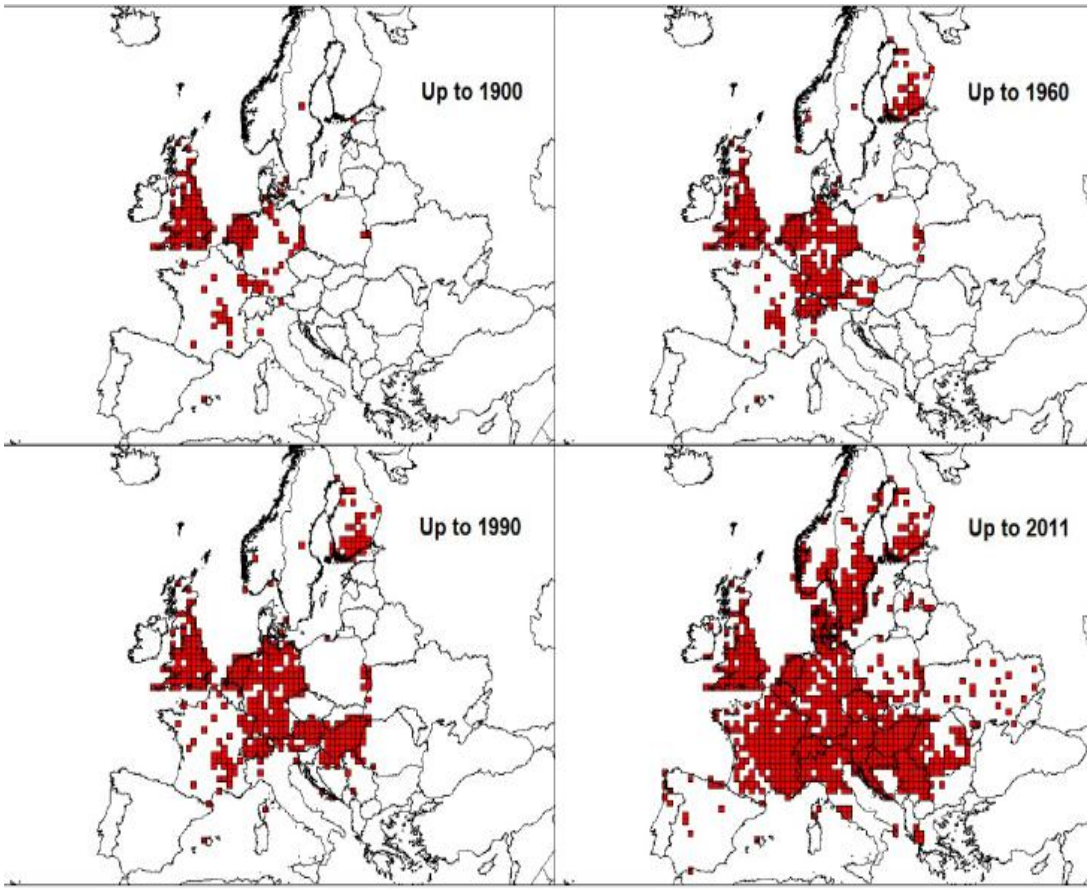

Fig. 7 - The temporal and spatial spread of *Ambrosia artemisiifolia* across Europe (from Bullock *et al*., 2010).

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.

3.1 Morfology

Ambrosia artemisiifolia (Fig. 8) is an herbaceous annual plant belonging to the family of *Asteraceae*; it mainly grows in arid and very bright environments, on gravelly, sandy, silico-argillaceous soils, with pH from 5 to 7 and with medium low humidity of soil. It is an opportunistic species that thrives successfully where the original vegetation has been removed. *A. artemisiifolia* generally grows up until 500 m altitude, but it was also found at 1834 m above sea level (Val Chisone, TO, Piedmont; see Bouvet *et al*., 2013).

Fig. 8 - Ragweed seedling.

Ambrosia artemisiifolia has an erect and robust stem, with dense hairiness, sometimes reddish, superiorly very branched, whose height ranges from 20 cm up to 2 meters (Dechamp and Meon, 2002). The leaves are pinnate, velvety, with a short petiole, and a uniform green on both pages, with similar appearance to mugwort's, wherewith it can be confused (hence the species' name). It is distinguishable from the latter for the most branched stem and less clear leaves underneath.

The root is weakly taprooting and very branched in the upper part (Ferrero and Maggiore, 2000). *A. artemisiifolia* is a monoecious species, indeed in the same plant both male and female flowers are present. The flowers are unisexual (male and female flowers are separated in different flower heads). The 8-15 cm long yellow-green male flower heads are gathered in racemes, in the terminal portion of the branches (Fig. 9-10).

Fig. 9 - Racemes in field plants (http://www.backyardnature.net/n/h/ragweed.htm).

Fig. 10 - Detail of male flower heads and male flower (http://drhuiallergist.com/allergy/allergens-and-avoidance/hay-fever-fall-weed-pollen-in-new-york/).

The seed, 3 mm of lenght, is fusiform with a variable number of spinule (4-8) inserted toward the apex. It spreads mainly through the movement of carry over soil, across the major roads (using vehicles and rainwater), runoff (especially in the hilly areas) and streams (Casarini, 2002).

In relation to temperature, *A. artemisiifolia* has different rates of germination. According to the experiments carried out by D. Brandes and J. Nitzsche (2006), the plant would have a wide range of germination temperature, between 7°C and 28°C and the first seedlings appear in April and May, depending on the temperature conditions (Ditommaso, 2004). Flowering is stimulated by less than 12 hours of solar illumination, it begins in late July and can last until the beginning of October, with a peak in August and September.

This period is characterized by a considerable production of pollen (Fig. 11), a plant can produce billions of small sized spherical pollen grains (20-30 µM) with short spines to be carried by the wind for more than 65 km (anemophilous pollination).

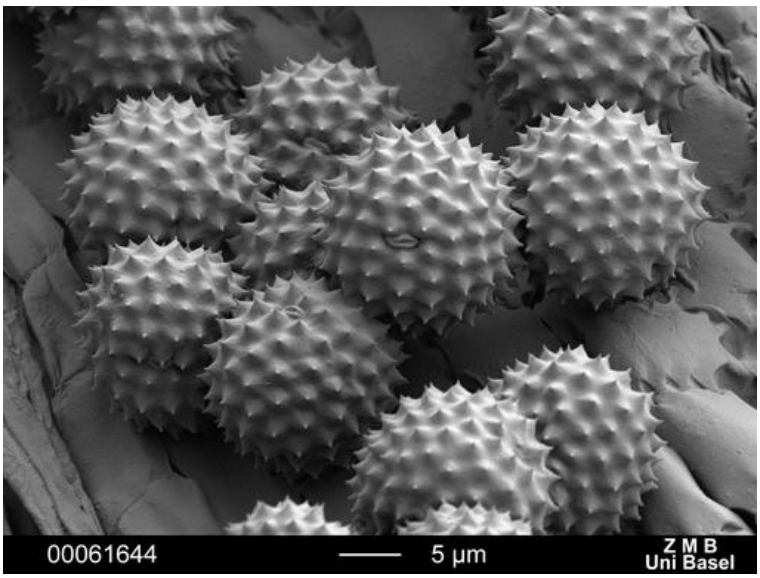

Fig. 11 - Transmission electron micrograph showing *Ambrosia artemisiifolia* pollen grains. (http://www.agrar.steiermark.at/cms/beitrag/11132767/98170585/).

Atmospheric concentrations of Ambrosia pollen are monitored across Europe (Fig. 12) by a network of sites using volumetric spore traps (Smith *et al*., 2013).

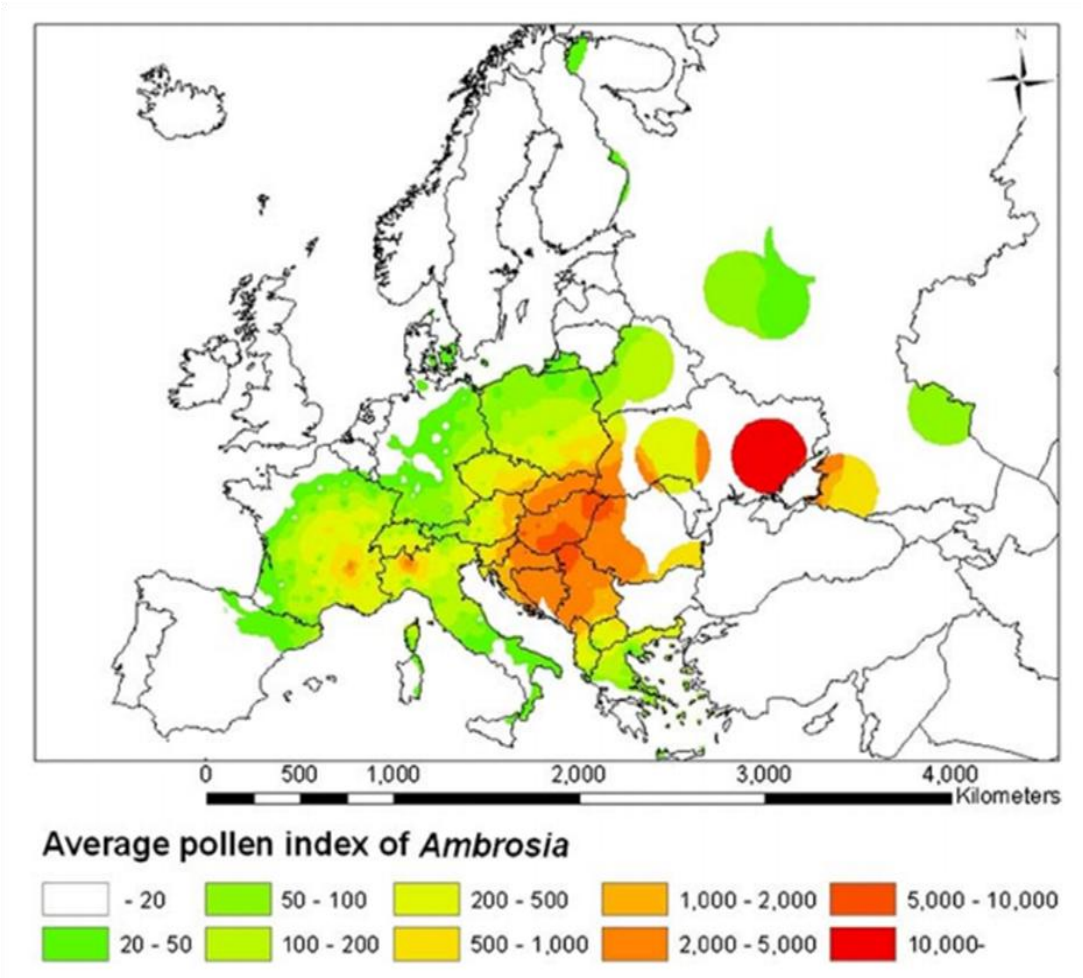

Fig. 12 - A spatial assessment of the density of naturalised Ambrosia populations with flowering potential. The map is based on the mean annual pollen index of Ambrosia from 368 stations in Europe, simple interpolation, buffer zones of 200 km and presence/absence information in Flora Europea. Taken from Skjøth *et al*. (2013).

As for the study of ragweed plant distribution, the ultimate goal of pollen monitoring is to predict ragweed expansion by developing models taking into account pollen concentrations and climate change (Smith *et al*., 2013).

In October the seed formation begins: on average a plant produces 3000 seeds (but it can reach up to 60000), which preserve their viability up to 40 years (Ciotti and Maspoli, 2005).

3.2 *Ambrosia artemisiifolia* allergens

In *Ambrosia artemisiifolia* L. pollen, ten allergenic molecules have been identified and listed in the official IUIS allergen database, to date (Amb a 1, 3, 4, 5, 6, 7, 8, 9, 10, 11. Tab. 2).

Tab. 2 - The table (from database ALLERGOME, IUIs, SDAP) reports the main features of each allergen: name, isoallergens and / or variants, biological function, molecular mass and theoretical isoelectric point (MW, pI), IgE antibody reactivity evaluated by RAST1 test, identification code of protein sequence in the UniProt (Universal Protein Resource) database.

Allergene	**Varianti**	**Mass (Da)**	**pI**	biological function	reactivity	Acc. N
Amb a 1	Amb a 1.0101	42,709	5.58	Pectate lyase	97%	P27759
	Amb a 1.0201	43,665	6.63			P27760
	Amb a 1.0202	43,637	6.63			E1XUL3
	Amb a 1.0301	42,928	5.72			P27761
	Amb a 1.0302	42,928	5.72			P27761 (variant L48Y)
	Amb a 1.0303	42,928	5.72			P27761 (variant H392R)
	Amb a 1.0304	42,913	5.79			E1XUL4
	Amb a 1.0305	42,963	5.79			E1XUL5
	Amb a 1.0401	42,843	5.61			P28744
	Amb a 1.0402	42,311	5.32			E1XUL9
	Amb a 1.0501	44,082	6.00			P27762
	Amb a 1.0502	44,042	5.79			E1XUM1
Amb a 3	Amb a 3.0101	11,375	6.11	Plastocyanin	51%	P00304
Amb a 4	Amb a 4.0101	15,518	4.88	Defensin-like protein		D4IHC0
Amb a 5	Amb a 5.0101	4,979	8.19		10-20 %	P02878
Amb a 6	Amb a 6.0101	12,789	8.93	Lipid transfer protein (LTP)	21%	O04004
Amb a 7	Amb a 7.0101	no seq		Plastocyanin	20%	
Amb a 8	Amb a 8.0101	14,245	4.79	Profilin	35%	Q2KN24
	Amb a 8.0102	14,1	4.88			Q2KN23
Amb a 9	Amb a 9.0101	9,311	4.17	Polcalcin	10-15 %	Q2KN27
	Amb a 9.0102	9,294	4.15			Q2KN26
Amb a 10	Amb a 10.0101	17,799	4.25	Polcalcin-like protein		Q2KN25
Amb a 11	Amb a 11.0101	43,157	6.43	Cysteine protease	60%	V5LU01

The *Ambrosia artemisiifolia* L. major allergen is Amb a 1, a polypeptide of about 40 kDa, belonging to the pectate lyase family; it has been localized in the subpollen particles of the cytosol (Bacsi *et al*., 2006) and at the level of the wall of the pollen grain, in particular in the layer of the intine cellulose and in the cavities of exine (Howlett *et al*., 1973).

Approximately 97% of allergic patients to pollen of *A. artemisiifolia* has IgE antibodies directed against Amb a 1 (King *et al*., 1964; Gadermaier *et al*., 2008). In these subjects, the percentage of specific IgE for this allergen is 13% of total IgE (Wopfner *et al*., 2005).

Several studies have led to the recognition of five isoallergens (Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5) characterized by an homology in their sequence greater than 87%, showing that Amb a 1 is a family of proteins (Rafnar *et al*., 1991).

Based on IgE binding studies performed in the 1970s, Amb a 3 was classified as a minor allergen with a sensitization prevalence of 30–50% (Adolphson *et al*., 1978). Amb a 3 is a 11 kDa glycoprotein belonging to the plastocyanin protein family which are copper-containing plant proteins involved in electron transport. Another allergenic molecule with similarity to plastocyanins was identified in ragweed pollen and termed Amb a 7, with a sensitization prevalence of 15-20%. Similar (89% sequence similatity) to Art v 4 is instead Amb a 8 a protein belonging to the profilin family.

Regarding Amb a 4, sensitization frequencies to this protein differ among Austrian (39%) and Italian and Canadian (20%) patients. Amb a 4 is very similar to Art v 1, the major allergen of *Artemisia vulgaris*.

Among minor allergens there are Amb a 5 (5 kDa, 10-15% of allergic patients recognize this protein), Amb a 6 (10 kDa, belonging to the family of LTP with a sensitization prevalence of 21% among ragweed sensitized patients; Roebber *et al*., 1983), Amb a 9 (9 kDa) and 10 (17 kDa). The latter two protein are

panallergens belonging to cystathionine beta synthase family proteins recognized by 10 to 15% of ragweed pollen-allergic individuals (Gadermaier *et al*., 2014).
Amb a 11 is a novel allergen identified by Bouley and collaborators in 2014, is a cysteine protease with a mass of 37 kDa (Bouley *et al.,* 2015).

4. Aim of the thesis

In a context of climate changing, it is very important to understand what factors the allergenicity of pollen depends on. Specifically it should be very interesting to define if allergenicity is dependent on environmental conditions, acting as non-meiotically heritable factors, or genetic/epigenetic heritable elements. Pollen allergenicity is mainly caused by the presence of major allergens. Amb a 1 family proteins are usually accounted as the major allergens released from pollen of ragweed plants.
The objectives of this thesis were to study the allergenicity variation among pollen samples from different ragweed plants/populations and to identify the mechanisms and factors contributing to the allergenicity by investigating the IgE-immunoreactivity and the intra and inter-population variability of Amb a 1 isoforms. To this aim, seeds from ragweed Canadian, French and Italian populations, were collected and used to grow plants both "in standard natural conditions", where temperature (T), relative humidity (RH) and light (L) changed during plant development, and in "controlled conditions" where environmental parameters (T, RH, L) were maintained constant over the plant life cycle. The two applied growth conditions allowed the understanding of the relative contribution of heritable and non-meiotically heritable factors (environmental factors) in determining pollen allergenicity. The application of a proteomic approach for the analysis of Amb a 1 isoforms was instead important to investigate the qualitative and quantitative variations of these allergenic proteins and thus to understand the

mechanism through which the genetic and environmental factors govern pollen allergenicity.

Before the experiment, ragweed plants from Canada, France and Italy, were first studied and genetically characterized to define the origin of Italian populations and to confirm that the genetic variability was very high within population but not significant among populations (manuscript n. 1).

5. Results and discussion

5.1 Genetic characterization of the ragweed populations selected for this study

A detailed knowledge of the population genetics of ragweed is needed to correctly explain the results of allergenicity analysis made on the pollen of this species. While many studies about population genetic have been conducted in Canada and in France (Genton *et al*., 2005) till today, no data regarding Italian ragweed populations are available in literature yet. The aim of this first part of the research was to acquire such data, focusing on the origin of Italian populations and confirming that genetic variability is higher inside each population than among the all of them. Results of the experiments carried out in this first part of the research are reported in the manuscript below.

Manuscript n. 1: submitted (2015)

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

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Running head : Invasion of *Ambrosia artemisiifolia* in Italy

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 193 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.

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 use herbarium records 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 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.

Material and Methods

Population sampling

Seeds from 18 different *A. artemisiifolia* populations were collected from: 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 (Table 1). 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.

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

Microsatellite analysis

Molecular markers and specifically 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.

Genetic data analysis

The proportion of polymorphic loci (P), the number of alleles (observed and effective; Na and Ne), the Shannon’s index (I), the mean number of rare allele per

locus (Ra), the observed heterozygosity (H_O), the expected heterozygosity (H_E) across the populations and the gene flow estimate ($N_m = 1\text{-}F_{ST}/(4F_{ST})$) 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).

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.

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; N_e= effective number of alleles; Ra = Mean number of rare allele per locus. I= Shannon information index; 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; N_m = gene flow.

Pop	%P	N_a	N_e	Ra	I	H_O	H_E	F_{IS}	HW	n_{ds}	F_{ST}	N_m
CAN1	100.00	6.333	4.329	2.932	1.466	0.833	0.662	-0.261	*	2		
CAN2	100.00	6.667	5.715	3.196	1.598	0.611	0.704	0.211	**	4		
CAN3	100.00	5.667	4.545	3.005	1.503	0.667	0.713	0.053	*	4		
CAN4	66.67	4.667	3.270	2.195	1.098	0.444	0.505	0.113	**	4		
CAN5	100.00	6.000	4.640	2.801	1.401	0.667	0.611	-0.093	*	4		
Mean CAN	*93.33*	*5.867*	*4.500*	*2.826*	*1.413*	*0.644*	*0.639*	*0.029*				
Tot CAN											0.096	2.393

FRA1		100.00	7.667	6.256	2.901	1.693	0.667	0.706	0.007	***	4		
FRA2		100.00	7.667	5.123	3.183	1.628	0.556	0.698	0.167	**	6		
FRA3		100.00	6.667	4.793	3.005	1.468	0.611	0.616	-0.022	**	4		
FRA4		100.00	5.667	3.986	2.195	1.376	0.556	0.630	0.060	***	4		
FRA5		100.00	5.000	3.382	2.801	1.215	0.722	0.568	-0.223	***	2		
	Mean FRA	100.00	6.533	4.708	*2.817*	1.476	0.622	0.643	-0.002				
	Tot FRA											0.072	3.817
IT_P		100.00	4.667	3.336	2.938	1.169	0.411	0.556	0.154	*	4		
IT_MM		100.00	5.000	3.915	2.797	1.399	0.767	0.693	-0.097	*	4		
IT_BR		100.00	6.000	4.131	3.001	1.501	0.778	0.699	-0.148	**	4		
IT_MI		100.00	5.333	3.700	2.667	1.334	0.589	0.626	0.018	*	4		
IT_L		100.00	6.000	4.575	2.801	1.401	0.556	0.611	0.042	**	4		
IT_TO		100.00	8.333	6.072	3.750	1.870	0.861	0.804	-0.089	***	6		
IT_TV		100.00	7.000	5.805	3.535	1.735	0.972	0.782	-0.278	***	5		
IT_PU		100.00	5.667	4.257	3.015	1.493	0.944	0.727	-0.315	**	4		
	Mean IT	100.00	6.000	4.474	3.073	1.488	0.735	0.687	-0.089				
	Tot IT											*0.123*	*1.809*
Total	**Mean**	98.15	6.111	4.546		1.464	0.678	0.662	-0.042			0.120	1.844
	SE	1.85	0.262	0.237		0.063	0.027	0.024	0.022			0.003	

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 (starting from version 2.2); a recessive alleles model dealing with genotypic ambiguity associated with dominant markers was implemented using 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. 1 A, B).

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).

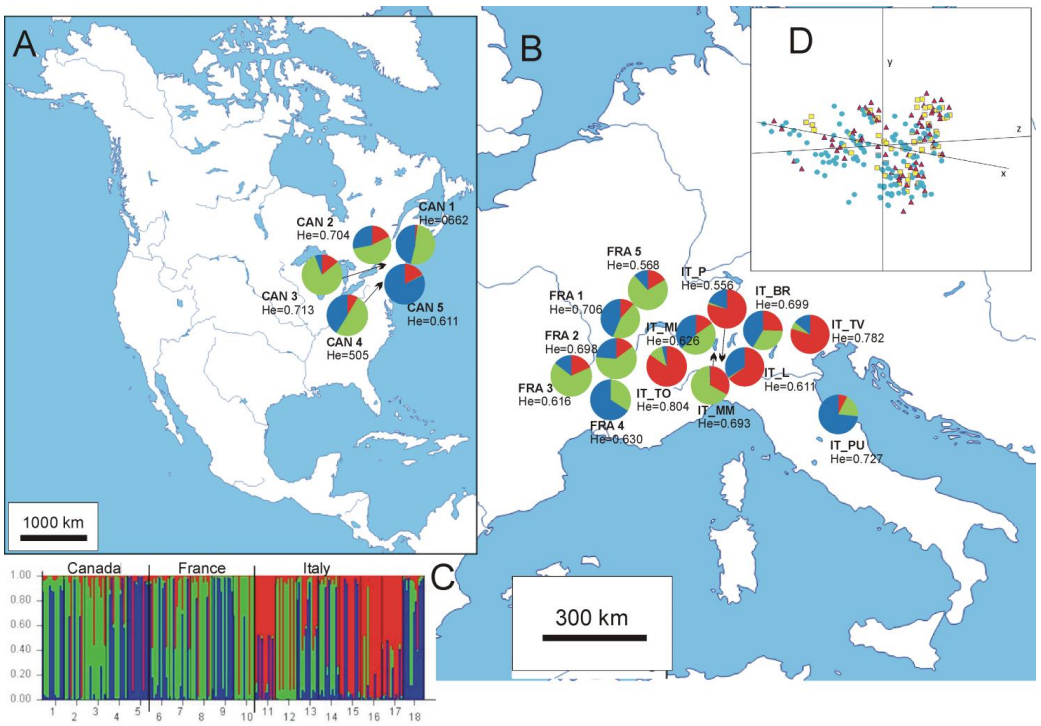


Fig. 1. A/B) Spatial genetic structure and population clusters (K=3) of *A. artemisiifolia* are inferred by the Bayesian cluster method implemented in STRUCTURE. At each location, pie charts in the maps indicate the mean proportion of membership of individuals at each location for the K = 3 genetic groups. 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).

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

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. 2 A). 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. 3 A-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. 2 B). We then displayed jitter and box plots (Fig. 3 C). Differences in the mean age of specimens between geographical areas were tested with the Kruskal-Wallis test.

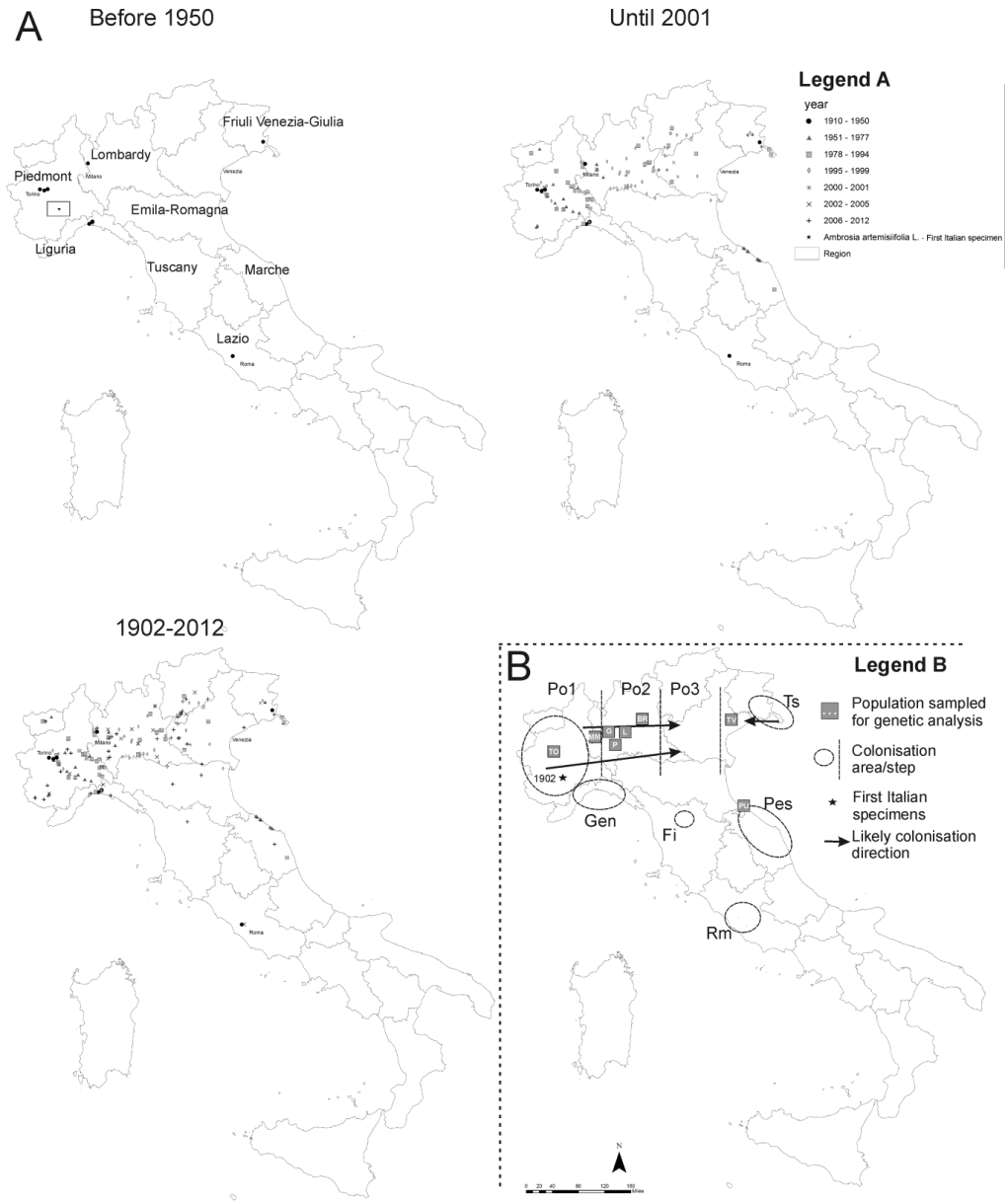
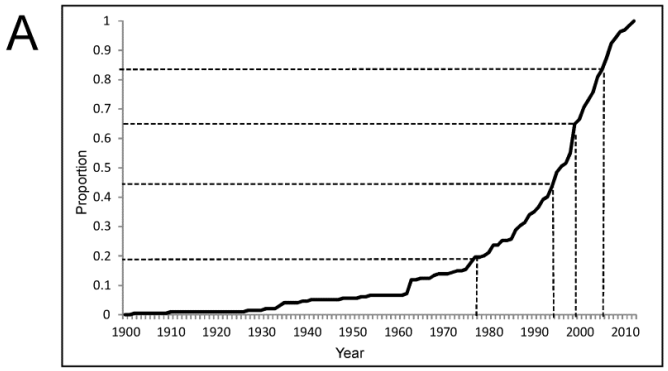


Fig. 2. A) Italian distributions of the *A. artemisiifolia* herbarium specimens that were collected before 1950, between 1951 and 2001, between 1902 and 2012. In the first illustration of Italy the

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).


A
Proportion
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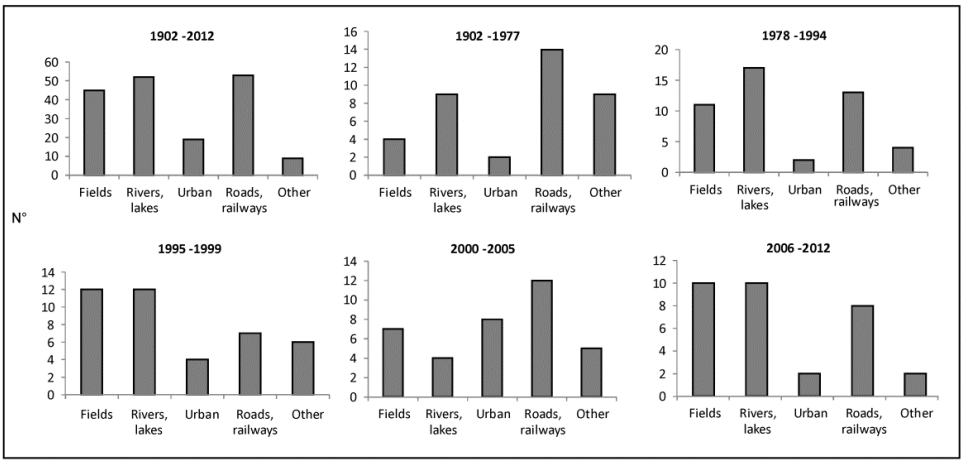
1900 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000 2010


B
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1902 -2012
1902 -1977
1978 -1994
1995 -1999
2000 -2005
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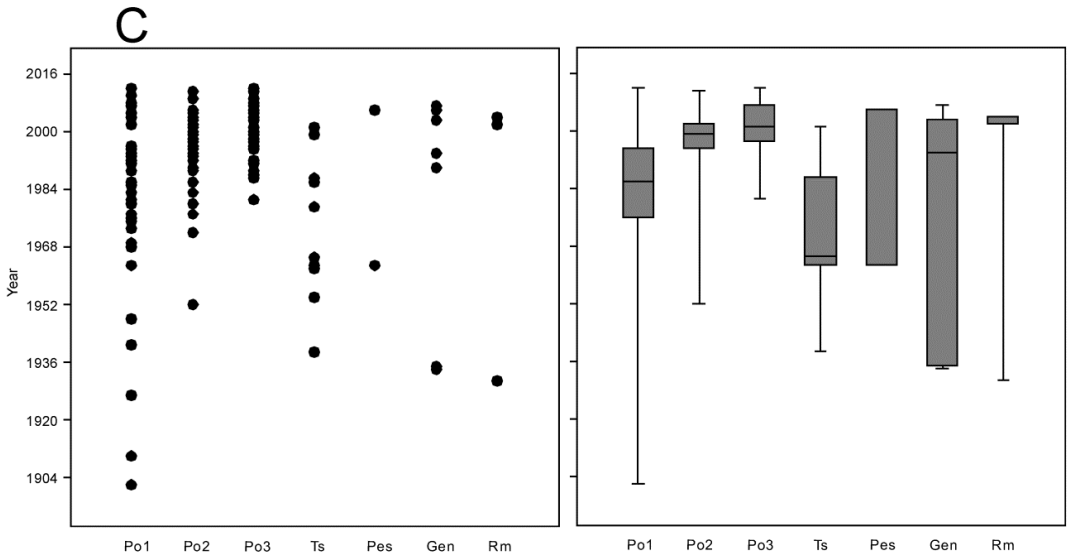
Fields
Rivers, lakes
Urban
Roads, railways
Other


C
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2016
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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).

Results

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. 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 effective number of alleles (N_e) varied from 3.270 CAN4 to 6.072 and 6.256 in IT_TO and FRA1, respectively. The Ra and I values were higher 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 low 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), even if IT populations accounted for the highest value. The overall gene flow (N_m) estimated from the mean F_{ST} value of each SSR primer was 1.844. 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.

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. 1 A, 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. 1 A, 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) French populations exhibit a more homogeneous pattern of membership coefficients to the three STRUCTURE

clusters than do Italian populations; b) French and Italian populations exhibit a degree of differentiation in the allelic pattern (light green and brown colours in the pie are scarcely represented in the French populations, which exhibit higher frequency of the violet colour); and c) as a general rule, the Italian populations exhibit a non-homogeneous allelic patterns.

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).

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 specimen examination, only 193 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. 3 A). 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. 2 A). The plant then spread toward the east, down the Po Valley (Fig. 2 B). However, in both the most eastern and the southern ranges of the plant's Italian distribution, distinct events of colonisation probably occurred close to a) Trieste (Friuli Venezia-Giulia

region), b) Pesaro (Marche region) and c) Rome (Lazio region). More recent colonisation events have occurred in Tuscany (close to Florence).

In Fig. 3 C, the jitter plot and box plots show the different temporal patterns of *A. artemisiifolia* specimens over the main geographic areas of species colonisation. The median age of specimens collected in the different geographic areas was statistically different according to the Kruskal-Wallis test (Supplementary Table ST3). In particular, along the Po plain, from west to east, the specimens were progressively more recent.

The finding of new specimens of this considered invasive species was infrequent until the '70s; the frequency of specimen collection then greatly increased during the '80s and '90s and continues at present. The sampling location of *A. artemisiifolia* specimens is related to the presence of roads and water networks, as well field areas (Fig. 3 B). In particular, *A. artemisiifolia* seems to use both indiscriminately, even if over time, based on the a priori temporal patterns (each comprising about 20% of the specimens), a closer relation to a certain habitat has occurred.

For the whole period of observations (1902–2012), the majority of specimens have been collected along roads/railways (29.8%), rivers/lakes (29.2%) and fields (25.2%). Habitat frequency comparisons between the whole period of observations (1902–2012) and temporal steps of invasion, individuated through the cumulative distribution of the number of herbarium specimens over time (Fig. 3 A-B), revealed the following significant differences: a) in the 1902–1977 period, Other habitats frequency is higher and Field habitat frequency is lower than during the 1902–2012 period (Df=4; Chi-square=17.83; $p<0.01$); b) in the 2000–2005 period, river and lake habitats exhibited lower habitat frequency than in the 1902–2012 period (Df=4; Chi-square=11.03; $p=0.026$).

Genetic variability and invasion time

The correlations according to the Kendall tau coefficient between time of residence of the 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.

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. These results 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). 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 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*., 2008). Our results confirm a strong propensity of *A. artemisiifolia* to be a generalist species, based on the analysis of its habitat spectrum through herbarium data.

In keeping with the genetic observations, the results from herbarium data suggest distinct introduction events, which have occurred in at least five different geographical areas: a) first in the western Po plain (specimen dated in 1902; Piedmont region) with a very likely expansion toward the east; b) then in the Rome (Lazio region), Genoa (Liguria region) and Trieste (Friuli-Venezia Giulia region) areas, with specimens from 1934 to 1939; more recently at Pesaro (Marche region), with specimens in 1963. Interestingly, these areas correspond to the most developed regions of Italy from a *commercial and industrial point of view:* Turin (and its surrounding) was one of the first industrial cities in Italy while Genoa, Pesaro and Trieste are important seaports. These areas have probably functioned as important introduction pathways for contaminated seeds of cereals and other agricultural crops from Canada and the USA into Europe (Buttenschøn *et al*., 2009).

The occurrence of *A. artemisiifolia* is sporadic and/or ephemeral toward the central and southern regions of Italy in the Mediterranean region (e.g. the city of Rome). Cunze *et al*. (2013), in their ecological niche modelling of *A. artemisiifolia* in Europe, hypothesised that "a possible reason for *Ambrosia'*s absence in the Mediterranean region, despite of predicted habitat suitability, may be that North American populations adapted to Mediterranean climatic conditions did not reach the adventive range (Europe) yet". In our opinion, the explanation lies in the differences at the ecosystem level, in the competition of better adapted species (Kueffer *et al*., 2013) and in bioclimatic filters (Haider *et al*., 2010). On the other hand, the general trend of increased "genetic potential" (in terms of H_O and H_E) that we found in populations with a higher residence time suggests a future increase in the ability of a population to adapt to new environmental characteristics.

Our findings, both from genetic analyses and herbarium data, are consistent with those of Genton *et al*. (2005); Gaudeul *et al*. (2011) and Gladieux *et al*. (2011) in that their genetic studies of *A. 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.

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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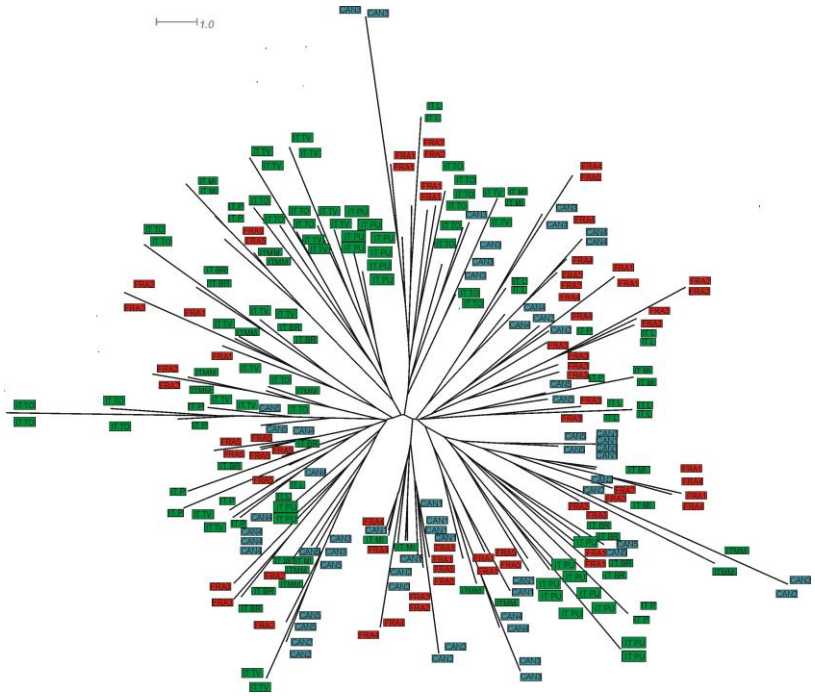
Supplementary materials



Suppl. Fig. S1: Split tree.

Table ST1: primers that were used

Locus	GenBank Accession n.	Primer sequences (5‘ - 3') (F: forward; R: reverse)	Size (bp)
Ambart 4	**FJ595149**	F:(*)CATACTAGTTTATACCCCGAGAGC R: CCGCGTGATTGAGTTTATGA	106 bp
Ambart 6	**FJ595150**	F:(*)CGCAATGTTATAATGCCTCCA R:TTCCGATGTTTCCATTCTGC	184 bp
Ambart 9	**FJ595151**	F:(*)CCCCTCGATTCCATATTGTC R:ATGACATCAAACGCCATCTG	296 bp
Ambart 18	**FJ595153**	F:(*)GATGTGGTGGTGGAGCTTTT R: TCATATGGACCCCATAGAAAGG	235 bp
Ambart 24	**FJ595155**	F:(*) CTGCCACCTCACGTTAAGTCT R: CGCCATAGCATTCCTTCAGT	159 bp
Ambart 27	**FJ595156**	F:(*) TCAGACACATATTCTCTTCTTCCTC R: TCCAACATCATAGCCATCAAA	194 bp

Table ST2: Italian Herbaria that were consulted in this study.

N° ID	**Herbarium**	**Adress**	**Answer**
1	Erbario Pirajno di Mandralisca; Museo Mandralisca	Via Mandralisca 13 90015 Cefalù (PA)	No answer
2	Biblioteca Casanatense	Via S. Ignazio 52 00186 Roma	No answer
3	Real Collegio Carlo Alberto; Casa Religiosa dei Barnabiti	Via Real Collegio 30 10024 Moncalieri (TO)	No answer
4	Liceo Ginnasio “Don Bosco”	Via San Giovanni Bosco 12 17021 Alassio (SV)	No answer
5	Liceo Ginnasio “Tiziano”	Via Cavour 2 32100 Belluno	No answer
6	Liceo Scientifico “Guglielmo Marconi”	Viale XX Settembre 140 54033 Carrara	No specimen
7	Liceo Scientifico “P. Giovio”	Via Pasquale Paoli 28 22100 Como	No answer
8	Istituto Tecnico Agrario “G.B. Cerletti”	Via 28 Aprile 1945, 20 31015 Conegliano (TV)	No answer
9	Liceo Classico “N. Machiavelli”; Museo di Storia Naturale	Via degli Asili 35 55100 Lucca	No answer
10	Istituto Tecnico Agrario Statale “D. Anzilotti”	Viale Ricciano 5 51017 Pescia (PT)	No answer
11	Liceo “Don Bosco”	Viale Michelangelo Grigoletti 3 33170 Pordenone	No answer
12	Liceo Ginnasio “E. Q. Visconti”	Piazza del Collegio Romano 4 00186 Roma	No answer
13	Istituto di Istruzione Superiore “C. Cavour”	Corso Italia 42 13100 Vercelli	No answer
14	Istituto Tecnico Commerciale e Linguistico “G. Cesare”	Viale L. Einaudi 66 70125 Bari	No specimen
15	Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali (Di.S.Te.B.A.)	Strada Provinciale Lecce-Monteroni 73100 Lecce	No specimen

	Università degli Studi del Salento		
16	Dipartimento di Scienze Botaniche, Ecologiche e Geologiche Università degli Studi	Via Piandanna 4 07100 Sassari	No specimen
17	Museo di Storia Naturale “Don Pietro Calderini”	Via Pio Franzani 2 13019 Varallo Sesia (VC)	No specimen
18	Erbario Augugliaro; Biblioteca Fardelliana	Largo San Giacomo 18 91100 Trapani	No specimen
19	Erbario Amidei; Biblioteca Guarnacci	Via Don Minzoni 3 56048 Volterra (PI)	No specimen
20	Erbario “Flora Montis Oropae”; Centro Studi del Giardino Botanico di Oropa	Via Santuario 480 13900 OROPA (BI)	No specimen
21	Istituto per l’Ambiente Marino Costiero, C.N.R. – U.O.S. Taranto	Via Roma 3 74123 Taranto	No specimen
22	Erbario Cammelli; Biblioteca Nazionale Centrale	Piazza dei Cavalleggeri 1 50122 Firenze	No specimen
23	Biblioteca Estense Universitaria	Largo Porta Sant’Agostino 337 41121 Modena	No specimen
24	Biblioteca Nazionale “Vittorio Emanuele III”	Piazza del Plebiscito 80132 Napoli	No specimen
25	Erbario Fiorentini; Biblioteca Statale	Via Santa Maria Corteorlandini 12 55100 Lucca	No answer
26	Museo Civico “Giuseppe Scarabelli”	Via Sacchi 4 40026 Imola (BO)	No specimen
27	Museo di Storia Naturale della Calabria e Orto Botanico	Contrada Rocchi 87036 Rende (CS)	No specimen
28	Dipartimento di Bioscienze; Università degli Studi di Parma	Via Università 12 43121 Parma (PR)	No specimen
29	Orto Botanico; Università degli Studi di Modena e Reggio Emilia	Viale Caduti in Guerra 127 41121 Modena (MO)	No specimen
30	Hortus Botanicus Catinensis; Dipartimento di Botanica - Università di Catania	Via Antonino Longo 19 95125 Catania (CT)	No specimen
31	Museo Biblioteca “Clarence Bicknell”;	Via Romana 39 18012	No

	Istituto Internazionale di Studi Liguri	Bordighera (IM)	specimen
32	Dipartimento di Arboricoltura, Botanica e Patologia vegetale, Sezione Botanica, Facoltà di Agraria; Università degli Studi "Federico II"	Via Università 100 80055 Portici (NA)	SPECIMEN
33	Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici "Monticelli-Cascina Stella"	26012 Castelleone (CR)	SPECIMEN
34	Dipartimento di Scienze Ambientali "G. Sarfatti"; Museo Botanico, piano 1; Università degli Studi	Via Pier Andrea Mattioli 4 53100 Siena	SPECIMEN
35	Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini"	Via E. Barsanti 18 61121 Pesaro	SPECIMEN
36	Museo Friulano di Storia Naturale	Via Marangoni 39-41 33100 Udine	SPECIMEN
37	Museo di Storia Naturale ed Archeologia	Via Piave 51 31044 Montebelluna (TV)	SPECIMEN
38	Centro di Scienze Naturali	Via di Galceti 74 50047 Prato	SPECIMEN
39	Biblioteca Angelica	Piazza S. Agostino 8 00186 Roma	SPECIMEN
40	Museo Biblioteca Archivio – Sezione Naturalistica	Via Museo 12 36061 Bassano del Grappa (Vicenza)	SPECIMEN
41	Centro Interdipartimentale dell'Orto Botanico; Università degli Studi della Tuscia	Via S. Camillo de Lellis 01100 Viterbo	SPECIMEN
42	Giardini Botanici Hanbury; Università degli Studi di Genova	Corso Montecarlo 43 La Mortola 18039 Ventimiglia (IM)	SPECIMEN
43	Museo Civico di Rovereto	Borgo Santa Caterina 41 38068 Rovereto (TN)	SPECIMEN
44	Dipartimento di Scienze della Terra; Università di Modena e Reggio Emilia	Largo S. Eufemia 19 41100 Modena (MO)	SPECIMEN

	(Modena)		
45	Museo Civico di Storia Naturale di Verona; Sezione di Botanica	Lungadige Porta Vittoria 9 37129 Verona (VR)	SPECIMEN
46	Museo Civico di Storia Naturale di Piacenza; Sezione di Botanica	Via Scalabrini 107 29121 Piacenza (PC)	SPECIMEN
47	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica	Via Ozanam, 4 25121 Brescia (BS)	SPECIMEN
48	Civico Museo di Storia Naturale di Trieste; Sezione di Botanica	Via dei Tominz, 4 34139 Trieste (TS)	SPECIMEN
49	Orto Botanico ed Erbario; Università di Bologna	Via Irnerio 42 40126 Bologna	SPECIMEN
50	Orto Botanico ed Erbario; Università degli Studi di Ferrara	Via Savonarola 9 44121 Ferrara	SPECIMEN
51	Museo Regionale di Scienze Naturali	Loc. Tache 11010 Saint Pierre (AO)	SPECIMEN
52	Übersee-Museum Bremen	Bahnhofsplatz 13 28195 Bremen	SPECIMEN
53	Erbario del Dipartimento di Biologia Vegetale; Università di Torino (TO)	Viale P. A. Mattioli 25 10125 Torino (TO)	SPECIMEN
54	Museo Regionale di Scienze Naturali	via Giolitti 36 10125 Torino (TO)	SPECIMEN
55	Museo Botanico	Via Orto Botanico 15 35123 Padova (PD)	SPECIMEN
56	Museo Civico di Storia Naturale di Milano	C.so Venezia 55 20121 Milano (MI)	SPECIMEN
57	Erbario Lombardo; Università di Pavia	Strada Nuova 65 27100 Pavia (PV)	SPECIMEN
58	Università degli Studi di Pavia; Facoltà di Scienze Matematiche, Fisiche e Naturali	Viale Taramelli 24 27100 Pavia (PV)	SPECIMEN
59	Museo botanico pisano; Dipartimento di Biologia	Via Luca Ghini 5 56126 Pisa (PI)	SPECIMEN
60	Orto Botanico; Comune di Lucca	Via del Giardino Botanico 14 55100 Lucca	No answer
61	Museo di Scienze Naturali; Università	Piazza dei Costanti 62032	No

	degli Studi di Camerino; Convento San Domenico	Camerino (MC)	specimen
62	Museo di Storia Naturale di Venezia	S. Croce 1730 30135 Venezia	No answer
63	Museo Orto Botanico; Campus Universitario, Università degli Studi	Via E. Orabona 4 70126 Bari	No answer
64	Centro Ricerche Floristiche dell'Appennino; Parco Nazionale del Gran Sasso e Monti della Laga – Università di Camerino, San Colombo	Via Provinciale km 4,2, 67021 Barisciano (AQ)	No answer
65	Naturmuseum Südtirol / Museo di Scienze Naturali dell'Alto Adige	Bindergasse 1 / Via Bottai 1 39100 Bolzano/Bozen	No answer
66	Dipartimento di Scienze Botaniche e Orto Botanico; Università degli Studi	Viale Sant'Ignazio da Laconi 11-13 09123 Cagliari	No answer
67	Struttura Autonoma Responsabile di Ricerca e Formazione di Scienze Ambientali SARRF Scienze Ambientali Palazzo Castelli Università degli Studi	Via Pontoni 5 62032 Camerino (MC)	No answer
68	Dipartimento di Scienze e Tecnologia per l'Ambiente ed il Territorio (DISTAT); Università degli Studi del Molise	Viale Mazzini 8 86170 Isernia	No answer
69	Dipartimento di Scienze Ambientali; Università degli Studi	Via Vetoio, Località Coppito 67100 L'Aquila	No answer
70	Dipartimento di Scienze della Vita "Marcello Malpighi", Sez. di Botanica; Università degli Studi	Via Ferdinando Stagno d'Alcontres, 31 98166 Messina	No answer
71	Dipartimento delle Scienze Biologiche, Sezione di Biologia vegetale; Università degli Studi di Napoli Federico II	Via Foria 223 80139 Napoli	No answer
72	Dipartimento di Biologia Ambientale e Biodiversità; Università degli Studi	Via Lincoln 2 90123 Palermo	No answer
73	Dipartimento di Biologia applicata Sez. Biologia vegetale e Geobotanica;	Borgo XX Giugno 74 06121 Perugia	No answer

74	Università degli Studi Dipartimento di Biologia, Difesa e Biotecnologie Agroforestali, Laboratorio di Botanica Ambientale ed Applicata; Università degli Studi della Basilicata	Via Ateneo Lucano 10 85100 Potenza	No answer
75	Dipartimento di Biologia Ambientale; Sapienza Università di Roma	Piazzale Aldo Moro 5 00185 Roma	No answer
76	Museo Tridentino di Scienze Naturali	Via Calepina, 14 38122 Trento	No answer
77	Museo di Storia Naturale “Antonio Orsini”; Musei della Cartiera Papale	Corso Mazzini 39 63100 Ascoli Piceno	No answer
78	Consiglio per la Ricerca e la Sperimentazione in Agricoltura C.R.A.; Unità di Ricerca per le Produzioni Legnose Fuori Foresta - MPAF	Strada per Frassineto 35 15033 CASALE MONFERRATO (AL)	No answer
79	Museo Naturalistico F. Minà Palumbo	Via Roma 72 90013 Castelbuono (PA)	No answer
80	Musei Provinciali	Borgo Castello 13 34170 Gorizia	No answer
81	Associazione Antares	Via Ronchi 78 20025 Legnano (MI)	No answer
82	Museo di Storia Naturale del Mediterraneo	Via Roma 234 57100 Livorno	No answer
83	Accademia Valdarnese del Poggio	Via Poggio Bracciolini 38/40 52025 Montevarchi (AR)	No answer
84	Erbario Paolucci; Museo di Scienze Naturali “L. Paolucci”	Via del Monastero 8 60027 Offagna (AN)	No answer
85	Ente Autonomo Parco Nazionale d’Abruzzo, Lazio e Molise	Viale Santa Lucia 67032 Pescasseroli (AQ)	No answer
86	Erbario “Irma Pierpaoli”; Stazione di Biologia Marina	Via A. Vespucci 13/17 73010 Porto Cesareo (LE)	No answer
87	Museo dei Fossili e di Scienze Naturali	Via A.C. Nobili 11 63020 Smerillo (FM)	No answer

88	Orto Botanico; Università degli Studi “Carlo Bo”	Via Bramante 28 61029 Urbino (PU)	No answer
89	“Hortus Siccus Pisanus”; Biblioteca Comunale	Via del Tribunale 8 52043 Castiglion Fiorentino (AR)	No answer
90	Biblioteche Riunite Civica e Ursino Recupero	Via Biblioteca 13 95124 Catania	No answer
91	Erbari Senesi; Biblioteca Comunale Chelliana	Piazza Carlo Cavalieri 9 58100 Grosseto	No answer
92	Erbario Fiorentini; Biblioteca Statale Palatina; Palazzo Pilotta	Piazza della Pilotta 9/A 43121 Parma	No answer
93	Erbario della Cattedra Ambulante; Archivio di Stato; Palazzo Farnese	Piazza Cittadella 29 29121 Piacenza	No answer
94	Erbario Meynier; Biblioteca Civica “Camillo Alliaudi”	Via CesareBattisti 11 10064 Pinerolo (TO)	No answer
95	Biblioteca Comunale “Rilliana”	Castello dei Conti Guidi 52014 Poppi (AR)	No answer
96	Erbario Antoir; Biblioteca Comunale Foresiana	57037 Portoferraio (LI)	No answer
97	Erbario Fiorentini; Biblioteca Statale	Via Santa Maria Corteorlandini 12 55100 Lucca	No specimen
98	Biblioteca dell’Accademia Nazionale dei Lincei e Corsiniana	Via della Lungara 10 00165 Roma	No answer
99	Seminario Vescovile	Via Seminari 9 13900 Biella	No answer
100	Santuario della Verna	52010 Chiusi della Verna (AR)	No answer
101	Abbazia Benedettina di San Martino delle Scale	Piazza Platani 11 90100 San Martino alle Scale (PA)	No answer
102	Museo Missionario Cinese e di Storia Naturale	Via Roma 127 74028 Sava (TA)	No answer
103	Istituto Tecnico Commerciale e per Geometri “Vanvitelli Stracca”	Via U. Trevi 4 60131 Ancona	No answer

104	Liceo Ginnasio “G. e Q. Sella”	Via Addis Abeba 20 13900 Biella	No answer
105	Liceo Classico “Dante”	Via Puccinotti 55 50129 Firenze	No answer
106	Istituto Tecnico Commerciale e per Geometri “P. Cuppari”	Via La Malfa 26 60035 Jesi (AN)	No answer
107	Liceo Classico Scientifico “R. Bonghi”	Viale Ferrovia 19 71036 Lucera (FG)	No answer
108	Istituto Tecnico Agrario “G. Garibaldi”	C. da Lornano 62100 Macerata	No answer
109	Liceo Classico “Virgilio”	Via Ardigò 13 46100 Mantova	No answer
110	Liceo Classico Statale “U. Foscolo”	Via Defendente Sacchi 15 27100 Pavia	No answer
111	Liceo Scientifico “G. Alessi”	Via R. D’Andreotto 19 06124 Perugia	No answer
112	Liceo Scientifico “G. Marinelli”	Viale Leonardo da Vinci 4 33100 Udine	No answer
113	Liceo Classico “M. Foscarini”	Cannaregio 4942 30131 Venezia	No answer
114	Istituto Tecnico Agrario “G. Ferraris”	Piazza della Vittoria 3 13100 Vercelli	No answer
115	Università degli Studi di Genova; Dipartimento di Scienze della Terra dell'Ambiente e della Vita	Corso Europa 26 16132 Genova (GE)	SPECIMEN
116	Giardino Botanico - Provincia Regionale di Agrigento	Piazza Aldo Moro 1 92100 Agrigento (AG.)	No answer
117	Settore Protezione Civile Provincia di Brindisi	Via Nicola Brandi 16 (rione Casale) 72100 Brindisi (BR)	No specimen
118	SardoLog	Via Leonardo da Vinci 32 09013 Carbonia (CA)	No specimen
119	Associazione Turistica Proloco Iglesias	Via Crispi 13 (Chiostro di S. Francesco) 09016	No answer

		Iglesias (CI)	
120	Museo Civico di Storia Naturale di Morbegno	Via Cortivacci 2 23017 Morbegno (SO)	SPECIMEN
121	Dipartimento di Biologia - Universita' degli Studi di Trieste	Via L. Giorgieri 5-9-10 34127 Trieste	No answer
122	Dipartimento AGRARIA - Università Mediterranea Reggio Calabria	Località Feo di Vito snc 89122 Reggio Calabria (RC)	No answer
123	Dipartimento di Scienze Agrarie, Alimentari e Ambientali - Università Politecnica delle Marche	Via Brecce bianche 60131 Ancona (AN)	SPECIMEN
124	Musei Civici di Reggio Emilia	Via Palazzolo 2 42121 Reggio Emilia	No answer
125	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"	Via Vittorio Emanuele 19 12051 Alba (CN)	SPECIMEN
126	Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma	Piazzale Aldo Moro, 5 00185 Roma - I	SPECIMEN
127	Centro Educazione Ambientale "Ecomuseo dei Monti Climiti"	Piazza Filippo Crescimanno 1 96010 Melilli (SR)	No answer
128	Erbario Centrale Italiano - Museo di Storia Naturale di Firenze	Via Giorgio la Pira 4 50121 Firenze (FI)	SPECIMEN
129	Erbario e Museo di patologia vegetale	Via De Sanctis 86100 Campobasso (CB)	No answer
130	Museo di Scienze Naturali di Cesena	Piazzetta Pietro Zangheri 6 47521 Cesena (FC)	No answer
131	Civico Museo Insubrico di Storia Naturale	Via Martinelli Foscarini 6 21056 Induno Olona (VA)	No answer

	SPECIES	MUNICIPALITY	LOCALITY	HABITAT	DATE	LEGIT	DETERMINAVIT	HERBARIUM
1	A. artemisiifolia L.	Vinadio	Lungo la strada poco a valle del paese Vinadio, Valle Stura	Roads	07/09/2005	M. Pascale	M. Pascale	Museo Regionale di Scienze Naturali (Torino)
2	A. paniculata Michsc.	Alba	Rinvenuto tra i semi di Lespideza siebaldi provenienti dal Giappone e coltivato nel giardino della scuola di viticoltura di Alba (CN)	Other	xx/xx/1902	T. Ferraris	T. Ferraris	Museo Regionale di Scienze Naturali (Torino)
3	A. artemisiifolia L.	Aosta	Ad est del lago della Citta di Aosta	Rivers, lakes	11/08/1992	M. Desfayes		Museo Regionale di Scienze Naturali (Aosta)
4	A. artemisiifolia L.	Valdieri	Margine di strada a Valdieri verso Andonno, Valle del Gesso (CN)	Roads	18/08/2008	M. Pascale	M. Pascale	Museo Regionale di Scienze Naturali (Torino)
5	A. artemisiifolia L.	Castiglione Saluzzo	Lungo la strada per Busca a Castiglione Saluzzo (CN)	Roads	10/08/2005	M. Pascale	M. Pascale	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
6	A. artemisiifolia L.	Borgo San Dalmazzo	Verso Borgo San Dalmazzo, Cuneo (CN)	Other	17/10/1968	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
7	A. artemisiifolia L.	Rivoli Torinese	Giardino privato di Luigi Colla a Rivoli Torinese (TO)	Other	15/08/1927	L. Colla	Vignolo-Lutati	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
8	A. artemisiifolia L.	Cuneo	Piazzale della nuova stazione a Cuneo	Urban	15/09/1975	C. Baccalario	C. Baccalario	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
9	A. artemisiifolia L.	Cuneo	Rotonda per Centallo in fraz. Madonna dell'Olmo, Cuneo (CN)	Roads	16/08/2004	M. Pascale	M. Pascale	Museo Regionale di Scienze Naturali (Torino)
10	A. artemisiifolia L.	Doberdo	Attorno il lago di Doberdo, lungo la strada Jamiano-Doberdo	Roads	17/09/1965	C. Zirmik	C. Zirmik	Civico Museo di Storia Naturale di Trieste; Sezione di Botanica (Trieste)
11	A. artemisiifolia L.	Ceretta	Incolto presso fraz. S. Maurizio C.se, Ceretta (TO)	Fields	10/08/1995	D. Mangapelo	D. Mangapelo	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
12	A. artemisiifolia L.	Torino	Lungo C.so Siracusa, Torino (TO)	Roads	22/08/1969	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
13	A. artemisiifolia L.	Torino	Nei prati residui tra Lingotto e Mirafiori, Torino sud	Fields	25/09/1963	U. Tosco	U. Tosco	Museo Regionale di Scienze Naturali (Torino)
14	A. artemisiifolia L.	Torino	Incolti presso il Fiume Stura di Lanzo, tra Via Settimo e Corso G. Cesare, Torino	Fields	16/07/1989	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
15	A. artemisiifolia L.	Torino	Ex alveo fluviale sulla destra orografica tra la Parrocchia della Madonna del Pilone ed il ponte sul Po, Torino	Rivers, lakes	30/07/1948	Vignolo-Lutati	Vignolo-Lutati	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
16	A. artemisiifolia L.	Pecetto	A lato della strada a Pecetto (TO) verso Sanglio, al confine con Trofarello	Roads	17/08/1986	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
17	A. artemisiifolia L.	Settimo Torinese	Margine stradale pietroso a Settimo Torinese (TO)	Roads	19/08/1996	L. Maggiora	L. Maggiora	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
18	A. artemisiifolia L.	Santena	A lato della strada a Santena (TO) verso Villastellone	Roads	02/11/1981	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
19	A. artemisiifolia L.	Santena	Lungo la circonvallazione di Santena (TO)	Roads	07/08/1973	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
20	A. artemisiifolia L.	Ceresole d'Alba	Ceresole d'Alba, Peschiera Branchio (CN)	Other	16/07/2007	A. Pistarino, F. Rota	A. Pistarino, F. Rota	Museo Regionale di Scienze Naturali (Torino)
21	A. artemisiifolia L.	Pralormo	Lungo la S.S. 29 presso Pralormo (TO)	Roads	31/08/1994	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)

22	A. artemisiifolia L.	San Sebastiano da Po	Lungo il fiume Po, S. Sebastiano da Po (TO)	Rivers, lakes	16/08/1977	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
23	A. artemisiifolia L.	Cisterna d'Asti	Lungo la strada, nella periferia di Cisterna d'Asti (AT)	Roads	29/08/1977	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
24	A. artemisiifolia L.	Saluggia	Boscaglia lungo la strada per Torino a Saluggia (VC)	Roads	07/09/2010	A. Pistarino R.	A. Pistarinp	Museo Regionale di Scienze Naturali (Torino)
25	A. artemisiifolia L.	Azeglio	Rive del lago presso Torbiera Moregna, Azeglio (TO)	Rivers, lakes	01/10/1992	Camoletto, A. Pistarino	R. Camoletto, A. Pistarino	Museo Regionale di Scienze Naturali (Torino)
26	A. artemisiifolia L.	Viverone	Margine di campo presso il Lago di Viverone (BI)	Fields	15/08/1991	L. Guglielmetto	L. Guglielmetto	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
27	A. artemisiifolia L.	Verrua Savoia	Terreno incolto nelle cava di sabbia sulle colline torinesi, Verrua Savoia (TO)	Fields	25/06/2005	L. Gallo	L. Gallo	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
28	A. artemisiifolia L.	Asti	Piazzale presso il cimitero, Asti	Urban	02/08/1977	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
29	A. artemisiifolia L.	San Marzano Oliveto	Lungo la strada di circonvallazione di San Marzano Oliveto (AT)	Roads	11/10/1968	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
30	A. artemisiifolia L.	Trino	Scarpate lungo la strada in fraz. Robella, Trino (VC)	Roads	02/09/1985	G. Varalda	G. Varalda	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
31	A. artemisiifolia L.	Casal Monferrato	Riva del fiume Po, presso la diga di Casal Monferrato (AL)	Rivers, lakes	22/07/1986	G. Varalda	G. Varalda	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
32	A. artemisiifolia L.	Casal Monferrato	Casale Monferrato (AL)	Urban	18/09/2002			Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
33	A. artemisiifolia L.	Acqui Terme	Lungo il fiume Bormida, Acqui Terme (AL)	Rivers, lakes	27/08/1976	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
34	A. artemisiifolia L.	Pontestura	Campi e incolti a destra del Po, Pontestura, Basso Monferrato (AL)	Fields	20/08/1993	F. Picco	F. Picco	Museo Regionale di Scienze Naturali (Torino)
35	A. artemisiifolia L.	Occimiano	Occimiano (AL)	Urban	16/09/1981	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
36	A. artemisiifolia L.	Valmacca	Lungo il fiume Po, Valmacca (AL)	Rivers, lakes	28/08/1976	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
37	A. artemisiifolia L.	Cogoleto	Via al Piano, strada Sciarborasca-Lerca, Cogoleto (GE)	Roads	29/08/2007	Gabriele Galasso	Gabriele Galasso	Museo Civico di Storia Naturale (Milano)
38	A. artemisiifolia L.	Ovada	Greto del torrente Orba, Ovada (AL)	Rivers, lakes	27/08/1976	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
39	A. artemisiifolia L.	Garzaia di Valenza	Incolti aridi sabbiosi ai margini della strada presso Garzaia di Valenza, Valenza Po (AL)	Fields	16/09/1986	I. Ostellino	I. Ostellino, R. Camoletto	Museo Regionale di Scienze Naturali (Torino)
40	A. artemisiifolia L.	Oleggio	Greto del Ticino, Oleggio (NO)	Rivers, lakes	02/08/1980	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
41	A. artemisiifolia L.	Galliate	Parecchi esemplari poco prima del ponte sul Ticino a Galliate (NO)	Roads	01/09/1981	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
42	A. artemisiifolia L.	Samarate	Sterrato a margine di strada a Samarate (VA)	Roads	15/09/2008	A. Scoppola	A. Scoppola	Centro Interdipartimentale dell'Orto Botanico; Universita degli Studi della Tuscia (Viterbo)
43	A. elatior L.	Castano Primo	Strada per Vanzaghello, Castano Primo	Roads	01/08/1941	Stucchi	Stucchi	Museo Civico di Storia Naturale (Milano)

44	A. artemisiifolia L.	Alluvioni Cambio	Su ghiaie e sabbia molto grossolane sulla sponda destra del fiume Po, Alluvioni Cambio (AL)	Rivers, lakes	23/08/1983	F. Sartori; V.Terzo; F.Bracco	V. Terzo	Erbario Lombardo; Universita di Pavia (Pavia)
45	A. artemisiifolia L.	Pegli	Greto del Rio Varenna, Pegli (GE)	Rivers, lakes	30/07/1935	Vignolo-Lutati Fontana	Vignolo-Lutati Fontana	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
46	A. artemisiifolia L.	Tortona	Zona arida lungo lo Scrivia, Tortona (AL)	Rivers, lakes	08/09/1996	A. Bertoldi	A. Bertoldi	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
47	A. artemisiifolia L.	Tortona	Sponda destra dello Scrivia, Tortona (AL)	Rivers, lakes	28/05/1986	F. Sartori; V. Terzo	F. Sartori; V. Terzo	Erbario Lombardo; Universita di Pavia (Pavia)
48	A. artemisiifolia L.	Cassano Spinola	Lungo il torrente Scrivia, Cassano Spinola (AL)	Rivers, lakes	16/08/1989	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
49	A. artemisiifolia L.	Isola S. Antonio	Abbondantissima presso il ponte sul Po, Isola S. Antonio (AL)	Rivers, lakes	06/09/1989	Giacinto Abbà	Giacinto Abbà	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
50	A. artemisiifolia L.	Bolzaneto	Greto del Rio Secca, Bolzaneto (GE)	Rivers, lakes	30/07/1935	Vignolo-Lutati Fontana	Vignolo-Lutati Fontana	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
51	A. artemisiifolia L.	Bolzaneto	Individui molto invasivi e molto varie per dimensione sul greto e sponde del Rio Secca, Bolzaneto (GE)	Rivers, lakes	30/08/1934	Vignolo-Lutati	Vignolo-Lutati	Erbario del Dipartimento di Biologia Vegetale; Universita di Torino (Torino)
52	A. artemisiifolia L.	Vignole Borbera	Greto del Fiume Borbera in loc. Variano Superiore, Vignole Borbera (AL)	Rivers, lakes	06/09/1989	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
53	A. artemisiifolia L.	Volpedo	Greto del Curone Monleale, Volpedo (AL)	Rivers, lakes	30/09/1980	Giacinto Abbà	Giacinto Abbà	Museo Regionale di Scienze Naturali (Torino)
54	A. artemisiifolia L.	Cesate	Nei pressi del campo sportivo di Cesate (MI)	Fields	10/09/2003	A. Romano	A. Romano	Museo Civico di Storia Naturale (Milano)
55	A. artemisiifolia L.	Castelletto di Branduzzo	Campo incolto, su terreno alluvionale argilloso-limoso, presso cava Busche, Castelletto di Branduzzo (PV)	Fields	09/06/2004	Graziano Rossi; A. Mondoni; A. Morini	Nicola Ardenghi	Universita degli Studi di Pavia; Facolta di Scienze Matematiche, Fisiche e Naturali (Pavia)
56	A. artemisiifolia L.	Milano	Dietro al cimitero di Via Seguro, Milano (MI)	Urban	20/10/1996	Gabriele Galasso	Gabriele Galasso	Museo Civico di Storia Naturale (Milano)
57	A. artemisiifolia L.	Milano	Sul marciapiede di via Gozzoli, presso via Monegherio, Milano (MI)	Urban	09/09/1992	Gabriele Galasso	Gabriele Galasso	Museo Civico di Storia Naturale (Milano)
58	A. artemisiifolia L.	Carbonara al Ticino	Nei dintorni del ristorante Il Vigile, Carbonara al Ticino (PV)	Urban	22/06/2006	Graziano Rossi	Nicola Ardenghi	Universita degli Studi di Pavia; Facolta di Scienze Matematiche, Fisiche e Naturali (Pavia)
59	A. artemisiifolia L.	Castellazzo, Bollate	Ruderato di Castellazzo, Bollate (MI)	Other	01/09/1977	Enrico Banfi	Enrico Banfi	Museo Civico di Storia Naturale (Milano)
60	A. artemisiifolia L.	Castelletto di Branduzzo	Ex-cava su suolo argilloso-limoso presso Castelletto di Branduzzo - Bressana, Bottarone (PV)	Fields	01/09/2006	A. Morini	A. Morini	Universita degli Studi di Pavia; Facolta di Scienze Matematiche, Fisiche e Naturali (Pavia)
61	A. artemisiifolia L.	Milano	Fossato del lato nord-est del Castello Sforzesco di Milano (MI)	Fields	28/09/2011	Gabriele Galasso	Gabriele Galasso	Museo Civico di Storia Naturale (Milano)
62	A. elatior L.	Magenta	Strada di Magenta, Landriano (PV)	Roads	01/07/1952	R. Lessi		Museo Civico di Storia Naturale (Milano)

63	A. artemisiifolia L.	Cernusco sul Naviglio	Cascina Gaggiolo, Cernusco sul Naviglio (MI)	Fields	01/08/2005	Silva Argentiero	Silva Argentiero	Museo Civico di Storia Naturale (Milano)
64	A. artemisiifolia L.	Vimercate	Margine sud-ovest del parcheggio della piscina comunale, Vimercate (MI)	Urban	01/09/1995	Paolo Rovelli	Paolo Rovelli	Museo Civico di Storia Naturale (Milano)
65	A. artemisiifolia L.	Merlino	Alzaia in loc. Presa can. Vacchelli, Merlino (MI)	Rivers, lakes	20/09/2002			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
66	A. artemisiifolia L.	Castel San Giovanni	Golena del Po a Pievetta, Castel San Giovanni (PC); quadr. 0922-2	Rivers, lakes	15/06/1999	E. Romani	E. Romani	Museo Civico di Storia Naturale di Piacenza; Sezione di Botanica (Piacenza)
67	A. artemisiifolia L.	Rivolta d'Adda	Incolti presso Rivolta d'Adda (CR)	Fields	25/08/1999			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
68	A. artemisiifolia L.	Calendasco	Trebbia presso fraz. Malpaga, Calendasco (PC); quadr. 0923-2	Rivers, lakes	15/06/1999	E. Romani	E. Romani	Museo Civico di Storia Naturale di Piacenza; Sezione di Botanica (Piacenza)
69	A. artemisiifolia L.	Crema	Greto sabbioso del fiume in loc. palata S. Maria, Crema (CR)	Rivers, lakes	05/10/1995			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
70	A. artemisiifolia L.	Crema	Incolto arido presso il ponte ferroviario di Crema (CR)	Fields	26/08/1995			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
71	A. artemisiifolia L.	Crema	Incolto arido presso il ponte ferroviario di Crema (CR)	Fields	17/08/1995			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
72	A. artemisiifolia L.	Urgnano	Terrazzi aridi nel Parco del serio, in loc. la Basella, Urgnano (BG)	Fields	24/08/2002	Luca Gariboldi	Luca Gariboldi	Museo Civico di Storia Naturale (Milano)
73	A. artemisiifolia L.	Ricengo	Greto del fiume nei pressi di Ricengo (CR)	Rivers, lakes	22/08/1995	Franco Giordana	Franco Giordana	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
74	A. artemisiifolia L.	Genivolta	Spiaggione d'invaso presso il colatore del fiume Oglio, Genivolta (CR)	Rivers, lakes	08/07/2001			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
75	A. artemisiifolia L.	Castelnuovo Bocca d'Adda	Lungo il fiume presso Cascina Brevia, Castelnuovo Bocca d'Adda (LO)	Rivers, lakes	17/09/1986	R. Cavani; V.Terzo; R.Zucchetti	R. Cavani; V.Terzo; R.Zucchetti	Erbario Lombardo; Universita di Pavia (Pavia)
76	A. artemisiifolia L.	Genivolta	Incolto in loc. c.na Marisa, Genivolta (CR)	Fields	28/07/2002			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
77	A. artemisiifolia L.	Gandosso	Parcheggio in loc. Pologne, Gandosso (BG)	Urban	25/08/1999	E. Marchesi	E. Marchesi	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
78	A. artemisiifolia L.	Orzinuovi	Incolto nel centro abitato di Orzinuovi (BS)	Fields	12/09/1983	Eugenio Zanotti	Eugenio Zanotti	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
79	A. artemisiifolia L.	Monticelli d'Ongina	Argine in via Maginot, Monticelli d'Ongina (PC); quadr. 0825-4	Rivers, lakes	15/06/1999	E. Romani	E. Romani	Museo Civico di Storia Naturale di Piacenza; Sezione di Botanica (Piacenza)
80	A. artemisiifolia L.	Spinadesco	Su sabbie in loc. c.na S. Angelo, Spinadesco (CR)	Fields	19/06/1999			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
81	A. artemisiifolia L.	Spinadesco	Incolto su ghiaia a Spinadesco (CR)	Fields	04/08/1998			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
82	A. artemisiifolia L.	Spinadesco	Incolto arido a Spinadesco (CR)	Fields	11/07/1998			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici "Monticelli-Cascina Stella" (Castelleone)
83	A. artemisiifolia L.	Cremona	Massicciata della stazione ferroviaria di Cremona centro (CR)	Railways	30/08/1999			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)

84	A. artemisiifolia L.	Malagnino	Su terra di riporto in loc. Ca de' Marozzi, Malagnino (CR)	Other	28/05/1999			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
85	A. artemisiifolia L.	Rogno	Lungo le rive del fiume Oglio, Rogno (BG)	Rivers, lakes	23/08/1998	Enzo Bona	Enzo Bona	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
86	A. artemisiifolia L.	Darfo	Negli incolti lungo l'Oglio tra il Monticolo e Sacca di Esine, Darfo (BS)	Fields	12/09/1990	F. Tagliaferri	F. Tagliaferri	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
87	A. artemisiifolia L.	Nave	Bordo strada di via Campagnole, Nave (BS)	Roads	13/10/2003	B. Lanzini	Stefano Armiraglio	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
88	A. artemisiifolia L.	Volongo	Campo incolto a Volongo (CR)	Fields	29/07/1998			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
89	A. artemisiifolia L.	Volongo	Incolto su ghiaia a Volongo, rg. Picenarda (CR)	Fields	08/08/1998	F. Bonali	F. Bonali	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
90	A. artemisiifolia L.	Capo di Ponte	Una sola pianta nella discarica presso lo svincolo della superstrada di Capo di Ponte (BS)	Other	12/08/2000	Enzo Bona	Enzo Bona	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
91	A. artemisiifolia L.	Capo di Ponte	Una decina di esemplari presso lo svincolo della superstrada a nord-est del passaggio a livello di Capo di Ponte (BS)	Roads	22/09/2003	Enzo Bona	Enzo Bona	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
92	A. artemisiifolia L.	Nuvolera	Lungo la Statale a Nuvolera (BS)	Roads	31/08/1994	S. Danieli	S. Danieli	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
93	A. artemisiifolia L.	Nuvolento	Incolto su una scarpata presso Nuvolento (BS)	Fields	10/11/1993	G. Roncali		Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
94	A. artemisiifolia L.	Montichiari	Argini del fiume Chiese presso c.na Camere, Montichiari (BS)	Rivers, lakes	12/09/2009	Graziano Rossi	Graziano Rossi	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
95	A. artemisiifolia L.	San Giovanni in Croce	Macerie a S. Giovanni in Croce (CR)	Other	26/09/2004			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
96	A. artemisiifolia L.	Casalmaggiore	Su ghiaia a Casalmaggiore (CR)	Other	13/10/1999			Stazione Sperimentale di Ecologia applicata e Centro Studi naturalistici Monticelli-Cascina Stella (Castelleone)
97	A. artemisiifolia L.	Gavardo	Incolti tra Gavardo, Gazzolo e Limone (BS)	Fields	05/11/1997	F. Tagliaferri	F. Tagliaferri	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
98	A. artemisiifolia L.	Muscoline	Lungo la strada in loc. San Quirico, Muscoline (BS)	Roads	15/09/1988	F. Tagliaferri	F. Tagliaferri	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
99	A. artemisiifolia L.	Muscoline	Lungo la strada in loc. San Quirico, Muscoline (BS)	Roads	15/09/1988	S. Danieli	S. Danieli	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
100	A. artemisiifolia L.	Cunettone di Salo	Su suolo di riporto ricco di macerie a Cunettone di Salo (BS)	Other	01/09/1992	Riccardo Guarino	Riccardo Guarino	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
101	A. artemisiifolia L.	Pozzolengo	Campagna tra Pozzolengo e Vaccarolo (BS)	Fields	03/09/1991	C. Perlotti	C. Perlotti	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
102	A. artemisiifolia L.	Sirmione	Campo incolto in loc. Colombare, Sirmione (BS)	Fields	23/09/1998	F. Barluzzi	F. Barluzzi	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)

103	A. artemisiifolia L.	Castelnuovo del Garda	Massicciata nella stazione ferroviaria di Castelnuovo del Garda (VR), quadrante 0530/4	Railways	29/05/2001	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
104	A. artemisiifolia L.	Rivoli Veronese	Deposito sabbioso umido sulla sponda destra dell'Adige a sud di Canale, a Il Palazzo, comune di Rivoli Veronese, Valle dell'Adige (VR), quadrante 0430/2	Rivers, lakes	25/09/2006	Alessio Bertolli, Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
105	A. artemisiifolia L.	Ravazzone	Una colonia isolata di ca. 50 piante nel piazzale erboso ruderale a circa 800 m a sud-est di Ravazzone, presso i capannoni abbandonati tra l'Adige e il Canale Biffis (TN), quadrante 0131/2	Urban	15/08/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
106	A. artemisiifolia L.	Avioassa	Sponda sabbiosa destra del fiume Adige a nord-ovest di Borghetto (biotopo), comune di Avioassa, Valle dell'Adige (TN), quadrante 0231/4. Rara	Rivers, lakes	14/09/2006	Alessio Bertolli, Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
107	A. artemisiifolia L.	Borghetto	Una cospicua popolazione nell'incolto sabbioso sulla sponda sinistra dell'Adige a Borghetto, all'altezza della piazza, Valle dell'Adige (TN), quadrante 0331/2	Fields	25/09/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
108	A. artemisiifolia L.	Borghetto	Sulla riva del fiume a Borghetto presso l'Adige (TN), quadrante 0331/2	Rivers, lakes	14/07/1999	Francesco Festi	Francesco Festi	Museo Civico di Rovereto (Rovereto)
109	A. artemisiifolia L.	Brentonico	Alcuni esemplari nell'incolto erboso a bordo strada nella parte ovest della fraz. Fontechel di Brentonico (presso il Castelo Nero), Monte Baldo (TN), quadrante 0131/4	Fields	30/09/2007	Alessio Bertolli	Alessio Bertolli	Museo Civico di Rovereto (Rovereto)
110	A. artemisiifolia L.	Verona	Lungo la pista ciclopedonale a Santa Lucia (Verona)	Roads	03/08/2009			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
111	A. artemisiifolia L.	Verona	Cimitero di Santa Lucia a Verona	Urban	24/07/2005			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
112	A. artemisiifolia L.	Verona	Massicciata ferroviaria presso lo scalo ferroviario subito a sud della stazione di Porta Nuova di Verona (VR), quadrante 0531/4	Railways	10/10/2001	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
113	A. artemisiifolia L.	Verona	Scalo merci della stazione ferroviaria di Porta Nuova di Verona	Railways	10/05/2001			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)

114	A. artemisiifolia L.	Lavini di Marco	Alcune decine di esemplari sul ghiaino del piazzale ruderale del capannone appena a est della stazione ferroviaria di Mori, Lavini di Marco (TN), quadrante 0132/1	Railways	09/10/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
115	A. artemisiifolia L.	Rovereto	Incolto erboso nella zona industriale in via Caproni, presso il ponte sul Rio Coste, Rovereto (TN), quadrante 0132/1. Un grosso esemplare ancora sterile	Fields	13/09/2007	Claudio Raffaelli	Claudio Raffaelli	Museo Civico di Rovereto (Rovereto)
116	A. artemisiifolia L.	Lavini di Marco	Su materiale di riporto (ruderale) nella ex cava Lastiei, Lavini di Marco (TN), quadr. 0132/1	Other	17/07/1998	Francesco Festi	Francesco Festi	Museo Civico di Rovereto (Rovereto)
117	A. artemisiifolia L.	Rovereto	Lungo la S.S. 12 presso i laghetti di Marco a Rovereto (TN), quadrante 0132/1	Roads	13/09/1987	Francesco Festi	Francesco Festi	Museo Civico di Rovereto (Rovereto)
118	A. artemisiifolia L.	Rovereto	Lungo la S.S. 12 presso i laghetti di Marco a Rovereto (TN), quadrante 0132/1	Roads	17/09/1987	Giorgio Perazza	Giorgio Perazza	Museo Civico di Rovereto (Rovereto)
119	A. artemisiifolia L.	Rovereto	Incolto ruderale sulla destra idrografica del torrente Leno a est del campo sportivo delle Fucine, Rovereto (TN), quadrante 0132/1. Alcune piante	Fields	24/09/2009	Claudio Raffaelli	Claudio Raffaelli	Museo Civico di Rovereto (Rovereto)
120	A. artemisiifolia L.	Nomi	Una decina di esemplari a bordo strada sul cavalcavia autostradale di Nomi, Valle dell'Adige (TN), quad. 0032/3	Roads	26/08/2008	Claudio Raffaelli	Claudio Raffaelli	Museo Civico di Rovereto (Rovereto)
121	A. artemisiifolia L.	Roncafort	Piuttosto abbondante nella pista da motocross su terreno sabbioso a 1 Km a NW di Roncafort, tra l'Interporto Doganale e la ferrovia (TN); quadr. 9832/4.	Other	06/09/2000	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
122	A. artemisiifolia L.	Roncafort	Pista di motocross su terreno sabbioso in una zona umida tra l'Interporto Doganale e la ferrovia, Valle dell'Adige (TN), quadr. 9832/4	Other	24/07/2000	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
123	A. artemisiifolia L.	Roncafort	Piuttosto abbondante nella pista da motocross su terreno sabbioso a 1 Km a NW di Roncafort, tra l'Interporto Doganale e la ferrovia (TN); quad. 9832/4	Other	30/10/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)

124	A. artemisiifolia L.	Mezzocorona	Massicciata presso la stazione ferroviaria di Mezzocorona (Valle dell'Adige, TN), quadrante 9732/4	Railways	26/09/1992	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
125	A. artemisiifolia L.	Verona	Lungo l'Adige presso loc. Boschetto (Verona)	Rivers, lakes	22/08/1981			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
126	A. artemisiifolia L.	Verona	Lungo l'Adige in loc. Boschetto (Verona)	Rivers, lakes	18/09/1989			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
127	A. artemisiifolia L.	Badia Calavena	Incolto lungo la strada presso il Passo Spin del Potero, Badia Calavena (VR), quadrante 0432/2	Fields	02/09/2001	Francesco Festi	Francesco Festi	Museo Civico di Rovereto (Rovereto)
128	A. artemisiifolia L.	Terragnolo	Bordo strada sulla destra del torrente Leno presso il bacino sotto Geroli, Terragnolo (TN), quadrante 0133/1	Roads	07/08/2003	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
129	A. artemisiifolia L.	Albiano	Stazione di pasturazione dei caprioli nel bosco sopra loc. Mosin, comune di Albiano (TN), quadrante 9833/1	Fields	12/08/2009	Francesco Festi	Francesco Festi	Museo Civico di Rovereto (Rovereto)
130	A. artemisiifolia L.	Oppeano	Campagne di Oppeano (VR)	Fields	08/09/2012			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
131	A. artemisiifolia L.	Viarago	Su terra di riporto all'inizio della strada per loc. Pedoc, Viarago presso Pergine (TN), quadrante 9933/2	Roads	05/09/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
132	A. artemisiifolia L.	Verona	Lungo l'Adige in loc. Boschetto (Verona)	Rivers, lakes	17/10/2001			Museo Civico di Storia Naturale di Verona; Sezione di Botanica (Verona)
133	A. artemisiifolia L.	Castello-Molina di Fiemme	Bordo strada nel fondovalle sotto Molina, comune di Castello-Molina di Fiemme (TN), quadrante 9734/2	Roads	29/08/2004	Claudio Raffaelli	Filippo Prosser, Claudio Raffaelli	Museo Civico di Rovereto (Rovereto)
134	A. artemisiifolia L.	Strigno	Piante ancora sterili lungo il torrente Chieppena tra Strigno e la confluenza con il torrente Lusumina (Valsugana, TN); quadr. 9935/1	Rivers, lakes	06/07/1996	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
135	A. artemisiifolia L.	Strigno	Zona erbosa ghiaiosa nella stazione ferroviaria di Strigno, Valsugana (TN), quadrante 9935/1	Railways	05/08/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
136	A. artemisiifolia L.	Malalbergo	Incolto nella Valle La Comune, Malalbergo (FE)	Fields	10/08/2011			Orto Botanico ed Erbario; Universita degli Studi di Ferrara (Ferrara)
137	A. artemisiifolia L.	Ravalle	Isole sabbiose del fiume Po tra Ravalle e Pontelagoscuro (FE)	Rivers, lakes	14/08/2006			Orto Botanico ed Erbario; Universita degli Studi di Ferrara (Ferrara)
138	A. artemisiifolia L.	Trento	Greto umido su silice lungo il torrente Chieppena, quasi alla confluenza con il fiume Brenta, Valsugana (TN), quadrante 9935/3	Rivers, lakes	04/09/1999	Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
139	A. artemisiifolia L.	Imer	Abbondante tra la rotonda all'ingresso della galleria del Vanoi e il torrente Cismon, nel comune di Imer, Primiero (TN), quadrante	Roads	29/09/2011	Alessio Bertolli, Filippo Prosser,	Alessio Bertolli, Filippo Prosser	Museo Civico di Rovereto (Rovereto)

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140	A. artemisiifolia L.	Rosa	Lungo la strada a Rosa	Roads	15/09/1991	M. Zuin		Museo Botanico (Padova)
141	A. artemisiifolia L.	Battaglia Terme	Lungo la ferrovia a Battaglia Terme	Railways	17/09/1997	R. Marcucci	S. Chiesa	Museo Botanico (Padova)
142	A. artemisiifolia L.	Monfalcone	Monfalcone, Ponzano. Foglie e rami opposti anche nella parte alta del fusto	Other	09/10/1986	F. Martini		Museo Friulano di Storia Naturale (Udine)
143	A. artemisiifolia L.	Marzocca	Lungo la S.S. Adriatica tra Marzocca e Marina di Montemarciano	Roads	01/10/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
144	A. artemisiifolia L.	Marzocca	Luoghi erbosi, su suolo prevalentemente argilloso, lungo la S.S. Adriatica tra Marzocca e Marina di Montemarciano	Fields	02/09/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
145	A. artemisiifolia L.	Marzocca	Lungo la S.S. Adriatica entro l'abitato di Marzocca	Roads	01/10/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
146	A. artemisiifolia L.	Senigallia	Lungo la S.S. Adriatica tra Senigallia e Marzocca	Roads	01/10/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
147	A. artemisiifolia L.	Fano	Luoghi erbosi incolti, su suolo sabbioso-argilloso, lungo la S.S. Aditica tra Ponte Metauro e Torrette di Fano, nei dintorni di Fano	Fields	21/09/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
148	A. artemisiifolia L.	Fano	Lungo la S.S. Adriatica presso il ponte sull'Arzilla, Fano	Roads	03/10/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
149	A. artemisiifolia L.	Pesaro	Lungo la S.S. Adriatica tra Pesaro e il Fosso Sejore	Roads	09/09/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
150	A. artemisiifolia L.	Pesaro	Luoghi erbosi, su suolo sabbioso-molassico, lungo la S.S. Adriatica presso il Cavalcavia Sottomonte, Pesaro	Fields	20/09/1963	A. J. B. Brilli-Cattarini		Centro Ricerche Floristiche Marche "A.J.B. Brilli-Cattarini" (Pesaro)
151	A. artemisiifolia L.	Codigoro	Sentiero erboso, su suolo ricco di nitrati, presso l'ex-zuccherificio di Codigoro (FE)	Other	24/09/1999			Orto Botanico ed Erbario; Universita degli Studi di Ferrara (Ferrara)
152	A. artemisiifolia L.	Albisano	Un grosso esemplare su macerie presso un nuovo edificio nell'abitato di Garda, nella prima via a est della strada per Albisano (VR), quadrante 0430/1	Urban	17/08/2006	Alessio Bertolli, Filippo Prosser	Filippo Prosser	Museo Civico di Rovereto (Rovereto)
153	A. artemisiifolia L.	Trieste	Lungo vicolo degli Scaglioni, Trieste	Urban	21/09/2001	A. Tremul		Museo Friulano di Storia Naturale (Udine)

154	A. artemisiifolia L.	Trieste	Su terreno da calcare, tra Prosecco e Santa Croce, alla periferia di Trieste	Other	25/08/1979	Dino Marchetti	Dino Marchetti; E. Ferrarini	Dipartimento di Scienze Ambientali G. Sarfatti; Museo Botanico; Universita degli Studi (Siena)
155	A. artemisiifolia L.	Sgonico	Sul fondo di una dolina del Carso triestino presso Gabrivizza, Sgonico (TS)	Other	08/08/1987	F. Fenaroli	F. Fenaroli	Museo Civico di Storia Naturale di Brescia; Sezione di Botanica (Brescia)
156	A. artemisiifolia L.	Staranzano	Staranzano (Monfalcone)	Other	04/10/1954	C. Zirmik	C. Zirmik	Civico Museo di Storia Naturale di Trieste; Sezione di Botanica (Trieste)
157	A. artemisiifolia L.	Sagrado	Nelle vicinanze del serbatoio d'acqua a Monte San Michele (Sagrado)	Other	28/09/1939	C. Zirmik	C. Zirmik	Civico Museo di Storia Naturale di Trieste; Sezione di Botanica (Trieste)
158	A. artemisiifolia L.	Magredo	Lungo via della Viotta, Magredo (Udine)	Roads	06/09/2001	C. Putelli		Museo Friulano di Storia Naturale (Udine)
159	A. artemisiifolia L.	Lovaria	Pioppeto in una cava di ghiaia lungo l'argalfalla, Lovaria (Comune di Pradamano)	Other	07/07/1962	S. Iamerimi		Museo Friulano di Storia Naturale (Udine)
160	A. artemisiifolia L.	Udine	Largo Cappuccini a Udine (F04)	Roads	10/09/1999	Sergio Rizzardini	F. Martini	Museo Friulano di Storia Naturale (Udine)
161	A. artemisiifolia L.	Voltri	All'interramento per il nuovo porto a Voltri (GE)	Urban	25/08/1990	L Cornara, B. Burlando	L Cornara, B. Burlando	Erbario Genova
162	A. artemisiifolia L.	Recco	Vicino all'ex sbocco del Torrente Treccanega sulla spiaggia di Recco (GE)	Rivers, lakes	28/09/2006	A. Schiappacasse	A. Schiappacasse	Erbario S. Peccenini (Genova)
163	A. artemisiifolia L.	Busalla	Area di servizio di Busalla dell'Autostrada Milano-Genova (GE)	Roads	15/09/2003	Simonetta Peccenini	Simonetta Peccenini	Erbario S. Peccenini (Genova)
164	A. artemisiifolia L.	Vinadio	Lungo strada a Vinadio (CN)	Roads	12/08/2008	Simonetta Peccenini	Simonetta Peccenini	Erbario S. Peccenini (Genova)
165	A. artemisiifolia L.	Santo Stefano Belbo	Abbondante popolamento alla sinistra del Belbo, presso il ponte a Santo Stefano Belbo (CN)	Rivers, lakes	13/08/1976	Giacinto Abbà	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"
166	A. artemisiifolia L.	Santo Stefano Belbo	Alcuni esemplari in luogo incolto sul lato destro del Belbo a Santo Stefano Belbo (CN; primo rinvenimento per le Langhe, ma il campione precedente è anteriore di 5 anni)	Fields	17/07/1981	Giacinto Abbà	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"
167	A. artemisiifolia L.	Alba	Un esemplare presso il nuovo campo sportivo di Alba, ma la località non è ben chiara (CN)	Fields	31/08/1983	Giacinto Abbà	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"
168	A. artemisiifolia L.	Santo Stefano Belbo	Alcuni esemplari in un luogo incolto fra le case e l'argine del Belbo a Santo Stefano Belbo (CN)	Fields	21/09/1994	Giacinto Abbà	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"

169	A. artemisiifolia L.	Vaccheria	5 cespi nella scuola di agraria a Vaccheria (CN), sulla sinistra del Tanaro nella regione Mogliasso	Other	20/08/1995	Traversa Flavio	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"
170	A. artemisiifolia L.	Magliano Alfieri	Oltre 200 piante sui ghiaioni a Tanaro/Neive, presso Magliano Alfieri (CN)	Other	10/07/2004	Traversa Flavio	Giacinto Abbà	Museo Civico Archeologico e di Scienze naturali "Federico Eusebio"
171	A. artemisiifolia L.	Gualtieri	Presso il Fiume Po a Gualtieri (RE)	Rivers, lakes	15/08/2007			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
172	A. artemisiifolia L.	Villalunga	Presso il Fiume Secchia a Villalunga (RE)	Rivers, lakes	13/08/2007			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
173	A. artemisiifolia L.	Villalunga	Presso il Fiume Secchia a Villalunga (RE)	Rivers, lakes	09/08/2007			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
174	A. artemisiifolia L.	Gualtieri	Presso il Fiume Po a Gualtieri (RE)	Rivers, lakes	08/09/2007			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
175	A. artemisiifolia L.	Boretto	Presso il Fiume Po a Boretto (RE)	Rivers, lakes	08/09/2007			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
176	A. artemisiifolia L.	Boretto	Presso il Fiume Po a Boretto (RE)	Rivers, lakes	23/10/2004			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
177	A. artemisiifolia L.	Boretto	Presso il Fiume Po a Boretto (RE)	Rivers, lakes	15/10/1995			Orto Botanico; Università degli Studi di Modena e Reggio Emilia
178	A. artemisiifolia L.	Appignano	In vegetazione nitrofila in contrada Verdefiore a Appignano (MC)	Fields	03/09/2006	Fabio Taffetani	Fabio Taffetani	Dipartimento di Scienze Agrarie, Alimentari e Ambientali - Universit? Politecnica delle Marche
179	A. artemisiifolia L.	Campi Bisenzio	Area ruderale presso l'osservatorio sul nuovo lago Maccione nell'oasi WWF Stagni di Focognano a Campi Bisenzio (FI)	Fields	22/09/2012	L. Cecchi	L. Cecchi	Erbario Centrale Italiano - Museo di Storia Naturale di Firenze
180	A. artemisiifolia L.	Quart	Abbondante negli incolti presso la zona umida a sud della statale, a ovest di Villefranche a Quart (AO)	Fields	28/09/2012	M. Bovio, M. Broglio, G. Trompetto	M. Bovio, G. Trompetto	Erbario Centrale Italiano - Museo di Storia Naturale di Firenze
181	A. artemisiifolia L.	Varazze	Greto in un tratto di secondo ordine del Torrente Tairo a Varazze (SV)	Rivers, lakes	17/07/1994	F. V. Vignolo-Lutati		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
182	A. artemisiifolia L.	Varazze	Pochi ma grandi individui nel greto di un tratto di secondo ordine del Torrente Tairo a Varazze (SV)	Rivers, lakes	01/08/1994	F. V. Vignolo-Lutati		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
183	A. artemisiifolia L.	Varazze	Pochi ma grandi individui nel greto di un tratto di secondo ordine del Torrente Tairo a Varazze (SV)	Rivers, lakes	21/09/1994	F. V. Vignolo-Lutati		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
184	A. artemisiifolia L.	Bolzaneto	Una minuscola pianta crescente fra le scorie a Rio Sarca, poco oltre Bolzaneto (GE)	Other	17/06/1934			Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma

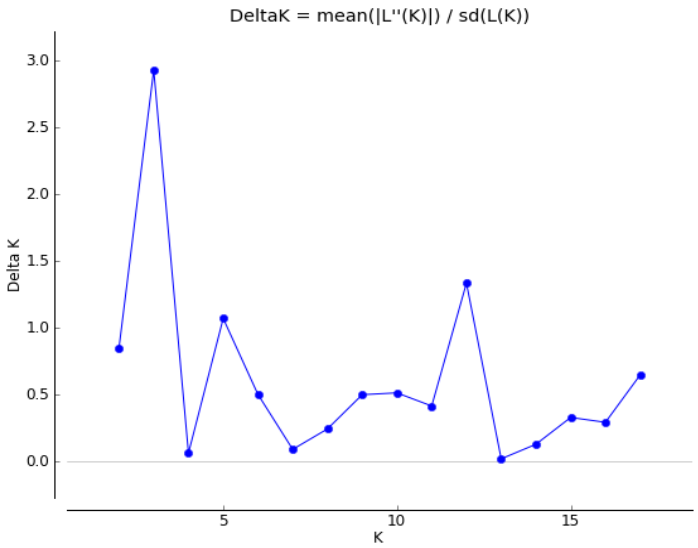
185	A. artemisiifolia L.	Bolzaneto	Piccolo individuo nel greto e dintorni del Rio Secca a Bolzaneto (GE)	Rivers, lakes	17/09/1994	F. V. Vignolo-Lutati		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
186	A. artemisiifolia	Roma	Piazzale Clodio a Roma (RM)	Urban	15/10/2002	Amalia Fezzi		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
187	A. artemisiifolia L.	Roma	Pochi individui su mucchi di macerie dei Prati Strozzi scaricate di fresco (circa un anno) presso la Siuliana, alle falde di Monte Mario, a Roma (RM)	Other	24/08/1931			Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
188	A. artemisiifolia L.	Roma	Spiazzo ruderale presso Piazzale Clodio a Roma (RM)	Urban	15/09/2004	B. Anzalone		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
189	A. artemisiifolia L.	Roma	Spiazzo ruderale presso Piazzale Clodio a Roma (RM)	Urban	15/09/2004	B. Anzalone		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
190	A. artemisiifolia L.	Roma	Spiazzo ruderale presso Piazzale Clodio a Roma (RM)	Urban	15/09/2004	B. Anzalone		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
191	A. artemisiifolia L.	Roma	Spiazzo ruderale presso Piazzale Clodio a Roma (RM)	Urban	15/09/2004	B. Anzalone		Museo Erbario, Dipartimento di Biologia Vegetale, Sapienza Università di Roma
192	A. marittima L.	Torino	Lungo i binari presso l'Ospedale Mauriziano a Torino (TO)	Railways	10/09/1910	L. Bassarino	L. Bassarino	Museo Regionale di Scienze Naturali (Torino)
193	A. maritima L.	Milano	Strada statale MI-GE, nella periferia di Milano (MI)	Roads	15/07/1972	G. Verri	G. Verri	Erbario Lombardo; UniversitÓ di Pavia (Pavia)
194	A. artemisiifolia L.	Gualtieri	Presso il Fiume Po a Gualtieri (RE)	Rivers, lakes	15/09/1995			Orto Botanico; Università degli Studi di Modena e Reggio Emilia

Table ST2: Italian Herbaria that were consulted in this study.

	Po_1	Po_2	Po_3	Ts	Ps	Gen	Rm
Po_1	0						
Po_2	**0,000**	0					
Po_3	**0,000**	**0,015**	0				
Ts	**0,046**	**0,000**	**0,000**	0			
Ps	0,306	0,1717	**0,032**	0,639	0		
Gen	0,803	**0,048**	**0,006**	0,519	0,962	0	
Rm	0,114	0,102	0,892	**0,022**	0,803	0,2363	0
H(chi^2)=47.69		Overall p < 0.000					

Table ST4: results of Kruskal-wallis test of median age of specimens collected in the different geographic areas. Po_1, Po_2 and Po_3 = three Po plan areas from west to east; Ts = Trieste area; Ps = Pesaro area;Gen = Genua area; Rm = Rome area.

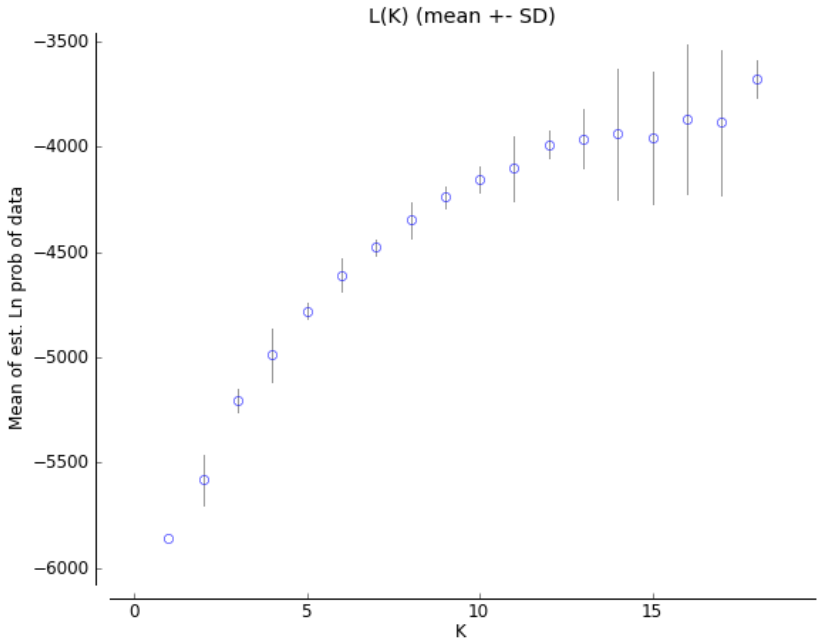
Supplementary file about K=3 results

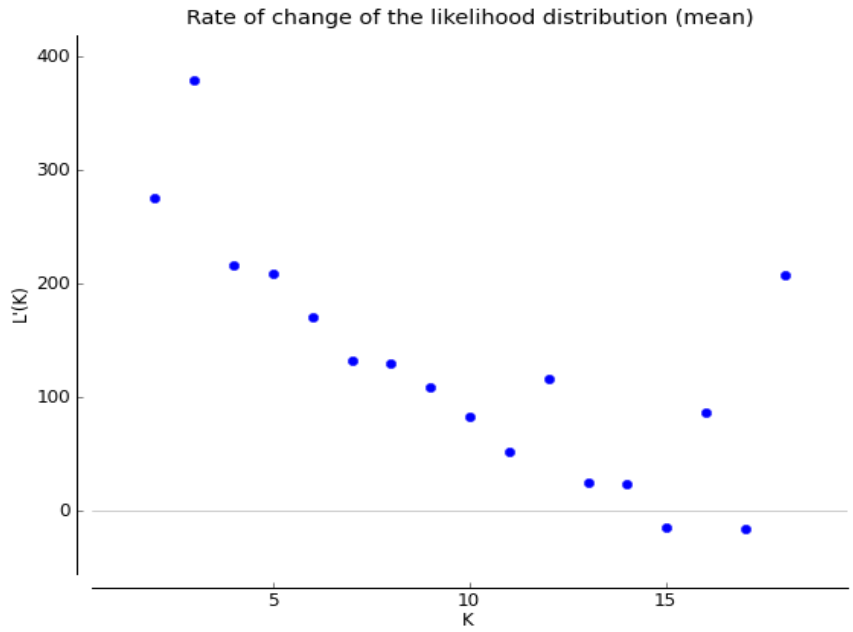


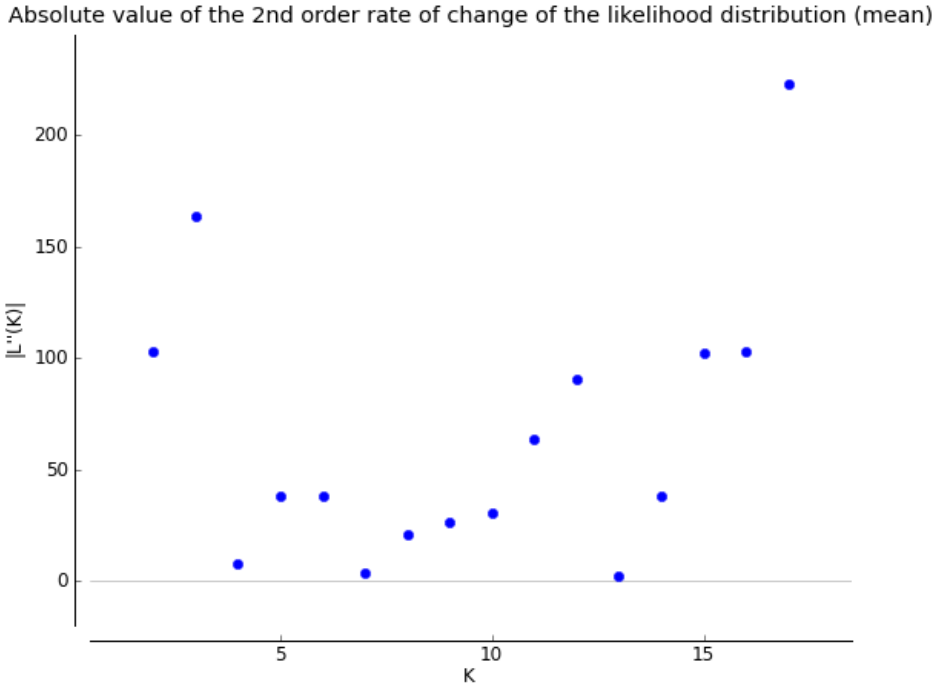
The Evanno table output

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-5857.940000	0.316930	—	—	—
2	10	-5581.810000	121.822562	276.130000	103.180000	0.846970
3	10	-5202.500000	55.770801	379.310000	163.448462	2.930717
4	13	-4986.638462	125.926424	215.861538	7.293077	0.057915
5	10	-4778.070000	35.778953	208.568462	38.198462	1.067624
6	10	-4607.700000	76.593110	170.370000	38.100000	0.497434
7	10	-4475.430000	36.712064	132.270000	3.140000	0.085530
8	10	-4346.300000	85.146018	129.130000	20.340000	0.238884
9	10	-4237.510000	52.669798	108.790000	26.110000	0.495730
10	10	-4154.830000	59.316553	82.680000	30.220000	0.509470
11	10	-4102.370000	153.861258	52.460000	63.220000	0.410890
12	10	-3986.690000	68.095398	115.680000	90.740000	1.332542
13	10	-3961.750000	139.237824	24.940000	2.020000	0.014508
14	10	-	308.839131	22.920000	38.240000	0.123819

		3938.830000				
15	10	-3954.150000	313.923205	-15.320000	102.020000	0.324984
16	10	-3867.450000	356.330023	86.700000	102.770000	0.288412
17	10	-3883.520000	346.475726	-16.070000	223.140000	0.644028
18	10	-3676.450000	90.140240	207.070000	—	—




Rate of change of the likelihood distribution (mean)
400
300
200
100
0
L'(K)
5
10
15
K


Absolute value of the 2nd order rate of change of the likelihood distribution (mean)
200
150
100
50
0
|L''(K)|
5
10
15
K

5.2 Analysis of ragweed pollen allergenicity

The aim of this second part of the thesis was to study the allergenicity variation among pollen samples from different ragweed plants/populations and to identify the mechanisms and factors contributing to the allergenicity by investigating the IgE-immunoreactivity and the intra and inter-population variability of Amb a 1 isoforms.

To the purpose, plants obtained from seeds of the populations characterized in the first part of the research were grown in standard and in controlled conditions; proteomic techniques were used to verify if differences in allergenicity were directly related to a different expression of Amb a 1 isoforms. Results of this experiment are reported in the following manuscript (n. 2)

Manuscript n. 2: in submission

Allergenic potency of common ragweed (*Ambrosia artemisiifolia* L.) pollen is mainly determined by environmental conditions during plant growth and flowering

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Abstract

Allergenicity of pollen is one of the main important factors influencing the prevalence and / or severity of allergic diseases. However, how genotype and environment contribute to determine ragweed pollen allergenicity is still to be established.

To throw some light on the factors governing allergenicity, in this work we grew 180 ragweed plants from seeds from three different Regions (Canada France and Italy) in two different environmental conditions: controlled and standard where, during all plant life cycle, temperature, relative humidity and light were maintained constant or varied according to environmental seasonal values, respectively. The two conditions were chosen on the basis of the hypothesis that plants from different locations grown in constant environmental conditions would have produced pollen with similar allergenicity only if the character had been regulated by environmental factors. Experimental results showed a statistical higher variability in allergenicity (variance=0.20) of pollen from plants grown in standard conditions in comparison to that measured for pollen from plant grown in controlled conditions (variance=0,01). The variability was due to differences among single plants, regardless of their origin, suggesting that the allergenic potency of pollen was principally determined by environmental factors and that the differences were not heritable through meiosis. The underlying regulation mechanisms of allergenicity were investigated by studying the qualitative and quantitative variations of the main isoforms of the major ragweed allergen Amb a 1. Electrophoresis and immunoblot analyses indicated that the variability in pollen allergenicity was mainly related to the total pollen content of Amb a 1 allergen and not to a different expression and reactivity of its single principal isoforms. In conclusion, on the whole the results suggests that environmental factors are mainly responsible for the high variability in pollen allergenicity detected in natural environments and that this variability is not ascribed to differences in expression/reactivity of individual

Amb a 1 isoforms but to a quantitative differences involving all the considered isoforms.

Introduction

Ambrosia artemisiifolia is an annual weed, native from North America and accidentally introduced to Europe, probably with seed imports, during 19th century (Chauvel *et al*., 2006). At the moment, in Europe, this alien plant is of great concern because it is one of the most prominent invasive species (Chauvel *et al*., 2006; Galzina *et al*., 2010; Gladieux *et al*., 2011). It has negative impacts on biodiversity, crop yields and human health causing respiratory allergies. (Peternel *et al*., 2006; Makra *et al*., 2005; Fumanal *et al*., 2007; Gadermaier *et al.*, 2014). At this regards *Ambrosia artemisiifolia* has been demonstrated to be one of the main allergenic species in Europe (Smith *et al*., 2013). The major allergen of ragweed pollen is Amb a 1. More than 90% of ragweed-sensitized subjects react to Amb a 1 in skin prick tests and at least 90% of the allergenic activity in ragweed pollen can be attributed to this protein (Gadermaier *et al*., 2008). Amb a 1 belongs to the family of pectate lyase and shows many genetic isoforms (Amb a 1.1, Amb a 1.2, Amb a 1.3, Amb a 1.4, Amb a 1.5). Pollen allergenicity, mainly due to the type and content of allergens, is widely recognized as a major determinant of health effects for sensitized patients and vary in pollen from single plants geographically and temporally (Cecchi, 2010). Lee *et al*. (1979), for example, demonstrated several-fold differences in allergenicity both among and within ragweed populations, although it was unclear whether these differences were genotypic or phenotypic. Several works demonstrated that pollution can influence the content of pollen allergens (Masuch *et al*., 1997; Gruijthuijsen *et al*., 2006; Aina *et al*., 2010). For instance Ghiani *et al*. (2012) reported an allergenicity increase of pollen from ragweed plants grown along polluted traffic roads. Climate change was also suggested to affect pollen allergenicity although, in this case, the research was

particularly addressed to the study and prediction of the increasing temperature and CO_2 concentration effects on pollen production and dispersion; only few studies were performed to define the role of climatic factors in altering pollen allergenic activity, which remains largely unexplored. Specifically it was for example shown that birch pollen content of the major allergen protein Bet v 1 increased at higher temperatures (Ahlholm, 1998) as well as ragweed Amb a 1 pollen content increased as function of rising atmospheric CO_2 concentration (Singer, 2005).

However, many other factors, both genetic and abiotic can influence allergen expression and it is still unknown if the antigen content is either function of environmental conditions or genetically determined. In addition pollen allergenicity is dependent not only on allergen amount but also on the type of allergens expressed; for example ragweed pollen allergenicity mainly depends on both the total content of Amb a 1 proteins and the presence of different Amb a 1 isoforms, whose single allergenic potential has not been completely elucidated yet.

In this work we studied the variation in Amb a 1 isoform expression and their IgE immunoreactivity among and within ragweed populations in order to determine the contribution of genetic and environmental factors to pollen allergenicity and to understand the underlying molecular mechanisms. Specifically, by growing ragweed plants at both standard natural conditions, where temperature (T), relative humidity (RH) and light (L) changed during plant development, and in controlled conditions where environmental parameters (T, RH, L) were maintained constant all over the plant life-cycle, we wanted to answer the following specific questions (i) is pollen allergenicity genetically determined or is it influenced by plant growth conditions? (ii) Are the differences among individuals related to differences in the quantitative/qualitative expression of single isoforms?

Materials and methods

Plant material

Seeds from 15 different ragweed populations (Tab. 1) were collected and used to obtain ragweed plantlets. To favor germination**,** collected seeds were subjected to a cold stratification (4°C) for 30 days and then sown in a culture medium, consisting of 7% plant agar. Petri dishes were placed in a growth chamber (20°C; 10 h dark/ 14 h light; 150 µmol m^{-2} sec^{-1}) and seeds were left to germinate. A total of 180 plantlets were transferred into 18 cm diameter pots containing universal soil. 90 plants out of 180 were grown in constant controlled conditions (25°C; 10 h dark/ 14 h light; 150 µmol m^{-2} sec^{-1}) whereas the remaining 90 were transferred into a greenhouse and grown in standard conditions, where temperature (T), relative humidity (RH) and light (L) changed during season and then during plant development. The trends of T and H during plant growth are reported in Fig. S1.
Plants were monitored every day and pollen was collected by covering the male inflorescences with a plastic envelope (ARASYSTEM®, Fig. S2). Sampled pollen was stored in 2 ml eppendorf containing silica gel at room temperature.

Tab. 1 Location of ragweed populations selected as source of seeds.

Code	Pop	Locality	State	N	E
1	MM	Magenta	Italy	45°27'15"	8°53'46"
2	L	Lodi	Italy	45°18'52"	9°31'05"
3	BR	Brescia	Italy	45°29'23"	10°11'47"
4	G	Greco	Italy	45°30'26"	9°12'39"
5	P	Pavia	Italy	45°11'43"	9°10'05"
6	26P18	Allex	France	45°44'52"	4°55'04"
7	39P04	Saint Germain les Arlay	France	46°45'56"	5°34'25"
8	01P01	Ambronay	France	45°59'35"	5°19'37"

9	26P21	Livron sur Drôme	France	44°46'02"	4°50'45"
10	26P19	Grane	France	44°44'58"	4°52'56"
11	LOT 18	Mirabel	Canada	45°39'45"	73°00'10"
12	LOT 878	Ste Clotilde de Chateauguay	Canada	45°11'24"	73°38'59"
13	LOT 6	L'Acadie	Canada	45°18'52"	73°21'19"
14	LOT 800	Ste Clotilde de Chateauguay	Canada	45°09'49"	73°40'17"
15	LOT 990	Ste Clotilde de Chateauguay	Canada	45°09'17"	73°41'02"

Immunochemical analysis

Patients' sera

Sera from 14 adult subjects previously selected for their ability to specifically detect ragweed allergens (Ghiani *et al*., 2012) were used to carry out all the immunochemical analyses. The serum pool was aliquoted and stored at -20°C until use.

Preparation of pollen protein extracts

Soluble protein extracts were prepared according to Aina *et al*. (2010) by suspending 0.1 g of pollen in 1 ml of bidistilled sterile water containing protease inhibitor (PMSF 1 mM). Samples were incubated on a rotating drum for 2 h at room temperature. The soluble fraction was isolated by means of two centrifugations at 13000 g for 10 min at 4°C and then stored at -20°C until use.

At least five independent extracts were prepared for each sample. Protein extracts were used for protein slot blot, 1D and 2D immunoblot analyses.

For Slot blot and 1D immunoblot analysis, samples were dissolved in SDS sample buffer [2% (w/v) SDS, 10% (v/v) glycerol, 1 mM DTT, 62.5 mM Tris-HCl, pH 6.8]. For 2D electrophoresis analysis, samples were purified with a clean-up kit

(Bio-Rad Laboratories®) and dissolved in IEF rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM Tris–HCl, pH 8.8, 20 mM DTT, 0.5% ampholyte mixture carrier, pH 3–10, 0.005% bromophenol blue]. Protein concentration was assayed according to Bradford (2) using bovine serum albumin (BSA) as standard.

Protein Slot blot

Slot blot technique was applied to assess the whole allergenicity of pollen samples. The analysis was carried out according to Ghiani *et al*. (2012). Briefly, equal volumes of protein extracts (3 µl) were bound to nitrocellulose membrane and first stained with Ponceau S staining solution [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to assess the amount of proteins loaded in each well. Membranes were then used to evaluate the immunoreactivity of the different pollen extracts to the sera mix from ragweed allergic patients. Protein extract from commercial pollen (Allergon) was used as standard to control staining variation when comparing measurements referring to different experiments.

Image analysis was applied to quantify immunochemical signals: the integrated optical density (IOD) of immunoreactive spots with respect to the IOD of standard (sample IOD/standard IOD) was measured. The mean results of five independent experiments were calculated and statistically analyzed.

1D and 2D immunoblotting

1D and 2D immunoblot analyses were performed to study the change in Amb a 1 isoform content and IgE reactivity among plants.

1D immunoblotting was carried out following the protocol reported by Aina *et al*. (2010). Briefly, equal volume of pollen extracts (15 µl/lane) were separated by 14% SDS-polyacrylamide gels according to Laemmli (1970). Gels were either stained with colloidal Coomassie Blue G-250 (0.1% Coomassie Blue G250, 170 g l^{-1} ammonium sulphate, 34% methanol, 3% phosphoric acid) or transferred to nitrocellulose membrane. Membranes were blocked with 5% (w/v) non-fat dry milk powder in TBS-T [20 mM Tris, 150 mM NaCl and 0.05% (v/v) Tween 20, pH

7.5] for 1 h and then incubated for 16 h at 4°C with a 1:10 dilution of the mixed sera from ragweed-allergic patients, and for control purpose, with sera from non-atopic adult individuals (1:10 dilution). Bound IgE were detected using an HRP-conjugated goat anti-human IgE antibody (1:15000 dilution; Sigma). Immunoreactive bands were visualized on an X-ray film (Kodak) using ECL Prime Western Blotting Detection (GE Healthcare®).

2D immunoblotting was performed according to Asero *et al*. (2014). Isoelectrofocusing (IEF) was carried out on 7 cm long immobilized pH gradient (IPG) strips (Bio-Rad®), providing a linear pH 4-7 gradient. Strips were rehydrated in 200 µl of rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, 0.5% ampholyte mixture carrier, pH 3-10, 0.005% bromophenol blue) containing 100 µg of protein sample. Passive rehydration (up to 10 hours) and IEF were performed at 20°C using a Protean IEF-Cell (Bio-Rad Laboratories®). After the first dimension separation, the IPG strips were equilibrated for 15 min against 6 M urea, 30% glycerol, 2% SDS, 0.375 M Tris–HCl pH 8.8, 2% DTT, in order to resolubilize proteins and reduce disulfur bonds. The –SH groups were then blocked by substituting the DTT with 2.5% iodoacetamide in the equilibration buffer for 15 min. After equilibration, strips were placed on the top of vertical 10 x 9 cm x 1.5 mm polyacrylamide gels (14% v/v). An agarose solution (0.5% low melting agarose in running buffer) was loaded to the top of the gel to lock strips, and electrophoresis was performed at 4°C in a Laemmli running buffer (25 mM Tris–HCl pH 8.3, 192 mM glycine, 0.1% SDS). Gels were run in the electrophoresis chamber (Mini-Protean electrophoresis system, Bio-Rad Laboratories®) in parallel and used for protein revealing or immunoblotting experiments. For proteins detection, gels were stained with colloidal Coomassie Blue G250 (0,1% Coomassie Blue G250, 170 g l^{-1} ammonium sulfate, 34% methanol, 3% phosphoric acid). For immunodetection experiments, gels were electroblotted (100 mA, overnight at 4°C) onto nitrocellulose membranes (0.45 mm, Bio-Rad Laboratories) by a Trans-Blot cell apparatus (Bio-Rad Laboratories) that contained transfer buffer (25-mmol l^{-1}

Tris, 192-mmol l^{-1} glycine, and 20% [vol/vol] methanol, pH8.3). Nitrocellulose filter saturation and sera-mix reaction were performed as reported above for 1D immunoblotting.

Amb a 1 isoform identification was made comparing the 2D maps with the ragweed reference map previously published by Asero *et al*. (2014). At least 3 independent samples for each selected plant were analysed.

Statistics

Data were analyzed by R program for Windows. ANOVA and Turkey's test, for multiple sample comparison, were applied when normality and homogeneity of variance were satisfied. Data, which did not conform to the assumptions, were transformed into logarithms.

Results

Total allergenicity of pollen from single plants

Slot blot technique was applied to assess the total pollen allergenicity of each of the 180 plants grown in controlled or standard conditions. Identical and comparable volumes of soluble pollen extracts were bound on a nitrocellulose membrane and subjected to immunoreaction with a sera mix from selected ragweed allergic patients. Figure 1 shows a representative membrane after immunodetection. Image analysis was applied to quantify immunochemical signals: the integrated optical density (IOD) of immunoreactive sposts with respect to the IOD of standard (sample IOD/standard IOD) was measured. At least 3 protein extracts from each plant were analyzed and the mean results of 5 independent experiments were calculated and statistically elaborated (Fig. 2). On average, the reactivity signal of pollen samples from plants grown in controlled conditions ranged from 0.89 to 1.22 with a variance value of 0.01 (Fig. 2 A). No statistical difference was found

among the mean IOD values calculated for Italian (1.07 ± 0.03), French (1.10 ± 0.04) and Canadian (1.03 ± 0.03) populations (P<0.01). Pollen from plants grown in standard conditions showed reactivity signals ranging from 0.56 to 2.19 with a variance (0.20) much higher (P<0.001) than that of pollen samples from plant grown in controlled constant conditions (Fig. 2 B). The IOD means of Italian (1.21 ± 0.12), French (1.09 ± 0.14), and Canadian (1.02 ± 0.06) populations were not statistically different for these plants too (P<0.05). Independently from seed origin, the mean reactivity signals were statistically classified in four significantly different (P<0.001) groups: (1) low: 0.56<IOD<0.75; (2) low-medium: 0.78<IOD<1.19; (3) medium-high: 01.27<IOD<1.50 and (4) high: 1.81<IOD<2.19.

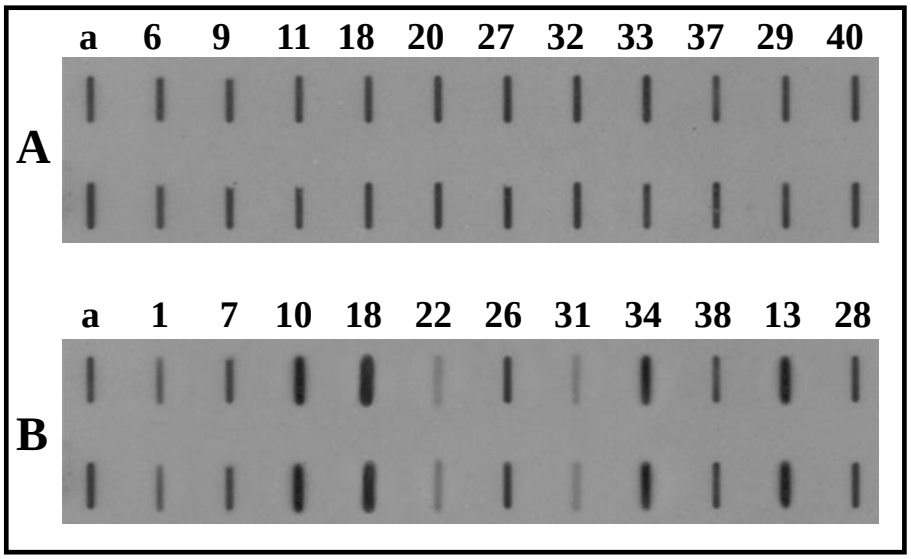

Fig. 1 Representative slot blot membrane probed with a pool of selected patient sera showing the total allergenicity of pollen samples collected from plants grown in controlled (A) and standard (B) conditions. Each sample was loaded in two replicates. (a) Standard (pollen from Allergon).

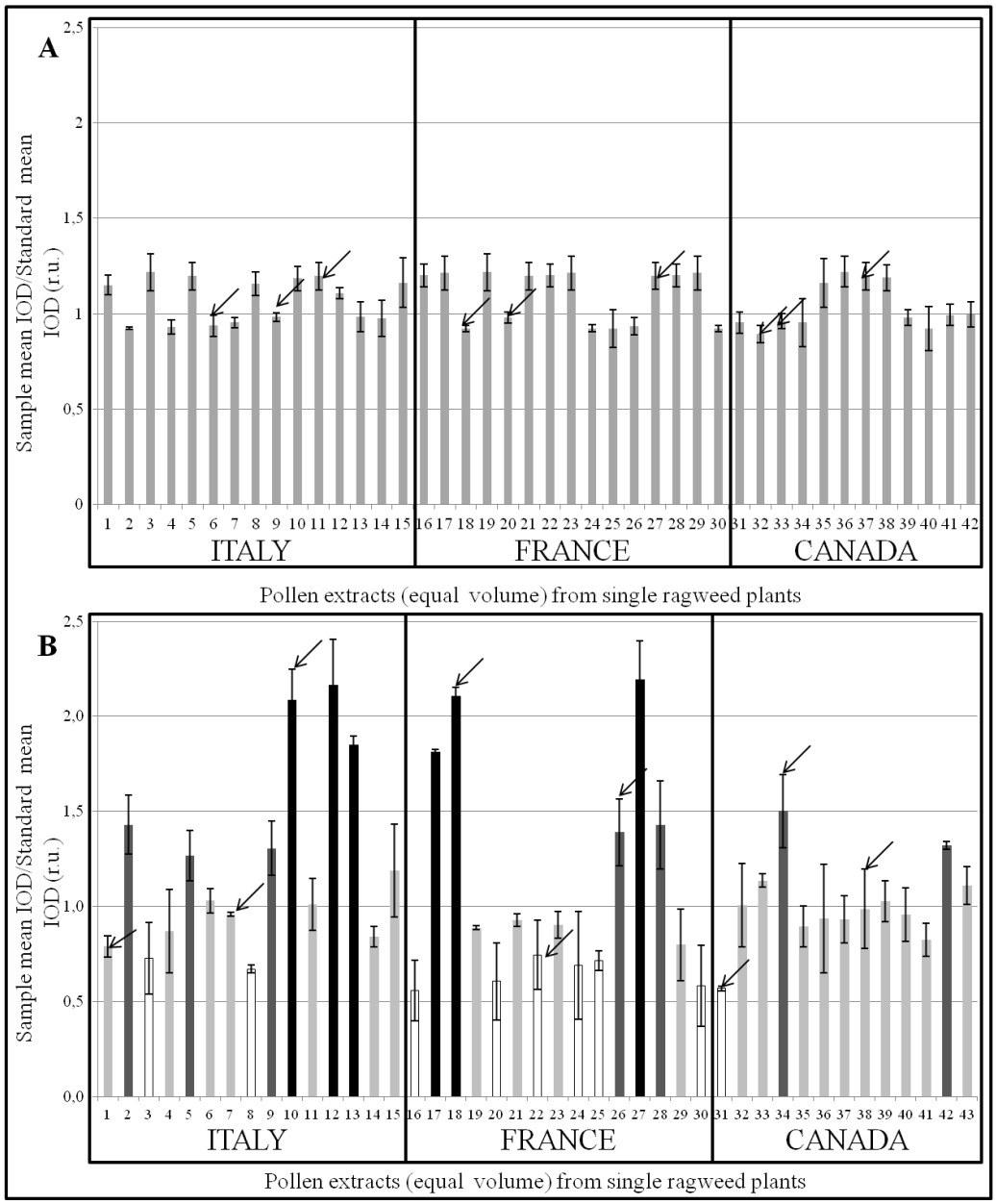

Fig. 2 Assessment of total pollen allergenicity through image analysis: the integrated optical density (IOD) of immunoreactive spots with respect to the IOD of the standard (sample IOD/standard IOD) was measured. The results reported are the mean of five independent experiments. (A) plants grown in controlled conditions and (B) plants grown in standard conditions. The four reactivity clusters obtain by statistical analysis are shown with different colors; white bars: low reactivity (0.56<IOD<0.75); light grey bars: low-medium reactivity (0.78<IOD<1.19); dark grey bars: medium- high reactivity (01.27<IOD<1.50); black bars: high reactivity (1.81<IOD<2.19).

Expression and IgE-reactivity of Amb a 1 isoforms in pollen from single plants

In order to understand if the high variability in total allergenicity found within plants grown in standard conditions was ascribed to different Amb a 1 amounts, 1D-SDS-PAGE and immunoblotting were carried out. Pollen from 9 plants, randomly chosen within the low, medium and high IgE reactivity groups resulting from the cluster statistical analysis of slot blot results, was analyzed. Additional 9 plants grown in controlled conditions were also considered as reference. Equal volumes of soluble protein extracts were loaded. Image analysis performed both on gels stained with Coomassie Blue and on the related immunoblot membranes, probed with the same sera mix used for slot blotting, confirmed the slot blot results and showed a statistical lower content of Amb 1 allergens ($P<0.05$) in samples with lower reactivity (Fig. 3) indicating a direct relation between Amb a 1 content and pollen allergenicity (Pearson correlation = 0.98 $P<0.001$ for standard group, 0.78 $P<0.05$ for controlled group).

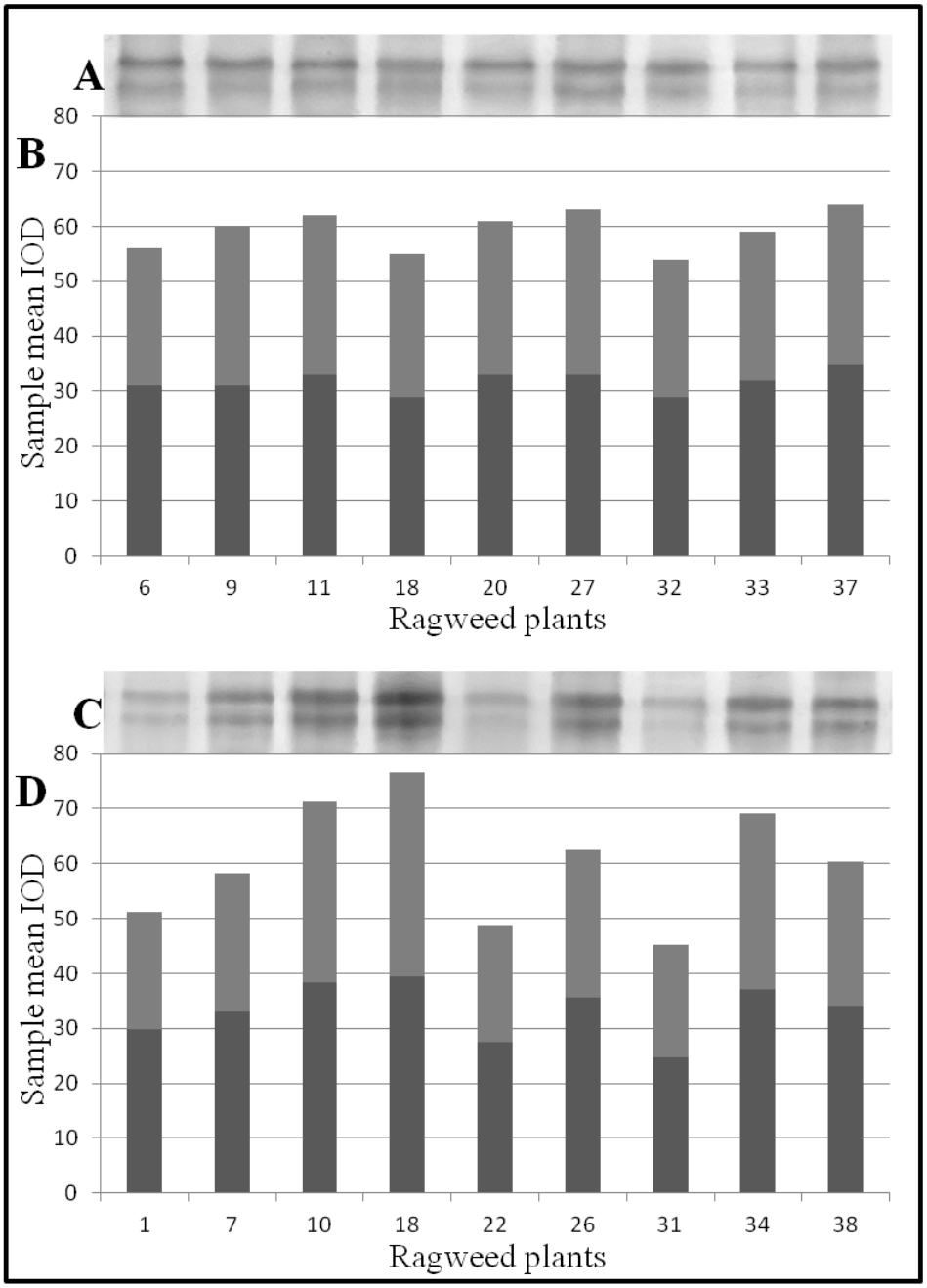

Fig. 3 Representative SDS-PAGE gels showing Amb a 1 proteins from pollen of plants grown in controlled (A) and standard conditions (C) and related mean IOD calculated on 5 independent extracts of pollen from each plant (B and D, respectively). Light and dark grey: upper and lower band, respectively. Amb a 1 content variance is 13 and 116 among plants grown in control and standard condition, respectively.

The same pollen samples were in parallel analyzed with 2D-SDS-PAGE and immunoblotting to understand if a differential expression and/or reactivity of the single Amb a 1 isoforms contributed to the high variability in IgE reactivity of pollen from plants grown in standard conditions. In this case equal amounts of proteins were loaded on gels. Figure 4 shows representative membranes after immunodetection and the related gels stained with Coomassie Blue. No consistent variation in relative spot presence/intensity on both gels and membranes was observed by comparing them with the reference map previously published by Asero *et al.* (2014). It suggested that the variability observed in pollen allergenicity was not ascribed to a different relative expression and IgE reactivity of the single isoforms considered.

On the whole electrophoresis and immunoblot analysis indicated that the variability in pollen allergenicity was mainly related to the pollen content of Amb a 1 allergen and not to a different expression and reactivity of its single principal isoforms.

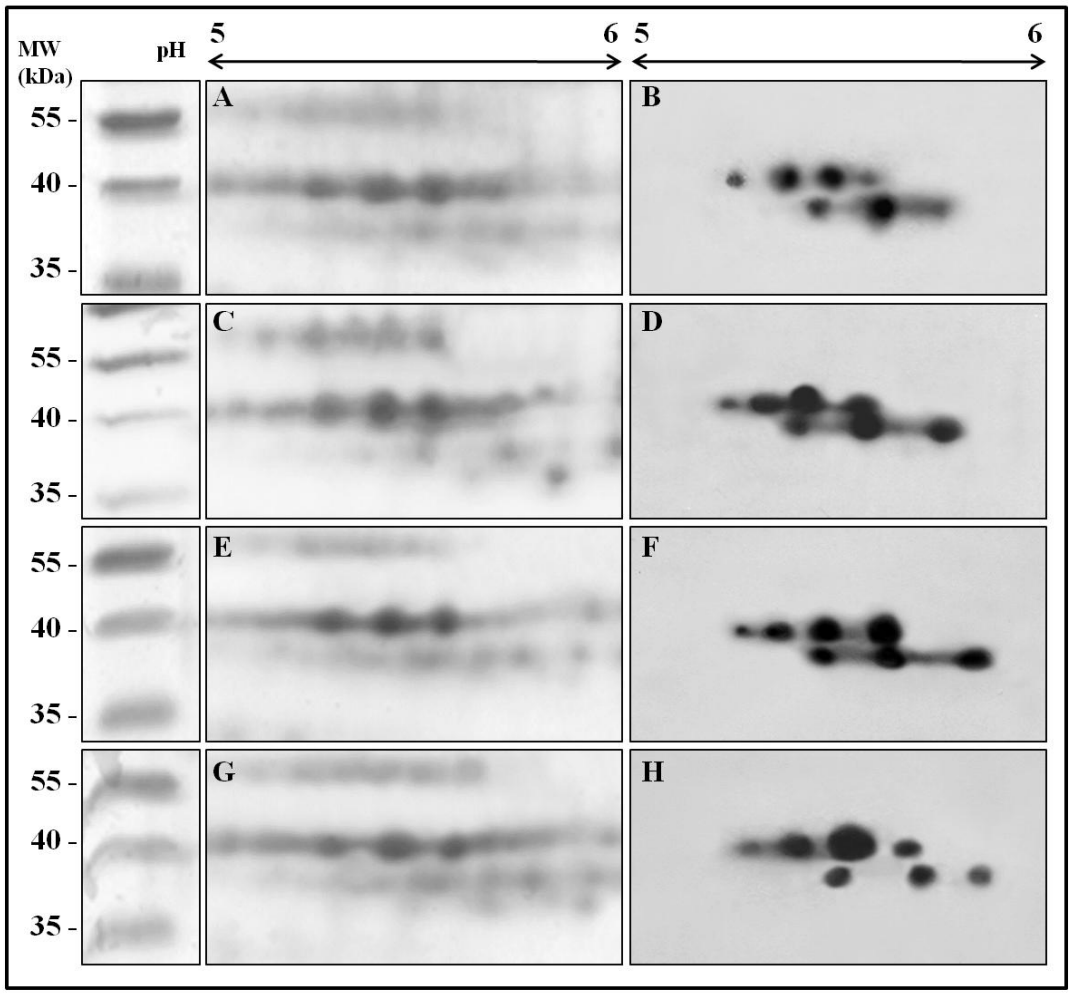

Fig. 4 Expression and IgE-reactivity of the Amb a 1 isoforms in pollen from plants grown in controlled (A-D) and standard conditions (E-H). A, C, E, G: two dimensional gels stained with colloidal Comassie Blue G-250; B, D, F, H: two dimensional immunoblot membranes probed with a pool of selected patients' sera.

Discussion

The introduction and naturalization of *Ambrosia artemisiifolia* L. to Europe has impacted on human health, because this plant produces large quantities of highly allergenic pollen representing one of the main causes of pollinosis in many regions (Smith *et al*., 2013). In Northern Italy, although common ragweed is present since

the beginning of the XX century, it has become the second cause of respiratory allergy only in the last two decades (Asero *et al.*, 2002; D'Amato *et al*., 2015). Among the factors influencing pollinosis, the allergenic potency of pollen is an important element to be taken into account. In fact, in addition to all the anthropogenic and environmental changes leading to plant diffusion and to the release of greater amounts of pollen into the atmosphere, pollen allergenicity can also influence the prevalence and/or severity of allergic diseases. However, how genotype and environment contribute to determine ragweed pollen allergenicity is still to be established.

In this work we demonstrated that variations in ragweed pollen allergenicity are mostly due to seasonal climatic variations (T, RH, L) occurring during plant development and particularly during flowering time which was single-plant-specific. We found that pollen from plants grown in constant conditions showed similar allergenic potency, whereas pollen from plants subjected to temperature, relative humidity and light seasonal changes showed a high variability in allergenicity. Moreover, we found that also flowers of the same plant produced pollen with different allergenic potency only when they developed under variable conditions (data not shown).

Previous studies on ragweed, demonstrated that the content of its major allergen Amb a 1 vary in plants from site to site and even from year to year at the same site (Lee *et al.*, 1979, Scea *et al*., 2008). Analogously for other allergenic plant species, the concentration of major pollen allergens was reported to differ among plants as function of climatic conditions. For example, Ahlholm (1998) indicated that birch pollen content of the major allergen protein Bet v 1 increased at higher temperatures and Saito and Teranishi (2002), by comparing the sugi major allergen Cry j 1 concentrations of four individuals of a clone growing at a low-altitude site and at a high-altitude site, found that the Cry j 1 concentration was higher in pollen collected at the low-altitude site. Specifically these authors speculated that differences in mean temperature of 1.1 and 1.5°C, would cause changes in Bet v 1

and Cry j 1 allergen concentration, respectively. However Goto *et al*. (2004), by studying a greater number of sugi clones, reported that the Cry j 1 concentration was controlled primarily by genetic factors.

Our results indicated a principal control of ragweed pollen allergenicity mediated by environmental factors leading to phenotypic modifications not meiotically heritable. However the low allergenicity variability that we found among plants grown in constant controlled conditions suggested that genotypic heritable differences existed among plants and influenced, although a far lesser extent, the final pollen allergenicity. It likely reflects the high gene flow existing among ragweed plants which determines a genetic variability very high among individual plants and very low among populations (Genton *et al*., 2005). Accordingly, in our experiment, allergenicity was single-plant-specific regardless of their origin. Interestingly also flowering time was single-plant-specific but the differences among plants were consistent and similar in constant and in standard conditions suggesting that this character, differently from allergenicity, was determined by genetic and/or epigenetic heritable factors. Moreover, in keeping with literature reporting an increase in pollen Amb a 1 concentration induced by climatic changes and/or pollutants (Singer *et al*., 2005; Kelish *et al*., 2014; Zaho *et al*., 2016), our experiment showed that environmental factors acted through a molecular mechanism regulating the pollen content of all the considered Amb a 1 isoforms. A preferential expression of specific Amb a 1 isoforms was, instead, not observed. This mechanism should involve an epigenetic control of pollen allergenicity as reported for many adaptation processes to environmental stresses. For instance Kelish and collaborators (2014) observed an increase of the main Amb a 1 mRNAs in pollen of plants exposed to high concentrations of CO_2 and under drought stress. We can speculate that the change in Amb a 1 pollen concentration could be a mechanism of adaptation acted by ragweed plants to favour their reproduction. Amb a 1 proteins belong, in fact, to the family of pectate lyases which activity is implicated in pollen tube growth emergence by initiating the loosening and

breaking of the pollen cell wall and also in pollen tube penetration in transmitting tissue (Shin *et al*., 2014). Unfortunately, although epigenome modulation in response to the environment potentially provides a mechanism for organisms to adapt, both within and between generations, at present neither the extent to which this occurs, nor the molecular mechanisms involved are still known (Shin *et al*., 2014).

Our data provide a first step in identifying factors that are involved in modulate pollen allergenicity. Future experiment aimed at investigating the direct relation between pollen allergenicity, single environmental parameters and epigenetic mechanisms, such as DNA methylation, are needed to gain further insight into the influence of environment changes on pollen allergenicity and into allergen physiological function for ragweed plants.

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Supplementary materials

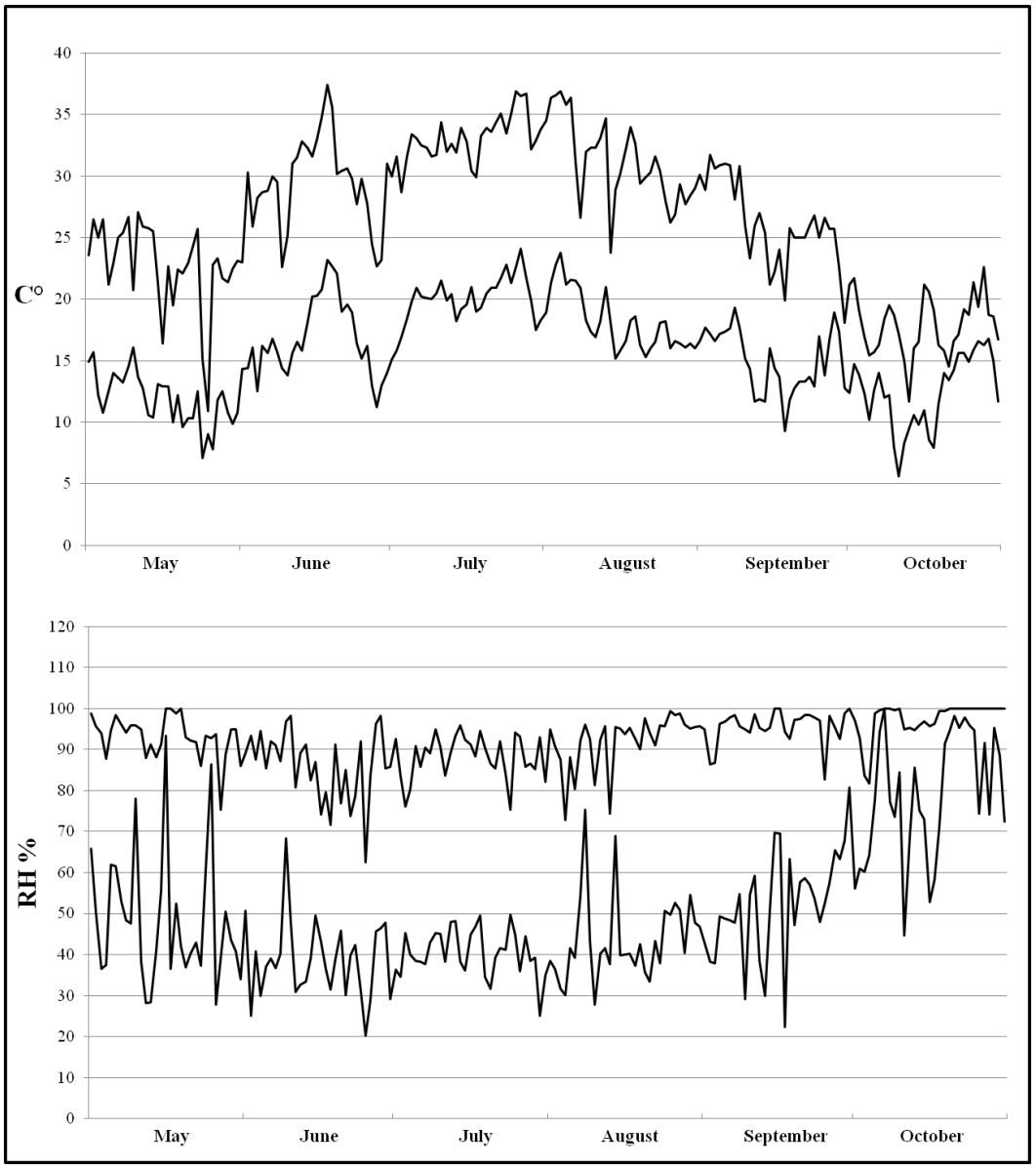

Fig.S1: trends of H (Humidity) and T (Temperature) during plant growth. ARPA data 2013.

Fig.S2: male inflorescences covered with a modified ARASYSTEM®

6. General conclusions

In the first part of the research, the Italian populations of *Ambrosia artemisiifolia* L. were genetically characterized, proving that the genetic variability is very high within populations, whereas it is very low among populations as already demonstrated for French and Canadian populations. In addition we demonstrated that Italian populations, as the French one, have a Nord American origin and that they are the result of multiple introduction of seeds happened during the last century.

In the second part of the thesis, the genetically characterized populations were used to study the variability in pollen allergenicity that is mainly due to the total concentration of the ragweed major allergen isoforms Amb a 1 in pollen. Our experiments demonstrated a moderate variability in total allergenicity between individuals grown in controlled uniform conditions; however, such variability was negligible when compared with that we found among plants grown under seasonal changes in temperature, relative humidity and light. This high variability was not due to the differential expression of the Amb a 1 isoforms but to a greater or lesser expression of all of them. Thus, data obtained in this thesis suggests that, although most individual plants within a population are genetically different, the total pollen allergenicity is similar in the different individuals and is only negligibly genetically determined. On the contrary, data suggests that environmental changes are the key factors governing allergenicity, probably through epigenetic non-meiotically heritable mechanisms regulating the expression of Amb a 1 proteins.

This work represents the first step toward understanding what environmental and/or genetic factors affect the allergenicity of pollen and how they act. To this purpose, in the future it will be useful to investigate the effect of the single environmental parameters on the allergenicity of pollen and to investigate the possible epigenetic mechanisms playing a key role in defining them. The obtained information will be essential to deeply understand and accurately predict how

climate changes, which represent a reality nowadays, can affect pollen allergenicity and, consequently human health.

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- Ciappetta S., Ghiani A., Gilardelli F., Bonini M., Citterio S., Gentili R. (2015). Assessing the invasion of *Ambrosia artemisiifolia* in Italy through the analysis of genetic variability and herbarium data. – submitted.
- Ghiani A., Ciappetta S., Gentili R., Asero R., Citterio, S. Allergenic potency of common ragweed (*Ambrosia artemisiifolia* L.) pollen is mainly determined by environmental conditions during plant growth and flowering. – in submission.
- Ciappetta S., Lommen S. T. E., Ghiani A., Asero R., Gentili R., Müller Shärer H., Citterio S. Pollen allergenicity of common ragweed is not affected by the plant exposition to the insect *Ophraella communa* LeSage in controlled conditions. – in preparation.

Congress communications

- Gentili, R., Ciappetta, S., Gilardelli, F., Ghiani, A., Colombo, F., Rodio, V., Guarino, M. F., Bonini, M., Citterio, S. The invasion of *Ambrosia artemisiifolia* L. in Italy: genetic variability, population structure and colonisation routes. 110° Italian Plant Biology Society – Pavia, Italy – 14/17 September 2015.
- Ghiani, A., Ciappetta, S., Gentili, R., Gilardelli, F., Citterio, S. Intra and inter population variabililty of ragweed Amb a 1 isoforms and their allergenicity. Annual meeting of work groups "Cellular and Molecular Biology" and "Biotechnology and Differentiation" of the Italian Plant Biology Society – Roma, Italy - 10/12 June 2015.
- Gentili R., Gilardelli F., Sgorbati S., Ghiani A., Ciappetta S., Citterio S. Distribution range of four invasive alien species in Italy: *Ambrosia artemisiifolia* L., *Reynoutria japonica* Houtt., *Prunus serotina* Ehrh., *Senecio inaequidens* DC. International congress of botany – Firenze, Italy – September 2014
- Gentili R., Gilardelli F., Ghiani A., Ciappetta S., Citterio S. Distribution range of four dangerous non-native species in Italy, 4th International Symposium on Weeds and Invasive Plants – Montpellier, France – 18/23 May 2014
- Gentili R., Gilardelli F., Ghiani A., Ciappetta S., Bonini M., Citterio S. Intensive grassland seeding to contrast *Ambrosia artemisiifolia* L. Third International Ragweed Conference – Rho, Italy – 3/4 April 2014.
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