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BIODIVERSITY MANAGEMENT AND CONSERVATION:

A COMPROMISE BETWEEN NATURE AND CULTIVATION

Dr. Mariaceleste Labriola

DOTTORATO DI RICERCA IN
Scienze e Tecnologie Vegetali, Microbiologiche e Genetiche
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COORDINATORE Prof. Aniello Scala

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December 31th, 2014

Dr. Mariaceleste Labriola

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Summary

In spite of the growing awareness of the importance of ecosystems and biodiversity to human welfare, loss of biodiversity and degradation of ecosystems continue to be a problem not easily solved in the short term. One of the most important threats to biological diversity is the introduction of alien species and the resulting impacts on ecosystems. Recently, the genetically modified organisms (GMOs) are also considered alien species since they are modified and introduced by humans and, as opposed to native species, they do not have a natural evolutionary background in the recipient environment. Since GMOs can represent a new and more serious threat to biodiversity, the conservation strategies need an ever more detailed knowledge about the range of consequences of such introductions, especially considering that woody and herbaceous crops, both GM and non-GM, have different biological and ecological characteristics.

The aim of this work is to investigate the possible interactions between cultivated plants and the surrounding environment from a genetic diversity conservation point of view; in particular, the potential wild-to-crop hybridization was examined. In order to highlight differences between woody and herbaceous plants we focused on two cropped species: *Populus* spp. and *Brassica* spp..

All sites were settled within the protected area of the Regional Park of Migliarino-San Rossore-Massaciuccoli (Pisa, Italy), because it presents the best context to study gene flow, i.e. crops nearby their wild relatives. We selected: i) a cultivated area with *Brassica napus* L. (oilseed rape), bounded by a mixed broadleaved alluvial forest and surrounded by a potentially candidate for breeding, *Sinapis arvensis* L. (wild mustard); ii) a wetland habitat with a scattered population of *Populus* spp.; and iii) a naturally-originated forest including *Populus* spp.. The last two areas are close to poplar plantations.

Leaves from adults and seeds from mothers (successively grown in laboratory) were collected to extract total DNA. Moreover, the sequencing of *trn*L chloroplast DNA (cpDNA) region and the genotyping with ten nuclear microsatellite (nSSR) loci of poplar were performed. The combination of the molecular information obtained with these two techniques permitted to identify individuals belonging to *P. alba* (white poplar), *P. x canescens* (gray poplar) and *P. nigra* (black poplar). As regards to *Brassica* spp., the analysis of ten nSSR revealed unique allelic variants, which allowed to distinguish oilseed rape from wild mustard.

In natural poplar populations, by means of suitable software, microsatellite data joined to geographical position of each individual, gave information on the spatial structure analysis of genetic diversity. The analysis revealed strong genetic isolation of black poplar and weak gene flow barriers between white and gray poplars.

Then, in order to detect the potential wild-to-crop hybridization, the paternity analysis was implemented by using both molecular and spatial data. In poplars, a female of *P. x canescens* produced progeny with individuals of nearby plantation belonging to "Triplo" clone (*P. x euramericana*). In Brassicaceae, the parents of seedlings were assigned by comparing all the genotypes and thanks to the specific allelic variants of the species. Most of the seedlings were the product of intraspecific crosses of *S. arvensis*, even though a smaller percentage originated from the interspecific crosses between individuals of wild mustard and oilseed rape.

Finally, for both genera, data from pollen, seed dispersal and distribution of wild relatives were intersected into maps together with vegetation and land use maps. The resulting maps highlighted the importance of a case-by-case study and outlined those sites within the protected area where cultivations could potentially influence wild relatives.

In conclusion, this work demonstrates that the simultaneous presence of conventional crops and wild relatives can favour their hybridization in the environment, and this event has to be carefully evaluated and taken in consideration if transgenic crops are planned in the near future. Even though gene flow is a natural event, it can negatively influence native populations by threatening their genetic integrity (i.e., by limiting local adaptation) and by affecting biodiversity at the level of communities and ecosystem processes. Thus, from a "conservation genetic" point of view, it is essential not to underestimate the possible hybridization events and consider conservation actions that take into account both the biological characteristics of the species and the surrounding environment (i.e., the width of buffer zones and physical barriers).

Riassunto

Nonostante la crescente consapevolezza dell'importanza degli ecosistemi e della biodiversità per il benessere umano, sia la perdita di biodiversità che il degrado degli ecosistemi continuano a rappresentare un problema di difficile risoluzione a breve termine. Una delle minacce più importanti per la diversità biologica è l'introduzione di specie aliene e i conseguenti impatti sugli ecosistemi. Recentemente, gli organismi geneticamente modificati (OGM) possono essere considerati come specie aliene in quanto risultano modificati e introdotti dall'uomo e, al contrario delle specie autoctone, non hanno subito un'evoluzione naturale nell'ambiente ricevente. Poiché gli OGM possono rappresentare una minaccia nuova e più seria per la biodiversità, le strategie di conservazione hanno bisogno di una conoscenza sempre più approfondita delle ripercussioni derivanti da tali introduzioni, soprattutto considerando che le colture legnose ed erbacee, sia GM che non GM, presentano diverse caratteristiche biologiche ed ecologiche.

Lo scopo di questo lavoro è quello di indagare le possibili interazioni tra piante coltivate e l'ambiente circostante dal punto di vista della conservazione della diversità genetica; in particolare, è stata esaminata la potenziale ibridazione *wild-to-crop*. Inoltre, per poter evidenziare le differenze tra specie legnose ed erbacee ci siamo concentrati su due specie coltivate: *Populus s*pp. e *Brassica* spp..

I siti di studio sono stati individuati all'interno dell'area protetta del Parco Regionale di Migliarino-San Rossore-Massaciuccoli (Pisa, Italia), perché presenta il contesto migliore per studiare il flusso genico, dal momento che i campi coltivati si trovano vicini a specie selvatiche parentali. Pertanto, abbiamo scelto: i) un'area coltivata con *Brassica napus* L. (colza), delimitata da una foresta alluvionale di latifoglie miste e circondata da *Sinapis arvensis* L. (senape selvatica), un potenziale candidato per l'incrocio; ii) un habitat umido con una popolazione sparsa di *Populus* spp.; e iii) una foresta d'origine naturale che comprende *Populus* spp.. Le ultime due aree sono vicine a piantagioni di pioppo.

Sono stati raccolti foglie e semi (fatti germinare successivamente in laboratorio) da madri per estrarne il DNA. Successivamente, sono stati effettuati sia il sequenziamento della regione *trn*L del DNA plastidiale (cpDNA) che la genotipizzazione con dieci microsatelliti nucleari (nSSR) di pioppo. La combinazione delle informazioni molecolari ottenute con queste due tecniche ha permesso di identificare gli individui appartenenti alle specie *P. alba* (pioppo bianco), *P. x canescens* (pioppo grigio) e *P. nigra* (pioppo nero). Per quanto riguarda le *Brassica*

spp., l'analisi effettuata con dieci nSSR ha rilevato varianti alleliche uniche, che hanno consentito di distinguere la colza dalla senape selvatica.

Nelle popolazioni naturali di pioppo, tramite l'utilizzo di opportuni softwares, i dati ottenuti con i microsatelliti, congiunti alla posizione geografica di ciascun individuo, hanno dato informazioni sulla struttura spaziale della diversità genetica. Quest'ultima ha evidenziato un forte isolamento genetico del pioppo nero e deboli barriere al flusso genico fra pioppo bianco e pioppo grigio.

Al fine di verificare la potenziale ibridazione tra piante selvatiche e coltivate, è stata eseguita un'analisi di paternità utilizzando sia dati molecolari che spaziali. Nei pioppi, una femmina di *P. x canescens* ha prodotto progenie con individui della vicina piantagione, che appartengono al clone "Triplo" (*P. x euramericana*). Nelle Brassicaceae, i parentali delle plantule sono stati assegnati grazie alle varianti alleliche specifiche di ciascuna specie e confrontando tutti i genotipi tra loro. La maggior parte delle piantine derivano da incrocio intraspecifico di *S. arvensis*, anche se una percentuale minore deriva dall'incrocio interspecifico tra individui di senape selvatica e di colza.

Infine, in entrambi i casi di studio, sono state incrociate le mappe di vegetazione e di uso del suolo con i dati provenienti dalle analisi di dispersione del polline, dispersione dei semi e distribuzione delle specie selvatiche. Le mappe così ottenute hanno sicuramente sottolineato l'importanza di un approccio *case-by-case* e hanno delineato i siti all'interno dell'area protetta dove le coltivazioni potrebbero potenzialmente influenzare i parentali selvatici.

In conclusione, questo lavoro dimostra che la presenza simultanea di colture convenzionali e parentali selvatici può favorire la loro ibridazione nell'ambiente, e che questo evento deve essere attentamente valutato e considerato nel caso in cui, in futuro, venissero utilizzate colture transgeniche. Anche se il flusso genico è un evento naturale, esso può influenzare negativamente le popolazioni autoctone, minacciandone l'integrità genetica (ad esempio, limitandone l'adattamento locale) e influenzando la biodiversità sia a livello di comunità che a livello dei processi ecosistemici. In definitiva, da un punto di vista di "conservazione genetica", è fondamentale non sottovalutare i possibili eventi di ibridazione e prevedere le azioni di conservazione che tengano conto sia delle caratteristiche biologiche delle specie che dell'ambiente circostante (ad esempio, la dimensione delle zone cuscinetto e delle barriere fisiche).

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Chapter 1
Introduction

The biodiversity conservation is an interdisciplinary science which aims to analyse and describe the diversity of living organisms, to understand the effect of human activities on them both at species and ecosystem level, to develop strategies useful in protecting and, whether necessary, restoring biodiversity (Primack et al. 2000).

Conservation *in situ* and *ex situ* are approaches directed to safeguard biodiversity at population or species level. The foundation of protected areas offers a wider protection, at ecosystem level, thus safeguarding numerous species or populations all together (Primack et al. 2000).

In spite of the growing awareness of the importance of ecosystems and biodiversity to human welfare, loss of biodiversity and degradation of ecosystems still continue on a large scale (Kumar 2010). In fact, nowadays most of the threats to biodiversity come from human activities. The harmful pressures are: destruction and fragmentation of natural habitats, their degradation, global climate change, overexploitation of species, diseases spread and introduction of alien species.

All these threats, in themselves and in combination, imply alteration of abiotic and biotic conditions in ecosystem equilibrium and thus in ecosystem services, subsequently described.

Thus, one of the major objectives of nature conservation is to preserve biological diversity and safeguards its benefits, i.e. ecosystem services (UNEP 1992).

1.1 Biodiversity and Ecosystem services

Millions of organisms, populating Earth, interact with one another in many ways, because of competition for food, space and resources in general. These fundamental linkages among organisms and their physical and biological environment constitute an interacting and ever-changing system that is known as an ecosystem.

As stated in the article 2 of the Convention on Biological Diversity (UNEP 1992) the ecosystem is “a dynamic complex of plant, animal and micro-organism communities and their nonliving environment interacting as a functional unit”. The diversity is a structural feature of ecosystems, as deducible from the definition of biodiversity as “the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems” (UNEP 1992).

Diversity can be considered at diverse levels including genetic, species, functional group, landscape and ecosystem diversity.

The genetic diversity refers to the variability of genes within a species, i.e. the total number of alleles for gene that can be found in one species. Species diversity within communities depends on the number of living organisms that are classified into different species. The diversity based on functional groups considers the distinguishable activity in habitats and role in food webs of each species. At a wider spatial scale, different habitats form landscapes, and the ecosystems can be larger units composed of several landscapes.

Humankind is a component of these ecosystems and depends on their benefits, i.e. ecosystem services (UNEP 1992), and on the network of interactions among organisms. Recent perturbations, driven principally by human activities, have added even greater complexity by changing, to a large degree, the nature of those environments (MA 2005).

Ecosystem services indicate ecological processes that humankind benefits from (Daily 1997) and, as well, the complexity of benefits derived, directly or indirectly, from ecosystem functions (Costanza et al. 1997) (Fig. 1.1).

The Millennium Ecosystem Assessment (MA 2005) recognizes four categories of ecosystem services.

i) Provisioning services are those products obtained from ecosystems, simply

Fig. 1.1 Ecosystem services that support humankind and its activities, i.e. raw materials, regulation of water flows, aesthetic information, food.

harvested from the wild without any management. They include:

- Food (e.g. fish, fruit)
- Water (e.g. for drinking, irrigation, cooling)
- Raw materials (e.g. fiber, timber, fuel wood, fodder, fertilizer)
- Genetic resources (e.g. for biotechnology, crop-improvement and medicinal purposes)
- Medicinal resources (e.g. biochemical products, natural medicine)
- Ornamental resources (e.g. artisan work, decorative plants, pet animals)

ii) Regulating services are the benefits obtained from the regulation of ecosystem processes:

- Air quality regulation (e.g. capturing (fine)dust, chemicals, etc)
- Climate regulation (e.g. C-sequestration, influence of vegetation on precipitation and temperature, etc.)
- Moderation of extreme events (e.g. storm protection and flood prevention)
- Regulation of water flows (e.g. natural drainage, irrigation and drought prevention)
- Waste treatment (especially water purification)
- Erosion prevention (e.g. vegetative cover prevents landslides)
- Maintenance of soil fertility (e.g. soil formation)
- Pollination
- Biological control (e.g. seed dispersal, pest and disease control)

iii) Cultural services are strongly bound to human values and behaviour, thus their perception is likely to differ among individuals and communities. They provide the nonmaterial benefits through:

- Aesthetic information (e.g. beauty or aesthetic value of various aspects of ecosystems)
- Opportunities for recreation and ecotourism
- Inspiration for culture, art and design
- Spiritual and religious values (e.g. in ecosystem components)

iv) Finally, supporting services include services that, by their functioning, support the normal functioning of ecosystems. They include some of the above-mentioned services, but differ from those because their perception occur over a very long time. For example, air and water purification, but also soil formation, fertility maintenance, and supporting plant production through seed dispersal and pollination; primary production, production of atmospheric oxygen, nutrient cycling, water cycling, and provisioning of habitat.

In conclusion, ecosystem services directly support humankind and its activities; thus, protecting ecosystems is also critical for economic development (Turner et al. 2007).

1.2 Threats to biodiversity: Invasive Alien Species

The second most important factor threatening biological diversity, after habitat destruction, is represented by Invasive Alien Species (IAS) (Sandlund et al. 1999) and their impacts can be considered as damaging as loss of habitat (IUCN 2000).

Alien species belong to all taxonomic groups, ranging from viruses to higher plants, invertebrates and mammals. They have invaded and affected native biota provoking many hundreds of extinctions (McNeely 2000). Both the IUCN (IUCN 2000) and the Convention on Biological Diversity (CBD; UNEP 1992) provide similar definition for alien species, as one that has been introduced outside its past, or present, natural range or dispersal, by direct or indirect human intervention. This designation emphasizes the importance of the ecological origin of the species as well as the facilitative role of humans in moving the species. To be alien, a species must overcome geographical barriers for human intervention otherwise impassable.

The introduction of species into new habitats are accidental (often invertebrates and pathogens by trade) and deliberate introductions (usually plants and vertebrates, for agricultural, forestry and ornamental scope) (Levin 1989). Once introduced, if the environmental conditions permit them to survive, two phases follow. The first is the naturalization, which occurs if the alien species survives and successfully reproduces to form a self maintaining populations. The next step is the invasion: after the establishment, alien species quickly expand their range and compete with native species for the conquest of resources.

The success of invasion increases with vulnerability of ecosystem. As worst case, long time of human disturbance have characterized urban and urban-fringe environments where, through the creation of vacant niches, IAS have been favoured (McNeely 2001). Moreover, higher vulnerability is associated to disturbed ecosystems; for example, the ecosystems degraded and stressed through processes of pollution, land clearance and intensive exploitation, agricultural ecosystems, and also the geographically or evolutionarily isolated ones (McNeely 2000).

The consequences of IAS can be observed at different scales.

The competition, for consuming or controlling access to a resource, can negatively affect species and it is more likely when the availability of resources is low and the niche overlap is high (Bøhn 2007). Interspecific competition may alter resource

utilization, leading to reduced density or in the worse case to competitive exclusion and extinction of one or more of the species involved (Hardin 1960).

Depending on the role within ecosystem functions that any native species fulfil, the impact on biodiversity loss due to IAS changes. The magnitude of the impact depends on redundancy of the ecosystem, i.e. the presence of more than one species that support the same perturbed function. Redundancy may counteracts disturbance and the loss of biodiversity if species in the ecosystem cover a variety of functional responses (Hooper et al. 2004; Winfree & Kremen 2009; Kumar 2010).

At a global scale, the most important effect of the numerous intentional or unintended introductions have negative impacts on ecosystems, since it leads to the homogenization of the distribution of species within ecosystems, thus reducing global biodiversity (Vitousek et al. 1997; Lövei et al. 2007).

It is difficult to list properties, characterizing invaders, but there are some regularities that allows to understand the success of some invasions while not of other ones. For instance, climatic compatibility and abundance of resources in the new ecosystem, both improve the chance of an invader succeeding (Williamson 1996).

In general, a species is more likely invasive if it is generalist (wide ecological niche) and the rate of reproduction is high. The following biological features of invaders are mostly referred to plants.

The genome size of invading plants is usually small and the invasiveness is related to small seed mass and short juvenile period. The vegetative reproduction facilitates invasion, but sexual reproduction can make more invasive a plant species if pollen and seed dispersal is mediated by generalized vectors; finally, species with numerous, relatively small, soil-stored seeds are pre-adapted for human dispersal, and hence invasion (McNeely 2000).

Managing IAS is an imperative, not simply for ecologists or conservation biologists, but also for the whole economy, since it involves the issue of global trade, agriculture, economics, health, water management, climate change, and genetic engineering (McNeely 2000; McNeely 2001).

First of all, facing the issue of IAS means preventing any potential establishment of alien species, by specific actions which involve public awareness and information, environmental risk assessment as well as both national and international regulations of trade.

When the invasion occurs, both the environmental and the economic costs of management can be prohibitive.

Once occurred, the responses of management to invasions are mitigation and adaptation.

Mitigation actions aim to eliminate the presence of an invader (eradication) or to reduce it, limiting both the spatial scale of invasion (containment) or the population levels under a certain threshold (suppression). Adaptation actions, on the other hand, try to minimize the consequences of invasions (McNeely 2000).

1.3 Genetically Modified Organisms (GMO) as Invasive Alien Species (IAS)

Modern biotechnology can introduce a greater number of genes into organisms than traditional methods of breeding and selection. The genetically modified organisms (GMOs) can be considered alien species since they are modified and introduced by humans and, as opposed to native species, they do not have a natural evolutionary background (McNeely 2000; Bøhn 2007).

The particular attention directed to GMOs arises from the long humankind experiences. Industrial and technological development, although beneficial in economic and well-being terms, had environmental costs, many of which outweigh the benefits (Harremoës et al. 2002).

Accordingly, all new potential environmental pressures need to be carefully assessed.

GMOs could represent a new and more serious threat to biodiversity than do non-modified species, especially because it is difficult to determine if the GMOs are more or less competitive (McNeely 2000).

Similarities and differences have been detected between IAS and GM plants. Both of them live a secondary spread after the introduction by humans; this stage occurs by means of the species itself (e.g. spread of pollen) and it is totally dependent on the rate of spread and its ability to establish (Bøhn 2007).

Moreover, the time of delay between the first introduction and the observed ecosystem changes can be very long, so that unintended ecosystem effects are difficult fact to handle. As GM crops have been introduced not so long time ago and usually located in absence of relatives, researches on long term effects of hybrids' invasiveness are lacking.

In a long-term perspective, IAS (both GM and non-GM) can have effects on several trophic levels, thus any evaluation of ecosystem consequences necessitates the understanding of complex ecological interaction; but studying these complex structure is not easy at all (Bøhn 2007).

Each ecosystem is characterized by resilience, i.e. the capacity to respond to a perturbation by resisting damage and recovering quickly, and some of impacts on natural ecosystems are reversible.

On the other hand, some perturbations are irreversible. For example, previous experiences have highlighted that eradication of IAS is successful only if precocious, otherwise it doesn't succeed. Similarly, carrying traits, that enhance fitness, makes huge the potential spreading of GMOs; thus, as IAS, they represent one possible irreversible disturbance of the environment (Bøhn 2007).

One of the important differences between IAS (non-GM) and GMOs concerns the public perception. A species, unusual for an environment, is visibly perceptible; but, GMO is not distinguishable from the equivalent non-GMO in the environment. This is true for hybrids between GM- and non-GM organisms, as well (Bøhn 2007).

Moreover, since the purpose of GMOs' introduction is for industrial production, it implicates a large scale and continuous utilization. On the contrary, non-GM IAS are often introduced as small numbers of individuals (Bøhn 2007).

The distribution of organisms is affected by limiting environmental factors. The modified traits, providing resistance to these factors, give higher fitness with GMOs. The consequence is a better survival in nature, which conveys in the expansion of geographical distribution because of an expansion of the ecological niche of GMOs. These considerations attract the attention to the constant interactions of human activities, as agriculture, and neighbouring ecosystems, and on the importance of studying the potential invasiveness of GMOs on a *case-by-case* basis.

In conclusion, both moral and utilitarian reasons suggest to protect biodiversity, since it is the base for the functioning of ecosystem services. Humankind is exploiting many of them in unsustainable ways. Thus, it is compulsory to test for the impacts of new kinds of activities, such as growing GM plants, on ecosystem services.

1.4 Woody versus Herbaceous Genetically Modified Plants (GMPs)

Biodiversity conservation must settle up with economic interests and technological development.

Indeed, the use of transgenosis as tool for producing transgenic crops is now widespread, although not commonly accepted (Farnum et al. 2007). The promises of transgenic crops are profound: pest resistance, tolerance to other biotic and abiotic stresses, healthier food, etc. But, despite these promises, there is a multitude of implications for food safety, environmental protection, animal welfare, socio-economic impacts, and regulatory aspects (Loader & Henson 1998; Freckleton et al.

2003). By 2012, 170.3 million hectares of biotech crops were grown, involving 28 countries worldwide (Fig. 1.2).

Nowadays, the bulk of the experimental studies appears to have been done on agronomic species (Farnum et al. 2007) rather than on trees. Certainly, the use of GM trees poses new and challenging concerns compared to GM crops at both spatial and temporal scales (Hamrick et al. 1992).

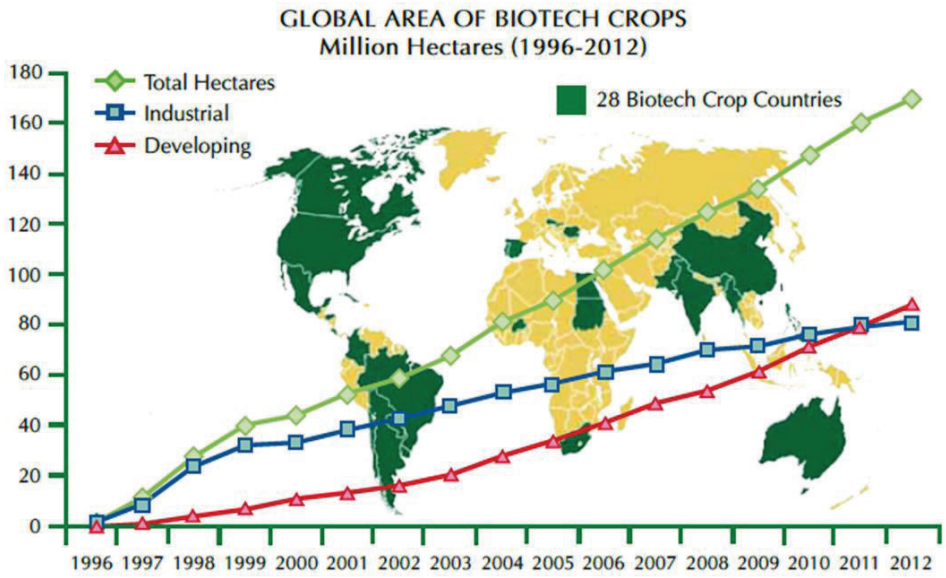

Fig. 1.2 A record 17.3 million farmers, in 28 countries, planted 170.3 million hectares (420 million acres) in 2012, a sustained increase of 6% or 10.3 million hectares (25 million acres) over 2011. Source: Clive James, 2012.

Trees are long-lived organisms, that can cover hundreds or thousands of hectares, with potential for spatially extensive gene flow (Hamrick et al. 1992; Slavov et al. 2002); they represent the dominant life form in many ecosystems (Gonzàlez-Martìnez et al. 2005) and have much longer generation intervals and crop cycle (Farnum et al. 2007). Moreover, many woody perennials are able to propagate vegetatively, they outcross considerably more than annual crops (Barrett 1998), and most of the forest-tree crop species hybridize naturally with wild congeners; all these factors highlight distinct biological features, which play major roles in determining GMO's impacts (Gonzàlez-Martìnez et al. 2005).

Both GM crops and trees hold modified traits related to tolerance/resistance to herbicides, insects and viruses, to abiotic stress, to functional properties.

Several crops as soybean, maize, cotton have been genetically modified to express different insecticidal proteins derived from the crystal proteins (*cry*) produced by *Bacillus thuringiensis* (*Bt*) strains (Baumgarte & Tebbe 2005).

Other traits affect the functional properties of the final product of crops. For example, oil composition have been changed in oilseed crops: higher oleic acid content in soybeans makes oil more stable during frying, or improving a desirable physical property in canola by increasing lauric acid content (Kok & Kuiper 2003).

As regards to GM trees, various modifications have been proposed (van Frankenhuyzen & Beardmore 2004; Farnum et al. 2007).

Herbicides tolerance, to glyphosate or glufosinate, was respectively achieved by using the *CP4* gene (from *Agrobacterium*) or the *bar* gene from *Streptomyces hygroscopicus* (van Frankenhuyzen & Beardmore 2004).

Insect resistance has focused on the use of delta-endotoxin genes (*cry*) from *Bacillus thuringiensis* (*Bt*) against lepidopteran pests (Wang et al. 1996).

For plant tolerance to abiotic stress (van Frankenhuyzen & Beardmore 2004), water-deficit and salt stress was obtained by inducing the over-expression of specific responsive protein (Wang et al. 2003); enhanced resistance to highlight and low-temperature stress was found out by over-expressing glutathione biosynthesis' enzymes (Foyer et al. 1995). Finally, Che et al. (2003) achieved transgenic plants able to grow in highly contaminated soils by insertion of the bacterial *merA* gene.

Traits related to wood quality are fundamental amongst the targets qualities of trees (van Frankenhuyzen & Beardmore 2004; Sticklen 2006; Harfouche at al. 2011). Removing lignin is a costly and energy-consuming component of the pulp and paper production process, that requires the use of polluting chemicals; that's why, reducing lignin content and/or modifying its composition for increased pulping efficiency have become essential objectives as an alternative to common processing technologies (Sticklen 2006).

The suppression of 4-coumarate:CoA ligase (4CL) or of cinnamoyl-CoA reductase resulted in a reduction in structurally normal lignin (Hu et al. 1999); down-regulation of other enzymes, such as caffeate O-methyltransferase or cinnamyl alcohol dehydrogenase (CAD), resulted in modified lignin structure (van Frankenhuyzen & Beardmore 2004).

Plant genetic engineering could increase biomass (Sticklen 2006), inducing the over-expression of the *gibberellin* (*GA*) *20-oxidase* gene from *Arabidopsis* (Eriksson et al. 2000) or inserting a single *Arabidopsis thaliana Flowering Locus C* (*flc*) gene, known to delay flowering, is able to shift the energy needed for reproduction into biomass growth, thus increasing the total amount of biomass (Salehi et al. 2005).

1.5 *Populus* spp.

1.5.1 Systematic and Evolution

The earliest fossil record of poplar leaves hails from the Late Palaeocene (58 million years ago); abundant fossil materials are available in many parts of the northern hemisphere (USA and Europe) and dated to Eocene (Manchester et al. 1986, 2006). Stated that the record are more complicated and difficult to interpret (Eckenwalder 1996), it seems that the section *Tacamahaca* Spach emerged in the Late Oligocene and the precursors of all extant sections were presumably present by the Miocene (Eckenwalder 1996; Cronk 2005). In particular, sec. *Aigeiros* Duby appeared in the Mid-Miocene and sec. *Populus* Duby in the Mid-Pliocene. However, further works are necessary to elucidate these evolutionary relationships (Dickmann & Kuzovkina 2008).

The current accepted classification of the genus *Populus* L. describes 29 species subdivided into six sections based on relative morphological similarity and crossability (Table 1.1; Eckenwalder 1996). In literature the number of species ranges from 22 to 85, excluding hundreds of hybrids, varieties and cultivars (Eckenwalder 1996). The main reasons of disagreement are the misclassification of natural hybrids and difficulties in drawing species boundaries (Eckenwalder 1996).

On the base of 76 morphological characters, a consensus cladogram showed that all sections are monophyletic; only the sec. *Tacamahaca* divided in two groups: the "balsam poplars" (e.g., *P. balsamifera* L. and *P. trichocarpa* Torrey & Gray) and the "narrow-leaved, thin-twigged" species (e.g., *P. angustifolia* James, *P. simonii* Carrière). Eckenwalder (1996) hypothesised that the genus firstly spread from North America or Asia in the Paleocene. After that, the precursors of subtropical sections (*Abaso* Eckenwalder and *Turanga* Bunge) separated by vicariance, while the sec. *Leucoides* Spach occupied temperate habitats. The other sections evolved in the Miocene.

Even though these sections are delineated by the occurrence of major hybridization barriers (Eckenwalder 1996), the placement of several taxa is still controversial. Moreover, recent molecular tools clearly contrast with some aspect of classification (Hamzeh & Dayanandan 2004; Cervera et al. 2005).

Cervera et al. (2005), for example, indicated that the most ancient section is the sec. *Populus* (alias *Leuce*). Analyzing RFLP of cpDNA, Smith and Sytsma (1990) demonstrated that the sec. *Tacamahaca* is polyphyletic and the sec. *Populus* is the terminal clade. On the contrary, considering the ITS region of the nuclear *rDNA* genes, Leskinen and Alstrom-Rapaport (1999) found *P. alba* L. (sec. *Populus*) as basal. The

classification of *P. nigra* L. within sec. *Aigeiros* is greatly debated, since it has a genetic affinity to species of sec. *Tacamahaca*. By means of RFLP analysis Smith and Sytsma (1990) found that cpDNA of *P. nigra* had similarity to species of the sec. *Populus*,

Table 1.1 Classification of the genus *Populus* modified by Eckenwalder (1996).

Section (synonym)	Species	Distribution
Abaso Eckenwalder	*Populus mexicana* Wesmael	Mexico
Turanga Bunge	*P. euphratica* Olivier	NE Africa, Asia
	P. ilicifolia (Engler) Rouleau	E Africa
	P. pruinosa Schrenk	Asia
Leucoides Spach	*P. glauca* Haines *sl*[a]	China
	P. heterophylla L.	USA
	P. lasiocarpa Olivier	China
Aigeiros Duby	*P. deltoides* Marshall *sl*[a]	N America
	P. fremontii S. Watson	USA
	P. nigra L.	Eurasia, N Africa
Tacamahaca Spach	*P. angustifolia* James	N America
	P. balsamifera L.	N America
	P. ciliata Royle	Himalayas
	P. laurifolia Ledebour	Eurasia
	P. simonii Carrière	E Asia
	P. suaveolens Fischer *sl*[a]	NE China, Japan
	P. szechuanica Schneider	E Eurasia
	P. trichocarpa Torrey Gray	N America
	P. yunnanensis Dode	Eurasia
Populus (*Leuce* Duby)	*P. adenopoda* Maximowicz	China
	P. alba L.	Europe, N Africa, Central Asia
	P. gamblei Haines	E Eurasia
	P. grandidentata Michaux	N America
	P. guzmanantlensis Vazques & Cuevas	Mexico
	P. monticola Brandegee	Mexico
	P. sieboldii Miquel	Japan
	P. simaroa Rzedowski	Mexico
	P. tremula L.	Europe, N Africa, NE Asia
	P. tremuloides Michaux	N America

[a] *Sensu lato*

while nuclear rDNA were distinct, suggesting a possible hybrid origin of *P. nigra*.

Many contradictions in the classification of the genus *Populus* are still unsolved. More molecular genetics and genomic data integrated with informative

morphological traits and the fossil record are inevitably indispensable (Slavov & Zhelev 2010).

1.5.2 Biology and ecology

The species of the genus *Populus* (aspen, cottonwood and poplars), collectively known as poplars, face a great diversity of habitats, with different climates and soils (Eckenwalder 1996; Dickmann & Kuzovkina 2001), playing significant ecological role as pioneer and dominant species (Braatne et al. 1992).

They are widely distributed over the boreal, temperate and subtropical zones of the northern hemisphere, including central Asia, northern Africa, Europe and North America (Slavov et al. 2010). They have also been widely planted throughout the world, including the southern hemisphere (Dickmann & Kuzovkina 2008).

Poplars commonly colonize disturbed sites and can form monotypic stands or mixed forests with other hardwood and conifer trees. They typically occur in or on the border of alluvial, riparian, and wetland habitats. Enduring temporary anoxic conditions, most of them are well adapted to seasonal flooding and high water tables; moreover, after the recession of surface water, the moist soil provides an ideal environment for seed germination (Braatne et al. 1996). For example, *P. nigra*, the European black poplar, is a typical pioneer tree species of the floodplain ecosystems, where it colonizes open areas being strictly heliophilous (Lefèvre et al. 2001). Its distribution area ranges from the Mediterranean through middle-north Europe, and from Ireland and England to western Asia.

In contrast to their wetland relatives, some poplars prefer dry and upland environments (i.e. sec. *Turanga*). The aspens (sec. *Populus*) grow in north-temperate uplands, ranging from wet mesic to xeric (Dickmann & Kuzovkina 2008). For example, *P. tremula* L. (European aspen) is an upland forest pioneer tree extending from plains into subalpine regions, occupying high altitudes in mountains (von Wühlisch 2009). It ranges from the British Isles, through Scandinavia and northern Europe, to the eastern Russia and China; in the south of its range a disjointed population grows on the Africa continent. The ecological role of these poplars is the colonization of upland areas disturbed by intense natural events; indeed, seeds are able to germinate readily on adequately moist and exposed mineral or ash-covered soil (Dickmann & Kuzovkina 2008).

Species of the genus *Populus* present wide phenotypic variance in morphology and reproductive features even within populations (Fig. 1.3; Slavov & Zhelev 2010).

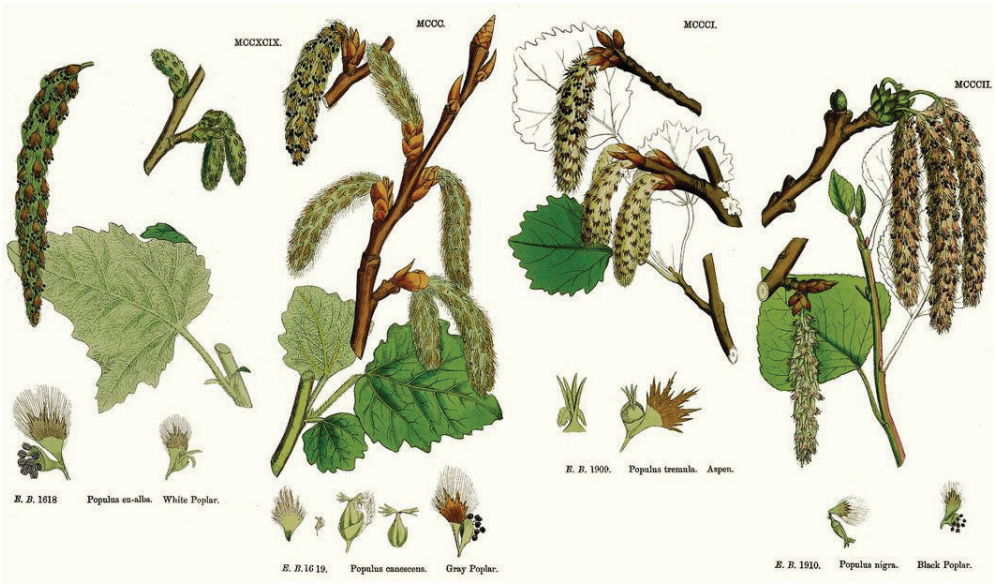

Fig. 1.3 Examples of phenotypic variance in leaves and in reproductive organs of different species of *Populus*. From left to right: *P. alba, P. x canescens, P. tremula, P.nigra.*
Source: Boswell 1868.

They are generally single trunked (Slavov & Zhelev 2010). The growth habit depends on quite variable morphology of twigs and branches. If the slant is large, the branches may generate a spreading crown; but, on the other side, if the slant is small, a narrow upright or fastigiate crown raises. The bark of young poplars can be creamy or dirty white (i.e. *P. alba*, *P. nigra*), various shades of grey, grey-green (i.e. *P. tremula*), olive-green, orange-brown, red-brown or bronze in colour, and it often remains smooth for many years. The colour and the surface of the lower bark can change with age, becoming dark and furrowed (i.e. *P. nigra*, *P. alba*). Lenticels are prominent and diamond shaped (Dickmann & Kuzovkina 2008).

Leaves morphology is distinctive but very complex and variable among taxa. They originate from vegetative axial or terminal buds, which are long, conical and sharp, with basal scales absorbed with resinous exudates (Eckenwalder 1996). In general, they are alternate and simple, with pinnato-palmate venation (Eckenwalder 1996) and elongated or pointed apex. Leaves may be longer or wider and the shape may be linear, lanceolate, oblong, obovate, deltoid, cordate, rhombic, round, reniform or palmately lobed.

Leaf size among taxa depends on the adaptation to different environments. In the arid ones poplars have small (5-10 cm^2 in area) and pubescent leaves; in the humid tropics or subtropics, leaves area may reach 500 cm^2. Petioles vary in length (from 1

to 10 cm) and are sometimes laterally flattened, causing a peculiar fluttering of leaves (i.e. some taxa in sections *Aigeiros* and *Populus*). Some poplars have leaves persistently (i.e. *P. alba*) or primarily (young leaves) pubescent (Dickmann & Kuzovkina 2008).

The heterogeneity in leaf characteristics is also detected between juvenile and adult trees (heteroblasty) and within tree (seasonal heterophylly) (Slavov & Zhelev 2010). Within tree, preformed leaves belong to primordia that overwinter in vegetative buds and substantially differ in shape and size from the neoformed leaves, initiated during the growing season by the apical meristem; as stated by Eckenwalder (1996), the preformed leaf characteristics have higher taxonomic value.

Populus spp. are typically dioecious, with both male (staminate) and female (pistillate) flowers grouped in pendent catkins, lacking petals and sepals that elongate from axial reproductive buds. Each catkin carries about 30-200 small and inconspicuous flowers and, occasionally, can be hermaphroditic (Dickmann & Kuzovkina 2008).

The colour, the length and the number of stamens widely vary among species and ovaries contain two to four carpels. Once the fruits is mature, tiny and cottony seeds are split out the capsules and spread by wind. Poplar seeds show substantial variation among species and genotypes within a species; old trees can produce 30-50 million seeds in a single season (Braatne et al. 1996; Dickmann & Kuzovkina 2008).

The genus *Populus* is capable of both sexual and asexual reproduction. The prolific seed production together with strong cloning ability makes poplars suitable to re-establish in flooding habitats and invade new ones after disturbance events. (Dickmann & Kuzovkina 2008)

Under favourable conditions, the reproductive maturity of poplars comes within 4–8 years in intensively managed plantations and within 10–15 years in natural populations (Stanton 1996). Catkins typically appear before leaves in early spring (Braatne et al. 1996; Eckenwalder 1996). The timing of flowering is temperature-dependent, so that populations living at higher-elevations, at northern latitudes and more continental climates, flowers later (Braatne et al. 1996; Slavov & Zhelev 2010).

Once mature, pollen grains are dispersed by wind, reaching remarkable long-distances (Tabbener & Cottrell 2003; Lexer et al. 2005; Pospíšková & Šálková 2006; Vanden Broeck et al. 2006; Slavov et al. 2009). Considering whole population, the pollination period can last one or even two months (Braatne et al. 1996); thereafter the male catkins fall to the ground.

After fertilization, the female catkins extend and develop into capsules. In several weeks to a month or more the fruits mature, capsules ripen and dehisce before leaves are fully developed.

A great numbers of tiny seeds, enveloped in downy fluff, disperse from capsules by means of wind and the empty capsules abscise soon thereafter (Fig. 1.4).

Fig. 1.4 Mature capsules on female catkins on the left and downy seeds dispersed on the right.

Seeds potentially can cover long distances by wind dispersion (10 km or more; Slavov & Zhelev 2010) and secondary dispersal may occur moving by water thus extending the range of diffusion (Braatne et al. 1996; Slavov & Zhelev 2010). Under natural conditions, seeds remain viable for only a few days or weeks and germination occurs within 24 h (Braatne et al. 1996). Seedlings establishment is numerically successful when the environmental conditions are suitable, even though high levels of mortality are observed in the first year (i.e. up to 77–100%) (Braatne et al. 1996; Dixon & Turner 2006).

Even the tendency to form clones by vegetative propagation varies among poplars and represents one of the distinctive characteristics of the genus (Dickmann 2001).

Most of them, especially aspens and white poplars, propagates vegetatively through root suckering (Slavov & Zhelev 2010), by means of sprouting of adventitious shoots on shallow lateral roots (Dickmann & Kuzovkina 2008). Poplars may produce a dense clonal forest by suckering; they adopt this strategy once killed, but living trees are able to propagate, invading open areas, sometimes even 30 m far away from the parent tree (Dickmann & Kuzovkina 2008).

Another way to reproduce vegetatively under natural conditions is sprouting from the root collar, after that a tree has been felled. Sometimes surviving root collar sprouts may take on the appearance of mature trees.

Typical of certain riparian poplars (especially cottonwoods and black poplars; Slavov & Zhelev 2010) is the vegetative reproduction through rooting of shoots or of entire tree trunks. This strategy has a high ecologically value for riparian poplars, because they are allowed to establish along the banks of streams or on sandbars after water recedes. It occurs when lateral twigs are abscised (i.e. cladoptosis), or during storms and floods, when branches break off or trunks fall and then lodge in sediment and in moist soil (Braatne et al. 1996; Rood et al. 2003; Barsoum et al. 2004; Smulders et al. 2008). Whereupon, they root and form new trees (Rood et al. 2003).

The evolution of extant species of *Populus* are supposed to have been strongly affected by natural hybridization (Eckenwalder 1996; Hamzeh & Dayanandan 2004; Cervera et al. 2005). Barriers to gene flow are frequently ineffective, especially where species are sympatric, i.e. their natural or planted distribution overlaps. Based on studies of morphological traits and molecular markers, an extensive hybridization has been demonstrated both within and among sections (Table 1.2; Eckenwalder 1996; Slavov & Zhelev 2010).

Table 1.2 Examples of naturally occurring hybrids of *Populus* (modified by Eckenwalder 1996; Slavov & Zhelev 2010)

Hybrid	Scientific name
P. alba x *P. adenopoda*	*P.* x *tomentosa* Carrière
P. alba x *P. tremula*	*P.* x *canescens* (Aiton) Smith
P. angustifolia x *P. balsamifera*	*P.* x *brayshawii* B. Boivin
P. angustifolia x *P. deltoides*	*P.* x *acuminata* Rydb.
P. angustifolia x *P. fremontii*	*P.* x *hinckleyana* Correll
P. balsamifera x *P. deltoides*	*P.* x *jackii* Sargent
P. deltoides x *P. nigra*	*P.* x *canadensis* Moench
P. grandidentata x *P. tremuloides*	*P.* x *smithii* B. Boivin
P. trichocarpa x *P. deltoides*	*P.* x *generosa* Henry
P. trichocarpa x *P. fremontii*	*P.* x *parryi* Sargent

Interspecific hybridization is common within most *Populus* sections. Cottonwoods (sec. *Aigeiros*) readily hybridize both naturally and under controlled conditions, and hybrids show hybrid vigour. The most important one is commonly known as *P.* x *canadensis* Moench (syn. *Populus x euramericana* (Dode) Guinier); this hybrid originated in France about 1750 by natural hybridization of *P. deltoides* Marshall trees, introduced from North America, with the native *P. nigra* (Rajora & Rahman 2003).

Another example of spontaneous interspecific hybridization is the grey poplar (*P.* x *canescens* (Aiton) Smith), the hybrid between *P. alba* (maternal parent) and *P.*

tremula. It occurs in areas where the ranges of the parent species are sympatric and shows intermediate morphological characteristics and hybrid vigour. Moreover high level of backcrossing, especially with *P. alba* (Lexer et al. 2005), implied long-term effects for introgression of genes from one taxon into another or evolution of new species (Dickmann & Kuzovkina 2008).

Intersectional hybrids also occur spontaneously in nature. Most of the crossings are reported between taxa in *Aigeiros* and *Tacamahaca*, but hybridization also occurs among these sections with *Leucoides* as maternal parent (Smith & Sytsma 1990). On the other hand, it is difficult to meet successful matings across sectional lines in *Abaso, Turanga, Leucoides* and *Populus* (Stettler et al. 1980).

1.5.3 Importance and uses

The importance of poplars resides in their ecological, economical and genetic features.

They are commonly used both in urban areas and in rural areas as ornamentals, for landscaping, as windbreaks and field shelterbelts. Poplars provide many environmental benefits like moderation of climate, protection from wind, decrement of noise pollution and dust, and improvement of the air quality. They are also useful to prevent erosion of banks streams and rivers, maintaining a more natural landscape (Isebrands & Karnosky 2001). Poplars can be considered as 'foundation species', which drive forward the diversity and structure of a number of dependent communities (Whitham et al. 2006).

Since they can remove, degrade or contain chemical contaminants located in the soil by phytoremediation, they intercept contaminants before they reach the stream or recover polluted sites with heavy metals, pesticides, chlorinated solvents, hydrocarbons, excess nutrients and others pollutants (Balatinecz & Kretschmann 2001).

The last but not the least, poplars contribute to carbon sequestration and provide a natural mitigation strategy in the storage of carbon dioxide (CO_2) in biomass and soil (Isebrands & Karnosky 2001).

In regard to the economic point of view, spontaneous hybridization among sympatric and introduced species has facilitated the domestication of the genus and get off poplar breeding programs (Bisoffi & Gullberg 1996). Therefore clonal selection of hybrids have been used into commercial culture for centuries (Stettler et al. 1996) and is largely due to the relative ease of vegetative propagation, compared with sexual reproduction (McIvor & Hurst 2013).

They are an economically important source for production of lumber and pulp (Sticklen 2006) and recently, for production of biofuel feedstock (Rubin 2008; Slavov & Zhelev 2010).

Many breeding programs are focused on this genus in order to increase wood production, for example by providing adaptive properties for various soil and climate conditions, excellent rooting ability of stem cuttings, and resistance to pathogens. Poplars are able to produce large quantities of wood (Slavov et al. 2010), thanks to the rapid juvenile growth, both in natural and planted stands, the easy production of hybrids and the easy vegetative propagation; thus, poplar's hybrids are ideal for short-rotation intensive culture (Dickmann & Kuzovkina 2008). Italy, with an area of more than 6000 ha, is actually the European country with the widest land area planted with poplar in short-rotation culture (Sabatti & Nardin 2008).

Important long-term breeding programmes have a long history in Europe (Italy, France, Belgium, Netherlands), where the most widely used species are *P. deltoides, P. nigra, P. trichocarpa* and occasionally *P. maximowiczii* Henry.

For example in Netherlands, four Euramerican clones (*P. deltoides x P. nigra* in different combinations) named 'Polargo', 'Albelo', Degrosso' and 'Sanosol' have been developed and now commercially released all over Europe (de Vries 2008).

In Italy, a long-term breeding program is being carried out since the 1980s by the "Unità di Ricerca per le Produzioni Legnose Fuori Foresta (ex-Istituto di Sperimentazione per la Pioppicoltura) – CRA (Consiglio per la Ricerca e la Sperimentazione in Agricoltura)". A large number of native *P. nigra* and the best *P. deltoides* females in controlled crossing or open pollination (Fabbrini 2010), are used to produce improved populations of both species. Through semi-reciprocal recurrent selection of parents, the program aims to obtain parents of high breeding value to generate valuable *P. x canadensis* hybrids (Vietto 2008).

To date, the "Unità di Ricerca per le Produzioni Legnose Fuori Foresta" has provided several clones, available for commercialization in Europe; the crossings included *P. deltoides, P. nigra*, or their varieties, or their hybrids (i.e. Taro, whose maternal parent is *Populus sp.*'71-043', namely *P. deltoides* Bartr. '51- 119' × *P. x canadensis* Mönch 'I-262').

The transformability, clonability by vegetative propagation from root and stem cuttings, rapid growth, a relatively short juvenile period (Slavov & Zhelev 2010), and extensive genomic resources, including an available genome sequence (Tuskan et al. 2006), make poplar an extraordinary model to investigate the potential for transgenic biotechnology (Strauss et al. 2004). They can be useful to demonstrate gene

functions, and to carry out basic research in genomics and in physiological processes (Strauss et al. 2004; Slavov et al. 2010) also considering that the genome of *P. trichocarpa* has already been completely sequenced (Tuskan et al. 2006). So, recombinant-DNA technology can modify endogenous genes, already present in the tree genome, improving certain traits, such as fiber quality and quantity, while exogenous genes can be transferred from unrelated organisms to confer entirely novel traits, such as resistance to herbicides, diseases or pests (van Frankenhuyzen & Beardmore 2004; Eriksson et al. 2000).

1.5.4 Genetics

Poplars are generally diploids, with two sets of 19 ($2n=38$) chromosomes (Blackburn & Harrison 1924; OECD 2000). Polyploid individuals (i.e. triploids and tetraploids) are rare but existing; the earliest discovery of a triploid forest tree was a clone of *P. tremula* (Müntzing 1936).

The genome is very small – six time smaller than *Zea mays* L. and 40 times smaller than *Pinus taeda* L.. In 2004, the sequencing of the genome of *P. trichocarpa* was completed by a worldwide team of researchers (Tuskan et al. 2006). The *Populus* genome measures about 485 megabases of DNA. The nuclear genome contains more than 45000 protein-coding genes. Analysis on the chloroplast and mitochondrial genomes revealed that they included 101 and 52 genes, respectively. Until now, several genes and gene families have been identified and are linked to lignocellulose wall formation, secondary metabolism, disease resistance, membrane transport and phytohormone biosynthesis and regulation (Dickmann & Kuzovkina 2008).

As already mentioned, the variation of the wide distributed *Populus* spp. and the chances to produce novel genotypes through hybridization are enormous. Indeed, the genetic variation for neutral molecular markers reaches high levels, since poplars have obligatorily outcrossing mating systems, large population size and extensive pollen and seed dispersal.

The first studies of the population genetics of the genus *Populus* (Table 1.3) were carried out by allozyme markers and Restriction Fragment Length Polymorphisms (RFLP) and highlight several aspects. The levels of polymorphism (average number of alleles per locus, *A*) and expected heterozygosity under Hardy-Weinberg equilibrium (H_e; Nei 1973) are consistent with the mean values for long-lived woody species (A = 1.8, H_e = 0.15) and are higher than those for plants in general (A = 1.5, H_e = 0.11; Hamrick et al. 1992). The differentiation among populations, measured by F_{ST} (Wright 1965), is weak. This result is in accordance with the direct analysis of gene flow, which

indicate an extensive and long-distance pollination in *Populus* (Tabbener & Cottrell 2003; Pospíšková & Šálková 2006; Vanden Broeck et al. 2006; Slavov et al. 2009).

Table 1.3 Allozyme and RFLP diversity and differentiation in *Populus* (modified by Slavov & Zhelev 2010).

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}	References
Turanga	*P. euphratica*	20[b]	3	85	1.8	0.10	0.24	0.592	-	Rottenberg et al. (2000)
Aigeiros	*P. deltoides*	33[b]	9	84	1.2	0.06	-	-	-	Rajora et al. (1991)
	P. fremontii	22[b]	21	-	1.5	-	0.08	-	0.064	Marty (1984)
	P. nigra	36[c]	4	47	1.5	0.18	0.15	-0.175	0.074	Martinsen et al. (2001)
		8[b]	3	146	-	-	0.16	0.113	0.063	Legionnet and Lefèvre (1996)
Tacamahaca	*P. angustifolia*	36[c]	10	281	1.4	0.10	0.08	-0.236	0.022	Martinsen et al. (2001)
	P. balsamifera	17[b]	5	248	-	-	0.04	0.061	0.014	Farmer et al. (1988)
	P. trichocarpa	18[b]	10	456	1.2	-	0.09	-	0.063	Weber and Stettler (1981)
Populus	*P. grandidentata*	14[b]	-	96	1.4	0.07	0.08	0.125	-	Liu and Furnier (1993)
	P. tremula	37[c]	-	75	1.8	0.12	0.13	0.077	-	Liu and Furnier (1993)
	P. tremuloides	11[b]	6	233	1.7	0.15	0.17	0.153	0.014	Easton (1997)
		10[b]	5	41	1.7	0.33	0.23	-0.427	-	Lopez-de-Heredia et al. (2004)
		26[b]	7	222	2.3	0.52	0.42	-0.238	-	Cheliak and Dancik (1982)
		15[b]	8	200	2.7	0.13	0.24	0.462	0.068	Hyun et al. (1987)
		10[b]	9	347	2.6	0.22	0.22	0.017	0.003	Lund et al. (1992)
		17[b]	6	156	2.4	0.32	0.29	-0.102	0.030	Jelinski and Cheliak (1992)
		13[b]	-	118	2.8	0.19	0.25	0.240	-	Liu and Furnier (1993)
		41[c]	-	91	2.7	0.21	0.25	0.160	-	Liu and Furnier (1993)
	Median[d]	18	7	146	1.8	0.17	0.170	0.077	0.047	

[a] N_{loci} is the number of loci used; N_{pop} is the number of populations sampled; N is the number of genets (or trees) analyzed; A is the average number of alleles per locus detected in each population; H_o is the observed heterozygosity; H_e is the expected heterozygosity (Nei, 1973); F_{IS} is the fixation index as reported in the study or calculated as $F_{IS} = (H_e - H_o)/ H_e$; F_{ST} is the among-population differentiation (Wright, 1965).

[b] Allozyme markers.

[c] Restriction Fragment Length Polymorphisms.

[d] Because relatively few studies were included, medians were calculates in order to minimize the influence of extreme values.

The median value of F_{ST} for the genus (0.047) is about two times lower than the mean for long-lived woody species (F_{ST} = 0.084) and almost five times lower than that for plants in general (F_{ST} = 0.228; Hamrick et al. 1992).

More recently, the growing use of variable microsatellites has facilitated and increased population genetic studies in *Populus* (Table 1.4). Because of high mutation rates, microsatellite analysis are not comparable to the previous ones (by allozyme and RFLP markers).

High levels of observed and expected heterozygosities place into the range of values reported for other angiosperm (Brondani et al. 1998; Streiff et al. 1998) and gymnosperm (Elsik & Williams 2001) trees. Unlike allozyme and RFLP analysis, the departures from Hardy-Weinberg equilibrium due to heterozygote deficiency are slightly more common, probably because of the higher rates of null alleles at microsatellite loci (Slavov & Zhelev 2010). As for allozyme and RFLP markers, the microsatellite analysis reveal a typically weak differentiation among populations (Slavov & Zhelev 2010).

Table 1.4 Microsatellite diversity and differentiation in *Populus* (modified by Slavov & Zhelev 2010)

Section	Species	N_{loci}	N_{pop}	N	A	H_o	H_e	F_{IS}	F_{ST}/R_{ST}	References
Aigeiros	*P. deltoides*	10	-	20	5.2	0.23	-	-	-	Rahman and Rajora (2002)
	P. fremontii	4	-	20	5.3	0.60	0.52	-0.146	-	G.T. Slavov (unpublished data)
	P. nigra	6	22	574	-	0.78	0.73	-0.077	0.047	Imbert and Lefèvre (2003)
		12	-	60	11	0.80	0.82	0.030	-	Pospíšková and Šálková (2006)
		7	17	921	-	0.74	0.76	0.027	0.081	Smulders et al. (2008)
Tacamahaca	*P. angustifolia*	4	-	28	4.3	0.45	0.44	-0.033	-	G.T. Slavov (unpublished data)
	P. balsamifera	10	-	29	6	0.35	-	-	-	Rahman and Rajora (2002)
	P. trichocarpa	9	47	372	6.1	0.60	0.80	0.293	0.078/0.112	Ismail et al. (2009)
		10	2	282	17.5	0.71	0.77	0.058	-	Slavov et al. (2009)
Populus	*P. alba*	20	2	40	-	0.38	0.39	0.021	-	Lexer et al. (2005)
	P. tremula	19	1	169	6.4	0.37	0.38	0.027	-	Van Loo et al. (2008)
	P. tremuloides	20	2	40	-	0.47	0.50	0.055	-	Lexer et al. (2005)
		9	3	113	-	0.35	0.41	0.120	0.117	Suvanto and Latva-Karjanmaa (2005)
		25	12	116	-	0.50	0.62	0.197	0.015	Hall et al. (2007)
		4	4	159	7.4	0.56	0.72	0.201	0.032/0.041	Wyman et al. (2003)
		16	11	189	4.9	0.41	0.45	0.093	0.045	Cole (2005)
		4	-	266	8.8	0.47	0.67	0.300	-	Namroud et al. (2005)
	Median[b]	10	4	116	6.1	0.47	0.62	0.055	0.047[c]	

[a] Parameter designation is the same as in Table 1.3. R_{ST} is an analog of F_{ST}, which is based on the Stepwise Mutation Model (Slatkin, 1995).

[b] Because relatively few studies were included, medians were calculated in order to minimize the influence of extreme values.

[c] Based on F_{ST} values.

Further genetic association studies are needed to improve information on Single Nucleotide Polymorphism (SNP) data in *Populus* (DiFazio 2005; González-Martínez et al. 2006). Until now, nucleotide diversity seems to vary considerably among species and genes (Gilchrist et al. 2006), even though are still equivalent to levels found in other tree species (González-Martínez et al. 2006; Savolainen & Pyhäjärvi 2007).

As already mentioned above, interspecies hybrids are suitable to be used in short rotation coppices thanks to their fast growth and disease resistance traits. Hybrids are morphologically extremely variable and thus, species identification within the genus *Populus* is difficult. But, species identification of hybrids, including both identification of existing clones and the breeding of new ones, is pivotal for breeding activities, especially for the registration of high-performing clones (Schroeder et al. 2012; Schroeder & Fladung 2014). Therefore, genetic markers are needed (Schroeder & Fladung 2010; Schroeder & Fladung 2014).

A worldwide project was launched to differentiate living species on the base of a genetic approach (Degen and Fladung 2007), by means of DNA-based molecular marker systems ('DNA-barcoding;' Barcode of Life http://www.barcodeoflife.org; Schindel & Miller 2005).

In the Animal Kingdom, the DNA region of the mitochondrial cytochrome oxidase gene is suitable and now widely used for barcoding. The same one is not useful in plants, where it is highly invariant (Schroeder et al. 2012). Until now, the choice of a proper region in plants resulted much more difficult: for example, regions in plastid genomes are often not sufficiently variable for barcoding or are suitable for few plants (Newmaster et al. 2006; Schroeder et al. 2012).

The recent publication of the full genome of *P. trichocarpa* (Tuskan et al. 2006) and its relative small size, made poplar a model species for tree genomics. Indeed, testing barcoding regions in poplar species aims to improve easy-to-use and cheap genetic markers for quick species identification techniques, an important tool for breeding programmes (Schroeder & Fladung 2010).

Up to now, several genetic markers in different DNA regions have been tested. Liesebach et al. (2010) tested SSR markers and found out that they are not helpful for species identification in breeding projects. Schroeder and Fladung (2010) used 40 barcoding primer combinations over 20 chloroplast regions, both intergenic spacers and coding regions, in order to identify the best ones in differentiating poplar species; they found that three chloroplast regions were necessary to discern between seven poplar species, suggesting the use of multi-locus combinations as already stated by previous studies (Kress & Erickson 2008; Schroeder et al. 2012).

1.6 *Brassica* spp.

The family Brassicaceae (Cruciferae) holds over 338 genera and 3709 species (Al-Shehbaz et al. 2006) and includes crop plants grown worldwide (e.g. tribes Brassiceae and Cardamineae).

Several but not definitive works have been carried out for the phylogenesis of the family, but they are often inconsistent to each another. Conventionally, the Brassicaceae have been divided into 25 tribes and subtribes on the basis of relatively few characters (morphology, embryology) (Al-Shehbaz et al. 2006). Modern molecular analysis disclosed that morphological variation, by itself, is not able to provide phylogenetic distinction of several groups (Koch et al. 2001). Molecular phylogeny and re-evaluation of morphological characters accepted 34 tribes (Koch & Al-Shehbaz 2009; Warwick et al. 2010). Phylogenetic studies based on *ndh*F analysis recognized 25 tribes (Beilstein et al. 2006), which have been confirmed by different researches ITS-based (Bailey et al. 2006) and mtDNA-based (Warwick et al. 2010). The remaining 9 tribes were based on ITS-based phylogenetic studies (Al-Shehbaz & Warwick 2007). Lately, Warwick et al. 2010 found 44 tribes analysing the ITS region of DNA sequence data of nuclear ribosomal.

The tribe *Brassiceae* holds 47 genera and 235 species (Warwick et al. 2010), belonging to six subtribes, and some of them are of primary interest for agricultural economy (e.g. *Brassica* L., *Sinapis* L., *Diplotaxis* DC, *Erucastrum* (DC)C. Presl, *Hirschfeldia* Moench, *Eruca* Mill. and *Raphanus* L.; OECD 2012; Koch and Al-Shehbaz 2009). Since the prehistoric times some of them have been cultivated (Koch et al. 2003) and selected to suit the needs of humans (OECD 2012). Nowadays, they are economically important for several uses, e.g. edible vegetable oil, condiments, vegetables (Koch et al. 2003). Oilseeds include *Brassica napus* L., *B. juncea* (L.) Czern., *B. rapa* L. and *B. carinata* A. Braun., while *B. juncea*, *Sinapis alba* L., *B. nigra* (L.) W.D.J. Koch represent the mustard condiment crops; lastly, the vegetable Brassicaceae include *B. napus*, *B. rapa*, *B. oleracea*, *Raphanus sativus* L.. Moreover, the family includes several weedy species, interesting with regard to cross-pollination with *B. napus,* i.e. *Sinapis arvensis* L., *Raphanus raphanistrum* L., *B. rapa* and *Hirschfeldia incana* (L.) Lagr.-Foss. (OECD 2012). Indeed, the main use is the oil production: recent estimates of agricultural land use showed that the *Brassica* oilseed crops occupy over 26 million hectares and are among the most important source of edible oil, providing 14% of the world's edible vegetable oil (OECD 2012).

Table 1.5 The "Nigra" and the "Rapa/Oleracea" lineages found through cpDNA (modified by OECD 2012).

Lineage	Species
"Nigra"	*Brassica nigra*
	B. fruticulosa Cirillo
	B. tournefortii Gouan
	Sinapis bubescens L.
	S. alba
	S. flexuosa Poir.
	S. arvensis
	Coincya cheiranthos (Vill.) Greuter & Burdet
	Erucastrum canariense Webb & Berthel.
	Hirschfeldia incana
"Rapa/Oleracea"	*Brassica rapa*
	B. oleracea
	B. rupestris-villosa complex
	B. barrelieri (L.) Janka
	B. deflexa Boiss.
	B. oxyrrhina (Coss.) Willk.
	B. gravinae Ten.
	Diplotaxis erucoides (L.) DC.
	D. tenuifolia (L.) DC.
	Eruca sativa Mill.
	Raphanus raphanistrum
	R. sativus
	Sinapis aucheri Boiss.

The tribe is distributed mainly in the Mediterranean region, in the south-western of Asia and South Africa; only four species of *Cakile* Mill. are native to North America (Koch & Al-Shehbaz 2009).

The earlier classification resulted from taxonomic studies (Schulz 1936), but nowadays it has been modified and criticised. Indeed, both molecular (Warwick & Black 1997; Warwick & Sauder 2005) and taxonomic recent researches have been carried out, reallocating taxa and establishing that only a few genera are monophyletic (Warwick & Black 1997). Warwick and Black (1991) tested only chloroplast DNA restriction sites together with cpDNA probes on 33 taxa of the subtribe Brassicinae and they clearly found two ancient and distinct evolutionary lineages within it: the "Nigra" and the "Rapa/Oleracea" lineages (Table 1.5), (OECD 2012).

1.6.1 *Brassica napus* L.

1.6.1.1 Systematic

The genus *Brassica* is classified as follows:

Order Brassicales (= Cruciales)
Family Brassicaceae (= Cruciferae)
Tribe Brassiceae
Subtribe Brassicinae
Genus *Brassica* L.

Fig. 1.5 Morphology of *B. napus.* Source: Boswell 1868.

B. napus (oilseed rape; Fig. 1.5) is of relatively recent origin resulting by the spontaneous hybridisation of an interspecific cross between ancient forms of *B. rapa* and *B. oleracea*, in the Mediterranean or the European west coastal regions, where both the two species occurred (Olsson 1960a). The consequent dispersal of the species is probably occurred throughout Europe in the 16th century, with the introduction to the Americas in the 17th and 18th centuries and the Far East in the 19th century (OECD 2012).

Firstly, Erickson et al. (1983) stated that *B. oleracea* was the maternal parent; but next analysis based both on SSR nuclear and plastid markers showed that *B. rapa* is the more likely plastid genome donor (Flannery et al. 2006). Moreover, the data supported the occurrence of several interspecific crosses which would explain multiple origins of *B. napus* (OECD 2012).

1.6.1.2 Biology and ecology

B. napus is cultivated in most European countries, throughout temperate regions and has both an annual (spring) and a biennial (winter) form. Oilseed rape crops are part of the European landscape since a very long time and coexist with the weedy, related species, *Sinapis arvensis* and *B. rapa*. Road and rail verges, field margins and disturbed soils are often colonized by feral populations of *B. napus*, even though their abundance and persistence differ greatly from country to country and between the two forms (OECD 2012). Oilseed rape prefers fertile and well-drained soils; growth is favoured by sunny days and cool nightsp (Duke 1983).

Oilseed rape have branched and erect stem, up to 1.5 m tall, often purple toward base (Duke 1983) The lower leaves are glaucous and form a rosette with petioles 10–30 cm long; from the rosette the flowering stalk emerges and bears a dominant, indeterminate main raceme, high up to 1 m (Duke 1983). The upper leaves are small, lanceolate, sessile and the way to clasp the stem represents a good feature to distinguish among some species of *Brassica*; in oilseed rape the upper leaves are partially clasping (OECD 2012).

The flowers are regular, bisexual and pale yellow; the siliques are ascending on slender pedicles and about 7 to 10 cm long with a beak about 0,5-3 mm long. Each silique contains two rows of seeds (from 10 to 30). Seeds are dark brown to black, and weigh 2.5 to 5.5 g per 1000 seeds (OECD 2012). Once ripe, the silique dehisces and disperses seeds.

Oilseed rape is 70% self-pollinating and 30% cross-pollinated.

The flowering proceeds from the lowest bud on the main raceme upward. After few days, flowers on the secondary branches open; in the evening they close and open again the following morning. On the third day the petals and sepals begin to wilt. As soon as the flower opens, if the weather is warm and dry, most part of the pollen is dispersed. Even though pollen is heavy and slightly sticky, it can be transported by wind thanks to the small size (30 to 440 µm). Moreover insects, in particular honey bees, can act as pollen vectors (Timmons et al. 1995). The stigma remains receptive from 3 days before to 3 days after the flower opens. Fertilization occur within 24h from the pollination and after that the ovary develops into a bivalve silique. Both the winter and the spring form have shattering mature pods which leave a large amounts of seeds on the ground (OECD 2012). Abiotic stresses could induce secondary dormancy in the shattered seed so that a small percentage can remain dormant and viable for 10 years or more (Lutman et al. 2003). Thus, *B. napus* is able to establish seed banks within cultivated fields (OECD 2012).

The outcrossing rate within fields ranges between 20 to 40%, mainly depending on the environmental conditions during flowering (Becker et al. 1992). Crossings between even distant fields can occur, since there are neither genetic nor morphological barriers to cross pollination (Becker et al. 1992); thus, pollen from neighbouring *B. napus* compete with the plant's own pollen to effect fertilization (OECD 2012).

Mechanisms preventing self-fertilization by inhibiting pollen tube growth of self-pollen on the stigma (i.e. self-incompatibility, SI) and in particular sporophytic SI system are widespread in the Brassicaceae (Ford & Kay 1985; Rahman 2005). Both its

parents (*B. rapa* and *B. oleracea*) have a sporophytic self-incompatibility system, but oilseed rape is generally self-compatible. Contrasting results showed (Olsson 1960b) the occurrence of naturally self- incompatibility in oilseed rape (Gowers 1989).

The occurrence of natural hybridization and introgression depends on a series of preconditions to fertilization, such as physical proximity of the parents, pollen movement and longevity, synchrony of flowering, breeding system of the parents, flower characteristics, pollen-style compatibility, and competitiveness of foreign pollen. Once the crossing occurs, the resulting hybrid must be fertile and have adequate fitness to backcross with the parent and thus, in turn, produce fertile progeny. But, introgression takes place only if there is pairing between parental chromosomes. The extent to which hybrid formation can occur differs widely among *Brassica* specie and generally, if there is an excess of rape pollen, chances of hybridization are significantly enhanced (Daniels et al. 2005; Scott & Wilkinson 1998). Several studies have been carried out on oilseed rape crossings.

Wei and Darmency (2008) reported that the hybrids between male sterile *B. napus* and *B. juncea, B. nigra*, *H. incana* and *R. raphanistrum* arose from few and small seeds and then seedling establishment was difficult under field conditions. Scheffler and Dale (1994) found that crosses between *B. oleracea* and *B. napus* are not easily produced, least of all in natural populations (Raybould & Gray 1993).

A wide study has been carried out by Chèvre et al. (2004), collecting data on potential natural cross and gene introgression from *B. napus*. In Europe and North America, 14 species related to *B. napus* have been identified. The highest levels of natural cross and introgression were found in the crossing between *B. napus × B. juncea* and vice versa, and *B. napus × B. rapa* and vice versa; *B. napus × H. incana* and *vice versa* (Lefol et al. 1996), showed high level of natural cross but low level of introgression.

Hybrids have not been produced naturally with *B. nigra, Sinapis arvensis* and *S. alba*, but only in the laboratory by means of embryo rescue (Daniels et al. 2005). Using hand pollination techniques, Moyes et al. (2002) obtained hybrids at low rate with *S. arvensis* as the maternal parent, although they did not produce viable pollen or seed. The evidence of possible spontaneous hybridisation between *Sinapis arvensis* (as the maternal parent) and *Brassica napus* has been firstly reported by Daniels et al. (2005); they obtained crosses at very low rate, even though wild mustard is the most common wild relative found associated with arable fields.

1.6.1.3 Importance and uses

Since the middle ages oilseed rape had been cultivated in Europe and only recently it have been spread in the world (OECD 2012). The increasing demand for edible oils and for biodiesel has consequences on the exploitation of this crop.

In the last ten years, the *Brassica* seed oil production has increased some 60% in the world (OECD 2012).

Winter oilseed rape (*B. napus*) is grown in both Western and Eastern Europe (Poland, Western Russia and the Ukraine); while, spring *B. napus* is used to replace the winter oilseed rape fields killed by cold.

Rapeseed oil was primarily used as a lubricant for steam engines or as a lamp oil, but then the *B. napus* and *B. rapa* oils became an important constituent of margarine. Indeed, breeding and selection, all over the world, successfully developed plants of *B. napus*, *B. rapa* and later *B. juncea* that produced nutritionally superior and more suitable to manufacture oils (Downey 1964). The new characteristic of this new natural oil , called 'canola oil', is the lesser content of erucic acid of the fatty acid total (OECD 2012).

As regard to the agroecosystem, *Brassica* species provide forage for many insects as well as wild life, but which limit the crop yield. Indeed, they produce a family of sulphur compounds (glucosinolates) which are both attractive and toxic for herbivores (OECD 2012).

1.6.1.4 Genetics

Oilseed rape is diploid, with two sets of 19 ($2n$ = 38) chromosomes. The direct cytology approach has been limited by small chromosome size of the Brassicaceae family. The whole genome sequence of *B. rapa* (A genome; Wang et al. 2011) and very recently of *B. oleracea* (C genome; Liu et al. 2014) are available at a web-based database (the *Brassica* database – BRAD; Cheng et al. 2011) and are providing a clearer picture of species interrelationships (OECD 2012). However, the *B. napus* genomes sequence has not been released, yet.

The genome size of the *Brassica* diploids is approximately 500-700 Mbp. Comparing the gene content of *Brassica* to *Arapidopsis thaliana* (L.) Heynh, Parkin et al. (2005) found high level similarity, up to 87% of sequence identity in the coding regions.

The cultivated *Brassica* shows significant genetic diversity, analyzed by nuclear RFLP (Restriction Fragment Length Polymorphisms) markers (Song et al. 1988). This evidence supports the theory of multiple centres of origin for *B. rapa* and *B. oleracea*,

which come from a different evolutionary pathway as respect to *B. nigra.* Then, *B. napus* and *B. juncea* have a polyphyletic origins, as resulting from different combinations of diploid morphotypes (OECD 2012).

B. napus is a relatively modern species, which results from human agricultural activities and thus, probably it has never existed in wild populations. For this reason, its genetic diversity is significantly less than that of the other diploid ancestor species and mostly depends on the genetic diversity of its progenitors.

1.6.2 *Sinapis arvensis* L.

1.6.2.1 Systematic

The genus *Sinapis* is classified as follows:

Order Brassicales (= Cruciales)
Family Brassicaceae (= Cruciferae)
Tribe Brassiceae
Subtribe Brassicinae
Genus *Sinapis* L.

Testing chloroplast DNA restriction sites, Warwick and Black (1991) found two distinct evolutionary lineages within the subtribe Brassicinae. The "Nigra" lineage included a distinct cpDNA subgroup formed by *Sinapis arvensis* together with *S. alba* and *S. flexuosa*; moreover *S. arvensis* showed a very close relationship with *Brassica nigra*, as already stated by other cytological, isozyme and nuclear DNA studies (Song et al. 1988; Warwick & Black 1991).

Fig. 1.6 Morphology of *S.arvensis.*
Source: Boswell 1868.

1.6.2.2 Biology and ecology

The annual forb *Sinapis arvensis* L. (wild mustard; Fig. 1.6) is a weed, living on calcareous and heavy soils (Rees & Brown 1991). It is susceptible to frost and it prefers mainly habitats with a high light intensity (Fogg 1950). Wild mustard is very common in arable land and widespread throughout Europe, North Africa, South East Asia while it has been introduced into America and Australia (Fogg 1950).

Seedlings of *S. arvensis* initially form a rosette and later develop into an erect plant. It presents a main branched stem 30-100 cm high, with stiff downward-pointing hairs, at least at the base. At the junction with the main stem, branches are often purple.

Leaves are alternate and hairy, particularly on the lower surface. Lower leaves are stalked, obovate, large in terminal segment with few smaller lateral lobes; the upper ones are smaller, sessile and roughly toothed.

Flowers are grouped in raceme and are very similar to other annual yellow-flowered mustards, so that it is often confused (Mulligan & Bailey 1975).

Pods spread from the stem on thick stalks; the siliques are ascending, glabrous and long 2,5-3,8 cm with an angular beak 0,6-1,3 cm long. Each silique contains two rows of seeds (4-8 seeds), which are round, about 1,5 mm across and black.

The reproduction occurs via seeds while the vegetative reproduction is absent (Fogg 1950; Mulligan & Bailey 1975).

Under favourable conditions, the flowering starts at the bottom of raceme, 6 weeks after plant's emergence and continues for other 6 weeks or more (Fogg 1950). The flowers are slightly protogynous and last for two days; because normally they are massed together, both self- and cross-pollination depend on insect visits (Fogg 1950). Self-pollination can take place both in rainy or dry weather (Fogg 1950). Cross-pollination is mediated by a wide variety of insects, mainly Hymenoptera and Diptera (Mulligan & Bailey 1975).

Seeds are not normally scattered too far away, except by the agency of birds or man. Indeed, several birds feed on wild mustard (Marshall et al. 2003). Some seeds are capable of germination as soon as they are mature, just after 4 days if the moisture is abundant (Mulligan & Bailey 1975). But under natural conditions germination is markedly periodic (Fogg 1950). The maximum occurs in spring, when the temperature is favourable and the dormancy of the previous year's seed ends; in summer, germination is presumably reduced by lack of water (Fogg 1950). Seed persistence and viability is known to reach at least 11 years, both when it is buried in soil or when air-dry (Fogg 1950).

As in the whole family, several studies demonstrated that *S. arvensis* have a single-locus sporophytic self-incompatibility (SSI) (Bateman 1955; Ford & Kay 1985). This SSI is controlled by the diploid (sporophyte) genotype of the parent (Hiscock & Taba 2003) and, in particular is genetically controlled by a complex and polymorphic locus (locus S, Schopfer et al. 1999). Up to now, the number of S-alleles found in natural populations ranges from 30 to 40 alleles (Hiscock & Taba 2003).

For natural hybridization and introgression See Par. 1.6.1.2

1.6.2.3 Importance and uses

From the strictly productive point of view, *Sinapis arvensis* is a serious weed of cultivated land, responsible for decline in crop yields and for costly chemical control measures (Mulligan & Bailey 1975). Indeed, the long soil seedbank life, the competitive growth habit, and high fecundity all contribute to the weedy nature of wild mustard and ensure that it remains a continuing problem (Mulligan & Bailey 1975).

The seeds were officinal and young plants were eaten in the past (Fogg 1950), but the presence of alkaloids makes a large quantity of seeds very toxic if ingested (Mulligan & Bailey 1975). In Europe, wild mustard has been used as a leafy vegetable, and oil from the seeds has been used for making soap, for cooking, and as a lubricant (Mulligan & Bailey 1975).

On the other hand, the biodiversity of agroecosystem is partially supported by wild mustard. Numerous phytophagous insects are closely associated; in particular, Marshall et al. (2003) identified 13 insect families, 37 species and 3 host-specific species related to *Sinapis arvensis*. Moreover, as mentioned before, this weed is highly relevant in bird diet (Marshall et al. 2001).

1.6.2.4 Genetics

The diploidy (2n = 18) of wild mustard was firstly discovered by Nagai and Sasaoka (1930) as reported by Fogg (1950) and confirmed later on by Bolkhovskikh et al. (1969) as reported by Mulligan and Bailey (1975).

To our knowledge, the most recent study on genetic variation of *S. arvensis* dates from Moodie et al. (1997). In accordance to previous studies using isozyme markers, Moodie et al. (1997) analyzed RAPD markers and detected high level of polymorphism both within and between wild mustard populations.

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Aim

The starting point which drove the purpose of this work is the increasing success of cropping GM organisms and their ecological consequences. In particular we were interested in those aspects that can affect biodiversity in natural habitat, and thus directing management practices and conservation.

The PhD project is part of the European funded LIFE+ Nature DEMETRA project (LIFE08 NAT/IT/000342).

The acronym DEMETRA stands for "DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops" and well explain the main purpose of the project.

DEMETRA aimed to create a monitoring instrument for genetically modified plants (GMPs) to support the Public Bodies, which would face with the presence of transgenic crops on their territories.

In particular, the quick monitoring index (QMI) took into account the level of risk posed by transgenic crops hypothetically used and their potential interactions with relevant biological, physical and climatic parameters of the study areas. All the project was carried out without employing GMPs.

Nowadays both agronomic/herbaceous species and trees are used in agriculture and the economic interests lying on them increase the attention of biotechnological improvement. Indeed, in the last decades, novel tools of direct gene transfer have been developed adding new possibilities to breeding practices (Jauhar 2006). Genetic engineering's tools have a great potential to speed up the process of crop improvement but, as with any new technology, are finding opposition from the public (Andow & Zwalen 2006).

The available techniques of genetic improvement of plants can be divided in: conventional breeding, cisgenesis and transgenesis (Schouten et al. 2006).

The first two ones are quite similar. Both of them involve only genes and their promoters already present in the species or in a crossable relative for centuries. Therefore, they neither alter the gene pool of the recipient species nor provide additional traits; changes in fitness are the same possibly occurring in conventional breeding or natural gene flow. However, it is necessary to consider that recombinant DNA technology is certainly not the same as meiotic recombination (Schouten et al. 2006). On the other hand, conventional techniques encounter some disadvantages, which slow down the genetic improvement process. When the genes of interest is inherited together with deleterious genes, successive generations of recurrent backcrossing are needed. Furthermore, the duration of reproductive cycles and

sometimes the complex reproductive characteristics of plants constrain all the process (van Frankenhuyzen & Beardmore 2004).

Both transgenesis and cisgenesis use the same genetic modification techniques, but the first one consists in introducing genes and their promoters from a species that is neither the recipient species nor a close relative. For example, genes from viruses, bacteria, animals and plants can be introduced by genetic engineering into plant genomes. This means that transgenesis can extend the gene pool of the recipient species by providing new traits otherwise not available to it (Schouten et al. 2006), and without compromising its desirable genetic background.

A novel trait might potentially spread through gene flow between crop and its wild relatives. Even though gene flow is a natural event and thus not hazardous in itself, the escape of transgenes should be seriously considered (Snow & Moràn-Palma 1997). Indeed, the novel trait could positively, negatively or even neutrally, alter the fitness of the recipient species (Farnum et al. 2007). This depends on the advantage the trait implies and, in any case, its frequency could increase until the wild genotype of the recipient species dies out in its pure form (Farnum et al. 2007; Ellstrand 2003).

For this reason, the nature conservation must consider that the loss of the genetic integrity of a species can threat its local adaptation (Talbot et al. 2012) and broadening the perspective negatively affect the biodiversity at the level of communities and ecosystem processes (Whitham et al. 1999; Talbot et al. 2012).

At the moment, the agriculture exploits both woody and herbaceous species and certainly, the consequences on the natural environment due to both kind of crop differ because of their distinct biological and ecological characteristics, especially considering that the extent, to which woody and herbaceous crops affect the environment, changes at both spatial and temporal scales (Hamrick et al. 1992).

Compared to the annual grasses, trees are long-lived organisms with a longer generation intervals (Slavov et al. 2002); it means that during the life-span they could interact with the environment for a longer time than do grasses. They are the dominant life form in many ecosystems, driving forward the diversity and structure of a number of dependent communities (Whitham et al. 2006). Moreover, trees stretch over hundreds or thousands of hectares (Gonzàlez-Martìnez et al. 2005), covering a wide range of diverse ecological conditions. Gene flow is spatially much more extensive than the grasses' one (Slavov et al. 2002) and thus it can maintain genetic connectivity even over large distances. Finally, many woody crop species can propagate vegetatively in addition to outcross markedly more than annual plants, and most of them hybridize naturally with wild congeners (Barrett 1998).

In this work, we focused on two cropped species (*Populus* spp. and *Brassica* spp.) in order to highlight the differences between consequences on the environment due to woody or herbaceous crops. We considered poplars since they are the widest short-rotation culture in Italy (Sabatti & Nardin 2008) and a growing biotechnological interest is focusing on them. On the other hand, we chose Brassicaceae since they represent the third most important source of vegetable oil worldwide and they are exposed to continuous attempts of biotechnological improvement (Hu et al. 2002).

The main aim of this work is to investigate the possible interactions between cultivated plants and the surrounding environment from the point of view of the genetic diversity conservation. To do that, the potential wild-to-crop hybridization was examined.

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Chapter 2
Potential threat due to crossing between poplar cultivations and wild relatives in Mediterranean environment

POTENTIAL THREAT DUE TO CROSSING BETWEEN POPLAR CULTIVATIONS AND WILD RELATIVES IN MEDITERRANEAN ENVIRONMENT

RUNNING TITLE: CROSSING BETWEEN NATURAL AND CULTIVATED POPLARS

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ABSTRACT

Most strategies for the genetic improvement and biotechnologies may be applied to the *Populus* spp. and their hybrids to increase productivity and adaptability. However, their weak reproductive barriers and spontaneous hybridization with natural populations may impact the sustainable deployment of new poplar cultivars. Deforestation and intensive management, with plantations of fast-growing tree species, may endanger tree species by the reduction or loss of their habitats, but also by loss of species integrity through hybridization and introgressive gene flow. Consequently, the implementation of conservation strategies requires the monitoring of gene flow in relation to habitat structure. In order to provide a knowledge base supporting sustainable forest management approaches for genetic diversity conservation, the objectives of this work were the characterization of spatial genetic structure in poplar stands, and the study of the potential crossing between natural and cultivated populations in the Mediterranean environment.

Two study areas (A1 and A2), near to poplar plantations, were settled in poplar stands within the Regional Park of Migliarino-San Rossore-Massaciuccoli (Pisa, Italy). The spatial genetic structure showed that three clusters can be identified in both A1 and A2. The differences between the spatial genetic structures depended on the environmental features of the two stands. The detection of hybridization (by paternity analysis) between the A2 and the poplar plantation, suggested the occurrence of a possible genetic exchange among natural stand and plantation.

Besides, the potential threat due to hybridization between poplar cultivations and its wild relatives in the Park was investigated. Based on the genetic information and using the spatial dataset available for the study area, we found that gene flow could affect important habitats for native poplar population. The assessment of this potential threat indicates that some management measures are required to mitigate the risk.

Introduction

Riparian habitats have increasingly relevance within the European biodiversity policy (Water Framework Directive 2000/60/EC), since degradation and threats to freshwater ecosystems are already widespread (Bravard et al. 1986; Kramer & Havens 2009; Gundersen et al. 2010; Clerici et al. 2013).

The importance of the riparian systems lies to the natural and social services they provide; they encompass a diverse mosaic of habitats, considered hotspots for biodiversity (Toner & Keddy 1997; Bornette et al. 1998; Hänfling et al. 2004) and are often characterized by high productivity (Clerici et al. 2013). Riparian zones are transitional areas between land and freshwater ecosystems connected to the main river by seasonal floodings, characterized by distinctive hydrology, soil and biotic conditions (Hänfling et al. 2004; Verry et al. 2004; Clerici et al. 2013). In literature, there is strong agreement with regard to the major role of riparian environments, as corridors in maintaining landscape connectivity (Machtans et al. 1996; Gillies & St. Clair 2008; Clerici et al. 2013), in supplying river bank stabilization and providing resistance to runoff during flood events (Bennett & Simon 2004; Clerici et al. 2013). Riparian forests are usually dominated by species of *Alnus* Mill., *Betula* L., *Populus* L. and *Salix* L. genera (EEA 2006).

Besides its keystone ecological role, the genus *Populus* has economical and genetic features, which make it one of the most exploited tree, especially in recent years with the enhanced worldwide demand for wood (Ellison et al. 2005; Sticklen 2006; Whitham et al. 2006). The resulting deforestation and intensively managed plantations of fast-growing species (Fenning & Gershenzon 2002; Frankenhuyzen & Beardmore 2004) may endanger tree species by the destruction or loss of their habitats but also by loss of their species integrity through hybridization and introgressive gene flow (Ziegenhagen et al. 2008; Primack 2010). Preserving forests should be an unavoidable purpose of natural resources management, since removal of trees can change the entire ecosystem (Ledig 1988; Rajora & Mosseler 2001).

In a changing environment, coping with these threats means rapid adaptation to new temporal and spatial conditions, and the adaptive value of living organisms depends on their genetic variation (Vornam et al. 2004; Gienapp et al. 2008; Kramer & Havens 2009; Paffetti et al. 2012). Hence, the preservation of genetic variation is important for the long term life and stability of populations (Vornam et al. 2004; Lefèvre et al. 2013). This is even more significant for forest tree species, as poplars,

since they are long-lived sessile organisms with a relatively belated sexual maturity (Finkeldey & Ziehe 2004).

Altering a wide variety of ecological and genetic processes (Young et al. 1996), the consequences of fragmentation and habitat loss are recently main topics in both evolutionary and conservation biology (Heino & Hanski 2001; Imbert & Lefèvre 2003). Fragmentation affects both the exchange of individuals and the gene flow (Young et al. 1996) among subpopulations of a larger metapopulation (Hanski & Gilpin 1991; Rajora & Mosseler 2001); fragmentation and habitat loss, together, lead to a considerable decline of populations and therefore to a greater susceptibility to perturbations or stochastic events (Lande 1988; Imbert & Lefèvre 2003; Vranckx et al. 2011).

When the genetic exchange (Slatkin 1985; Rajora & Mosseler 2001) is severely affected, then inbreeding and genetic drift may cause genetic diversity loss within subpopulations (Rajora & Mosseler 2001), with severe consequences on the entire gene pool of the population. Consequently, the implementation of conservation strategies requires the monitoring of gene flow in relation to habitat structure (Imbert & Lefèvre 2003).

All these issues hold true for native poplars, which are considered one of the most threatened forest tree species of old natural floodplain forests in the temperate zones (Lefèvre et al. 2001; Broeck et al. 2005).

In floodplain areas, river regulation measures, agricultural use and urbanization have dramatically degraded the environment (Dynesius & Nilsson 1994; Rajora & Mosseler 2001; Ziegenhagen et al. 2008). The synergy of decaying riparian forest and increasing habitat fragmentation seriously rises the opportunities of contact and therefore the production of hybrid seeds between native and cultivated poplars (Levin et al. 1996; Broeck et al. 2005).

In Europe, the intensive poplar cultivation began during the 1940s–1960s (de Vries & Turok 2001; Fossati et al. 2003; Broeck et al. 2005) and many questions about hybridization in *Populus* are still unresolved. Natural poplar hybrids are normally found where different species come each other into contact (Eckenwalder 1984a,b; Martinsen et al. 2001; Floate 2004; Broeck et al. 2005).

The interest in understanding gene flow dynamics is also related to the impacts of plantations on surrounding populations and ecosystems (DiFazio et al. 2004). Indeed, hybridization can also lead to the creation of new species, losing the native ones, and to the introgression from one species to another (DiFazio et al. 2004).

Several poplars and hybrid cultivars are economically important for production of lumber and pulp for applications in horticulture and phytoremediation and recently for production of biofuel and feedstocks (Rubin 2008; Slavov et al. 2010). The economic interest solicits the production and relative introduction of GM (Genetically Modified) poplar plantation for economic purpose (Burczyk et al. 2004), rising one of the major conservation issue (Rajora & Mosseler 2001), since GMOs (Genetically Modified Organisms) could act as exotic species, deteriorating the genetic diversity of native poplars.

In order to provide a knowledge base supporting sustainable forest management approaches for genetic diversity conservation, the objectives of our work were the characterization of spatial genetic structure in poplar stand, and the study of the potential breeding between natural and cultivated populations in the Mediterranean environment.

Materials and methods

Study area

We settled two permanent sites called "A1" and "A2", within the Regional Park of Migliarino-San Rossore-Massaciuccoli (MSRM) (Pisa, Italy). The A1 and A2 are 8 Km far away, and belong to different ecosystems (Fig. 2.1a).

The A1 is one of the most important wetland habitats in Italy, where single trees and small groups of *Populus* spp. are scattered along the shores of the Massaciuccoli Lake (Fig. 2.1b). The "Lago e Padule di Massaciuccoli" (Cod. Natura2000 IT5120021) covers a total area of 1908.01 ha, and it is a "Site of Community Importance" (SCI) according to the Directive 92/43/CEE. It is a lake of fresh water surrounded by helophytic formations (reeds and *Cladium* spp.), bog and upland vegetation. It is characterized by phytocenosis with *Cladium mariscus* (L.) Pohl and by other rare species, such as *Periploca graeca* L. (one of the few Italian stations), and *Drosera rotundifolia* L. a very rare upland species in the bog.

The A2 is large 7200 m^2 with flat morphology. It is a naturally-originated forest stand growing in proximity of Serchio River (Fig. 2.1c). This study area is in the SCI/SPA "Selva Pisana" (Cod. Natura2000 IT5170002) and it is included in the Regional Park. The A2 includes a mixed forest of white poplar (*Populus alba* L.), elm (*Ulmus minor* L.), narrow-leafed ash (*Fraxinus oxycarpa* Bieb.), black alder (*Alnus glutinosa* L.), grey poplar (*Populus x canescens* ((Aiton) Sm.)) and some individuals of peduncolate oak

(*Quercus robur* L.). This area has a structure similar to a high forest; however, individuals regenerated both from seed and from stumps are present.

We identified 32 and 30 *Populus* spp. individuals within A1 and A2, respectively. We morphologically determined the sex of each tree and their x, y coordinates were collected by GPS.

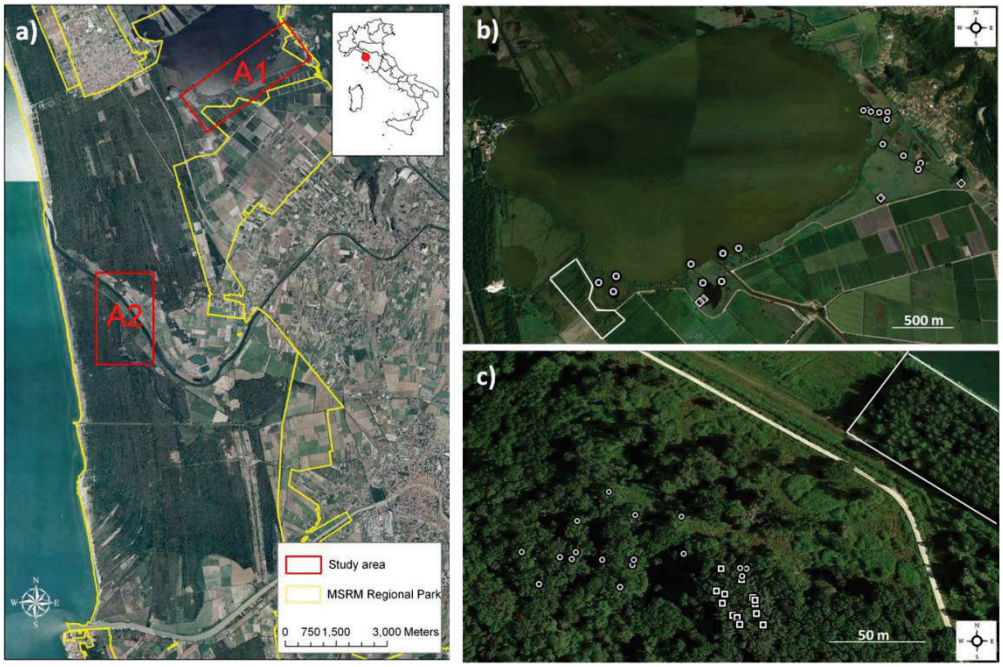

Fig. 2.1 a) Study area location. b) A1 (Massaciuccoli lake), ○ symbols represent the positions of *P. x canescens* trees and ◇ symbols of *P. nigra* trees, and white line defines the border of poplar plantation. c) A2 (Serchio river), □ symbols represent the positions of *P. alba* trees, ○ symbols of *P. x canescens* trees, and white line defines the border of poplar plantation.

The A1 and A2 were settled adjacent to poplar plantations (Fig. 2.1): i) A1 is located close to a private plantation of unknown clone; ii) A2 is located close to two plantations of "Triplo" clone (Italian Patent - Registro Nazionale dei Materiali di Base-Italia 17.11.75 G.U. n. 324 del 09/12/1975).

We installed two weather stations, near the study areas, for monitoring those parameters useful for investigating seeds and pollen dispersal, wind speed and direction.

Sample collection and molecular methods

In total, we collected leaves of 107 *Populus* spp. trees (32 from A1, 30 from A2, and 45 from cultivated poplars). We sampled seeds (100 per mother tree) from mature catkins of 9 and 5 mother trees in the A1 and in the A2, respectively. We sowed the seeds on filter paper and placed them in growth chambers at 25 °C (Fig. 2.2). Few days after germination, we harvested seedlings. Dr. Matthias Fladung and Georg von Wühlisch (Thünen Institute, Institute of Forest Genetics, Grosshansdorf, Germany)) shipped on dry ice to us the leaves of trees of the reference species *P. alba, P. x canescens*, *P. tremula* L., *P. deltoides* W. Bartram ex Humphry Marshall, *P. x euramericana* (Dode) Guinier (*P. x canadensis* Moench), *P. nigra* L., *P. trichocarpa* Torr. and A. Gray.

The plant material (leaves and seedlings) was stored at -20 °C until extraction. To extract total DNA from leaves and from seedlings (50–100 mg as starting material), we used the DNeasy plant kit (QIAGEN, Germany) following the manufacturer's specifications.

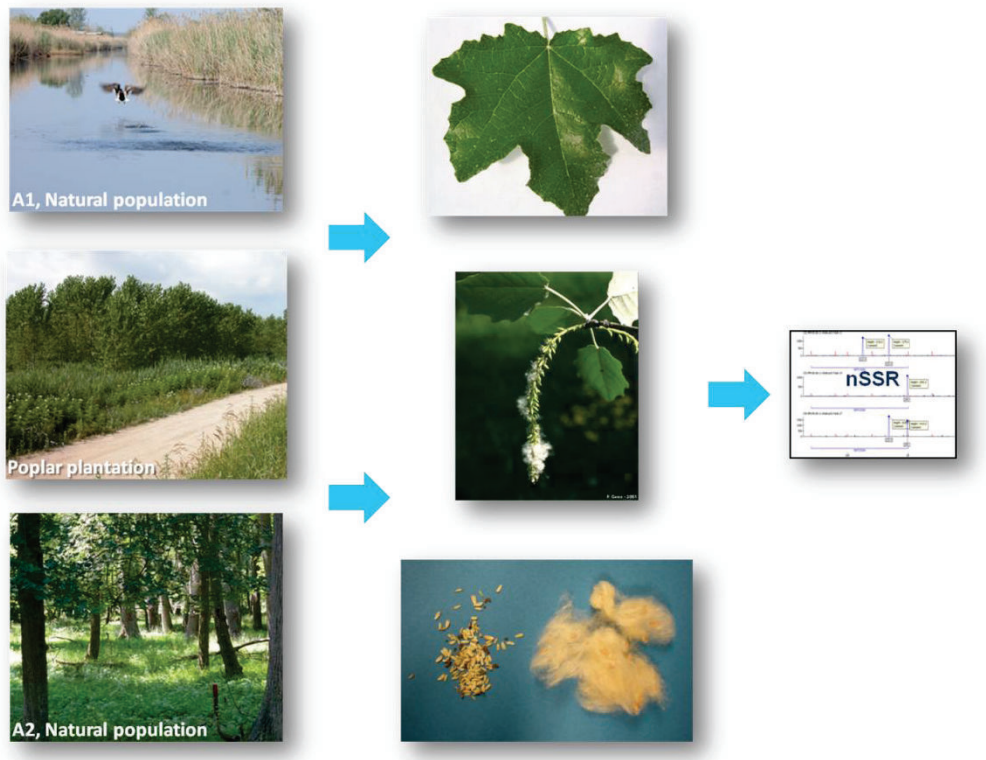

Fig. 2.2 a) Study areas and plantations; b) leaves from adult trees and seeds from mature catkins were collected; c) seeds sowed on filter paper up to germination; d) DNA from leaves and seedlings was used for nSSR analysis.

trnL chloroplast DNA (cpDNA) region sequencing

We performed polymerase chain reaction (PCR) in a final reaction volume of 20 µl. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 250 µM of each deoxynucleoside triphosphate, 1 µM of each primer, 1 µl (10 ng) of total DNA, and 1 U of Platinum Taq DNA polymerase (Life Technologies). To amplify the *trnL* cpDNA region, we used the universal primers c and d described by Taberlet and collaborators (1991). Prior to amplification, we incubated the amplification mixture for 60 s at 90 °C and for 90 s at 95 °C. Successively we used different cycles with the following temperature profiles: 1) 95 °C for 30 s, 60 °C for 30 s, 72 °C for 4 min, for 5 cycles; 2) 95 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min, for 5 cycles; and 3) 95 °C for 30 s, 50 °C for 30 s, 72 °C for 4 min, for 25 cycles (Paffetti et al., 2007). Amplification products were then incubated at 72 °C for 10 min. We used a GeneAmp® 9700 PCR System (Life Technologies, CA, USA) for PCR. We analyzed amplification products by gel electrophoresis on 1% (w/v) agarose gel (Life Technologies, CA, USA) at 10 V/cm for 2 h in Tris-acetate-EDTA buffer containing 0.5 µg/ml (w/v) of Gel Red (BIOTIUM Inc., CA, USA). The gels were photographed and analyzed with an UVP scanner (Photo-Capt, Vilbert Coormat, France). To purified the amplification products we used the PCR DNA purification Kit (QIAGEN, Germany) following supplier's instructions. We sequenced the purified amplification products in both directions on an Applied Biosystems® 3500 Genetic Analyzer (Life Technologies, CA, USA).

Genotyping

We genotyped samples using ten primer pairs of nuclear microsatellite (nSSR) loci (WPMS 15, ORPM 214, ORPM190, ORPM186, ORPM137, ORPM 86, ORPM 60, ORPM 30, ORPM 28, ORPM 26), and we performed PCR amplifications according to van der Schoot and collaborators (2000), Smulders and collaborators (2001), and Tuskan and collaborators (2004) as indicated on the web-site of the International Populus Genome Consortium: http://www.ornl.gov/sci/ipgc/ssr_resource.htm. We carried out the sizing of the PCR products on Applied Biosystems® 3500 Genetic Analyzer automatic sequencer (Life Technologies, CA, USA). To size the amplified fragments, we used the internal molecular size standard Liz 500 (Life Technologies, CA, USA) and the software Gene Mapper ver. 4.0 (Life Technologies, CA, USA).

Molecular analysis

To multiply align the *trnL* cpDNA sequences and the nucleotide sequences retrieved from the GenBank , we used the CLUSTAL-X program (Thompson et al. 1997). We verified and manually adjusted the alignments. We performed the BLAST probing of the DNA databases with the BLASTN option of the BLAST program (Altschul et al. 1997). Variable and informative sites were found using Molecular Evolutionary Genetics Analysis 6 software (Tamura et al. 2013).

We calculated the genetic diversity in the stands from nSSR data in SPAGeDi 1.3a (Hardy & Vekemans 2002), and the statistical significance with Jackknifed estimators (Sokal & Rohlf 1995) after 20000 permutations.

We determined the Wright's fixation index (F_{IS}) and the deviations from Hardy–Weinberg expectations in GENEPOP 3.3 (Raymond & Rousset 1995). We calculated the F-statistics and null alleles according to Weir and Cockerham (1984). Pair-wise comparisons of F_{ST} values were tested for significance, and critical values were adjusted for multiple tests with the Bonferroni correction. To examine the relationship between the genetic distance and the geographic distance, we performed a Mantel test on the matrix of F_{ST} values (50000 permutations) in GENEPOP 3.3.

We analysed the data using the Bayesian clustering methods implemented in STRUCTURE program and Geneland software. We used the model-based clustering algorithm implemented in v.2.3 of the STRUCTURE program (Pritchard et al., 2000; Falush et al., 2003, 2007) and the empirical statistic K (Evanno et al., 2005; Earl et al., 2012) to determine the number of sub-populations (K) in each plot studied. We used the default model parameters and varying K from 1 to 10 for both plots to run STRUCTURE. Each run consisted of 250,000 burn-in iterations and 1,000,000 data collection iterations, and was replicated 20 times. We inferred population structure using a Bayesian Monte Carlo Markov Chains method implemented in the Geneland package vers. 3.0 (Guillot et al. 2009) under the R Language and Environment for Statistical Computing software as described by Guillot and collaborators (2005 a, 2005 b, 2008). Ten independent Monte Carlo Markov Chains runs were performed by Geneland with the following settings: 1,000,000 iterations with 100 thinning intervals and a burn-in period of 250,000, using the correlated allele frequencies model. A map of posterior probabilities (membership) was obtained by Post- ProcessChain and PostTessellation functions into Geneland by tesselling the landscape at a resolution of

1 m. We calculated the null alleles for nSSRs in Geneland package vers. 3.0 (Guillot et al. 2008).

Paternity analysis

We conducted paternity assignment using all ten analysed nSSR loci through standard maximum-likelihood methods implemented in CERVUS 3.0 (Kalinowski et al. 2007). Critical likelihood values (LOD-scores) yielding 95% confidence in assignments were obtained using simulations. We simulated 100,000 offspring assuming an average mistyping error of 0.046 per locus, equal to the observed mother–offspring mismatch rate and very similar to the estimate from a mating model (0.044).

We calculated the allele frequencies from the whole population in CERVUS 3.0 and used in the simulations. To test the sensitivity of the point pattern analysis and kernel estimates to CERVUS (Marshall et al. 1998), we conducted additional analysis based on different CERVUS runs assuming different error rates, numbers of candidate fathers, proportion of sampled males and confidence levels.

Cartographic data

Topographic maps and thematic layers were acquired for spatial analysis using a Geographic Information System (GIS). Topographic maps at the scale of 1:10,000 were acquired from Tuscany Region. A land use land cover map (D.R.E.AM. 2002) at the scale of 1:15,000 was provided by the Regional Park. A forest type map at the scale of 1:10,000 was obtained by polygon delineation of vegetation maps produced by Tomei and collaborators (2003) and Sani and collaborators (2010). A map depicting the distribution of poplar plantation at the scale of 1:10,000 was provided by the Regional Park. Digital aerial images (year 2007) at the nominal scale of 1:10,000 (pixel size = 1 m) were also acquired. All these data were projected in a common coordinate system and registered in a geographic database. Natural and artificial objects (e.g., forest cover, tree plantations, river banks, motorway) that can act as barriers for pollen dispersal mediated by wind were identified using the topographic maps, the land use land cover map, and the forest type map. In addition, the distribution of rows of trees within the landscape mosaic was determined by polygon delineation of rows larger than 20 m on the basis of a visual interpretation of aerial images.

Results

Populus species identification

The natural poplars in A1 and A2 were firstly morphologically characterized and classified as belonging to *P. alba* and *P. nigra* species, and to hybrid *P. x canescens*. Grey poplar is a natural hybrid between *P. alba* and *P. tremula*, two ecologically divergent species that often hybridize in Europe. Both study areas are adjacent to poplar plantations (Fig. 2.1). In particular, the A1 is located near a plantation of unknown clone, and the A2 is adjacent to two "Triplo" plantations. "Triplo" is a artificial hybrid of *P. deltoides* (mother) and *P. nigra* (father), defined *P. x euramericana*.

The sequence analysis of the *trn*L cpDNA region, performed in several species of the genus *Populus*, showed some variable and informative sites that identify the different species. The length of the *trn*L intron is variable within the considered species, varying from 590 bp to 608 bp, the second *trn*L exon is always 50 bp long. The multiple alignment of the region among the different species resulted in a sequence 614 bp long. Twenty-seven variable sites were found in the *trn*L cpDNA region and distinguished the different species of *Populus* genus. The cpDNA haplotype variant is identical in *P. nigra* and *P. alba*, in agreement with Hamzeh and Dayanandan (2004). In particular, six deletion (position 20, 256, 257, 404, 405, and 406 respectively), one trasversion in position 198 (T/G) and one transition in position 279 (A/G) distinguish *P. alba* from *P. deltoides*.

The sampled trees in A1 and in the neighbouring plantation have the cpDNA haplotype of *P. alba* species, while individuals sampled in the A2 and in the nearby plantations are classified as *P. alba* and *P. deltoides* haplotypes (Table 2.1).

Allele/frequency per locus of most informative alleles and allelic variants per species within each locus in *P. alba*, *P. x canescens*, *P. nigra*, and *P. x euramericana*, are shown in Table 2.2.

Using the data from nSSR we designed a clustering approach in order to study how species have inter-individual similarity in genetically homogeneous populations. Therefore, we used the software STRUCTURE v.2.3.4 which divides individuals into K clusters with different frequencies of the marker. We chose K *a priori* and then we varied across different runs. Ten STRUCTURE runs have produced nearly identical membership coefficients for each K (data not shown). Contrary to the expected from morphological and cpDNA sequence data, at K = 2 *P. nigra* individuals are grouped together with those of *P. x euramericana*, and *P. alba* with *P. x canescens* trees (Fig. 2.3).

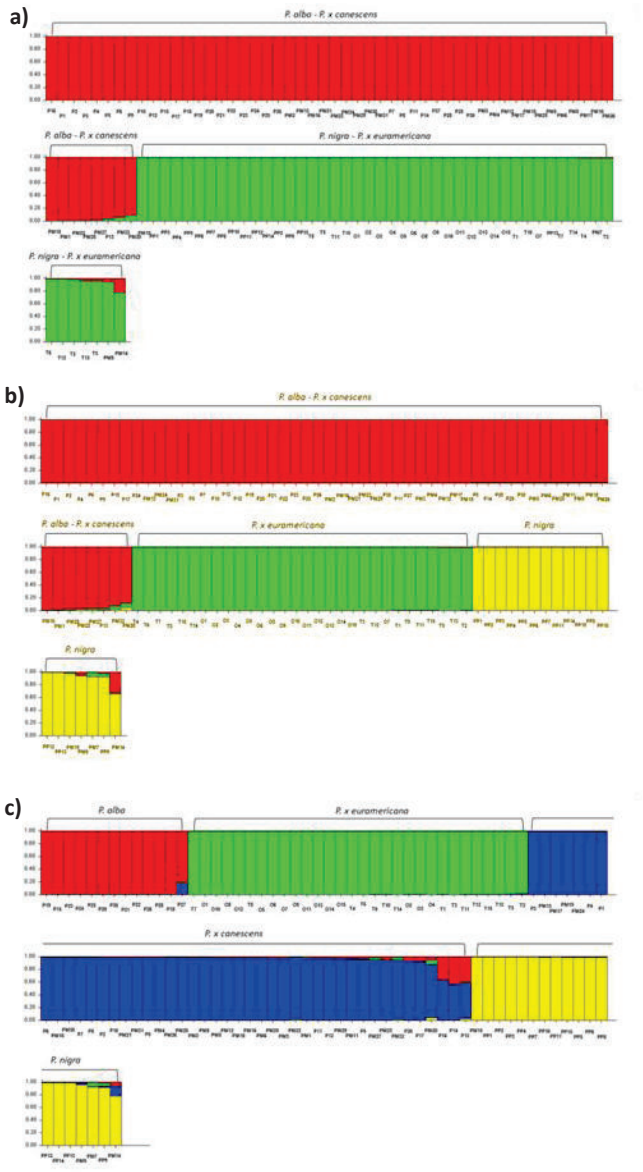

Fig. 2.3 Estimated species structure for 107 individuals with SSR markers. Each individual is represented by a vertical line, which is partitioned into K segments that represent the individual's estimated membership fractions in K clusters. Long black lines indicate the separation among *a priori* assigned groups. **a)** Red color indicates *P.alba – P. x canescens* group, green color represents *P.nigra – P. x euramericana* group. **b)** Red color indicates *P.alba – P. x canescens* group, green color represents *P. x euramericana* group and yellow color corresponds to *P.nigra* group. **c)** Red color indicates *P.alba* group, green color represents *P. x euramericana* group, blue color corresponds to *P. x canescens* group and yellow color to *P.nigra* group.

For K = 3, *P. alba* individuals are still grouped with *P. x canescens*, whereas with K = 4 they are broken down into two taxonomic entities (Fig. 2.3). At higher K values, the new groups are composed of individuals belonging to different clusters, making it difficult to identify the underlying classification criterion.

Table 2.1 shows the number of individuals belonging to different *Populus* species within both study areas. In particular, in A1 the trees belong to *P. x canescens* (18 males and 9 females), and to *P. nigra* (5 males); in A2, 13 males are *P. alba* individuals, while 5 females and 12 male are *P. x canescens* trees.

Sequence analysis and genotyping allowed the identification of each variety grown in poplar plantations (Table 2.1). It was confirmed in A2 that these are plantations of "Triplo" clone (*P. x euramericana*), while it was possible to identify the unknown clone grown in A1 plantation as a selection of *P. nigra*.

For K = 3, *P. alba* individuals are still grouped with *P. x canescens*, whereas with K = 4 they are broken down into two taxonomic entities (Fig. 2.3). At higher K values, the new groups are composed of individuals belonging to different clusters, making it difficult to identify the underlying classification criterion.

Table 2.1 shows the number of individuals belonging to different *Populus* species within both study areas. In particular, in A1 the trees belong to *P. x canescens* (18 males and 9 females), and to *P. nigra* (5 males); in A2, 13 males are *P. alba* individuals, while 5 females and 12 male are *P. x canescens* trees.

Sequence analysis and genotyping allowed the identification of each variety grown in poplar plantations (Table 2.1). It was confirmed in A2 that these are plantations of "Triplo" clone (*P. x euramericana*), while it was possible to identify the unknown clone grown in A1 plantation as a selection of *P. nigra*.

Table 2.1. ID poplar tree, sex and molecular identification by chloroplast DNA (cpDNA) haplotype and nuclear microsatellite (nSSR) genotyping of each poplar tree considered in A1 and A2, and in the adjacent plantations.

	ID poplar tree	Sex	cpDNA haplotype	nSSR genotyping	Species
A1 (Natural *Populus*)	PM1, PM2, PM3, PM4, PM5, PM6, PM10, PM13, PM16, PM17, PM18, PM19, PM20, PM21, PM24, PM28, PM29, PM30	♂	AT--AAAA	19/22	*P. x canescens*
	PM11, PM12, PM22, PM23, PM25, PM26, PM27, PM31, PM32	♀			
	PM7, PM8, PM9, PM14, PM15	♀	AT--AAAA	10/12	*P. nigra*
A1 (*Populus* plantation)	PP1, PP2, PP3, PP4, PP5, PP6, PP7, PP8, PP9, PP10, PP11, PP12, PP13, PP14, PP15	♂	AT--AAAA	6/12	*P. nigra*
A2 (Natural *Populus*)	P15, P18, P19, P21, P22, P23, P24, P25, P26, P27, P28, P29, P30	♂	AT--AAAA	7/7	*P. alba*
	P1, P2, P3, P5, P6	♀	AT--AAAA	14/22	*P. x canescens*
	P4, P7, P8, P9, P10, P11, P12, P13, P14, P16, P17, P20	♂			
A2 "Triplo" plantation	O1, O2, O3, O4, O5, O6, O7, O8, O9, O10, O11, O12, O13, O14, O15	♂	-GGGG---	6/6	*P. x euramericana*
A2 "Triplo" plantation	T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15	♂	-GGGG---	6/6	*P. x euramericana*

Table 2.2. Nuclear microsatellite markers employed in this study, alleles/frequency of most informative allele in *P. alba*, *P. x canescens, P. nigra,* and *P. x euramericana*.

Locus	Alleles/frequency			
	P. alba	*P. x canescens*	*P. nigra*	*P. x euramericana*
WMPS 15	188/0.038; 215/0.346	185/0.011; 188/0.144; 197/0.044; 200/0.311; 203/0.100; 215/0.033	212/0.500	212/0.267
ORPM 214		164/0.011	168/0.026; 170/0.868; 172/0.105	
ORPM 190			195/0.053	
ORPM 186	233/1.000	233/0.789	227/0.947	223/0.500; 227/0.500
ORPM 137	184/1.000	184/0.500	180/0.947	192/0.833; 194/0.167
ORPM 86				
ORPM 60	204/1.000	204/0.422	186/0.237; 213/0.026	
ORPM 30	217/0.500	215/0.344; 217/0.022; 221/0.011; 223/0.011; 225/0.100; 227/0.022; 229/0.189; 231/0.167	219/0.026; 239/0.026	239/0.267
ORPM 28		204/0.022	206/0.026; 208/0.158	
ORPM 26	215/0.154	207/0.022; 209/0.022; 215/0.122		

Spatial structure of the genetic diversity in the natural poplar populations

The spatial structure analysis of genetic diversity in natural poplar populations allowed to identify three clusters both in A1 and A2. In both areas the Log-likelihoods from runs of STRUCTURE with K ranging from 1 to 10 have a clear peak at K=3 (Fig. 2.4)

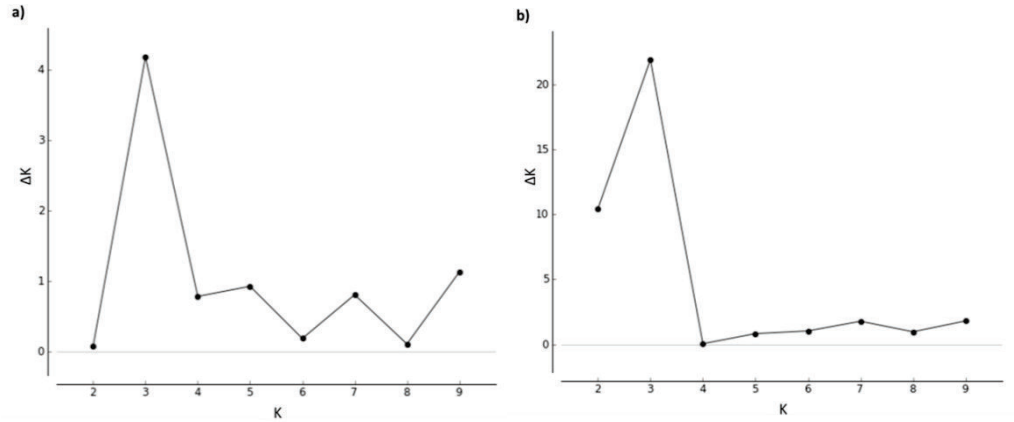

Fig. 2.4 Population substructure in A1 (a) and A2 (b) detected using the STRUCTURE program. Circles indicate values of the ad hoc statistic ΔK, which is based on the rate of change of the log-likelihood as K is increased (Evanno et al., 2005). ΔK tends to peak at the value of K that corresponds to the highest level of hierarchical substructure.

and spatially explicit in clustering analyses using GENELAND (Fig. 2.5).

In the Massaciuccoli Lake population (A1, Fig. 2.5a), as shown by the maps of posterior probabilities and values of F_{ST}, only the cluster 1 (F_{ST} = 0.162 and F_{ST} = 0.220 from cluster 2 and 3, respectively), comprising trees belonging just to the species *P. nigra*, is genetically isolated from the others. On the contrary, gene flow is evident between the other two clusters consisting of *P. x canescens* trees (F_{ST} = 0.075).

The results of spatial structure analysis of the Serchio river population (A2, Fig. 2.5b) show a subdivision into three clusters. Clusters are genetically isolated, as indicated by the maps of posterior probabilities and confirmed by the values of F_{ST}. The cluster 2, comprising trees belonging to species *P. alba*, is most genetically isolated (F_{ST} = 0.602) from cluster 3.

On the other hand, the cluster 2 has a lower divergence (F_{ST} = 0.235) from the cluster 1. The latter has a value of F_{ST} (0.257) very similar to the cluster 3, which consisted of individuals belonging to the species *P. x canescens*.

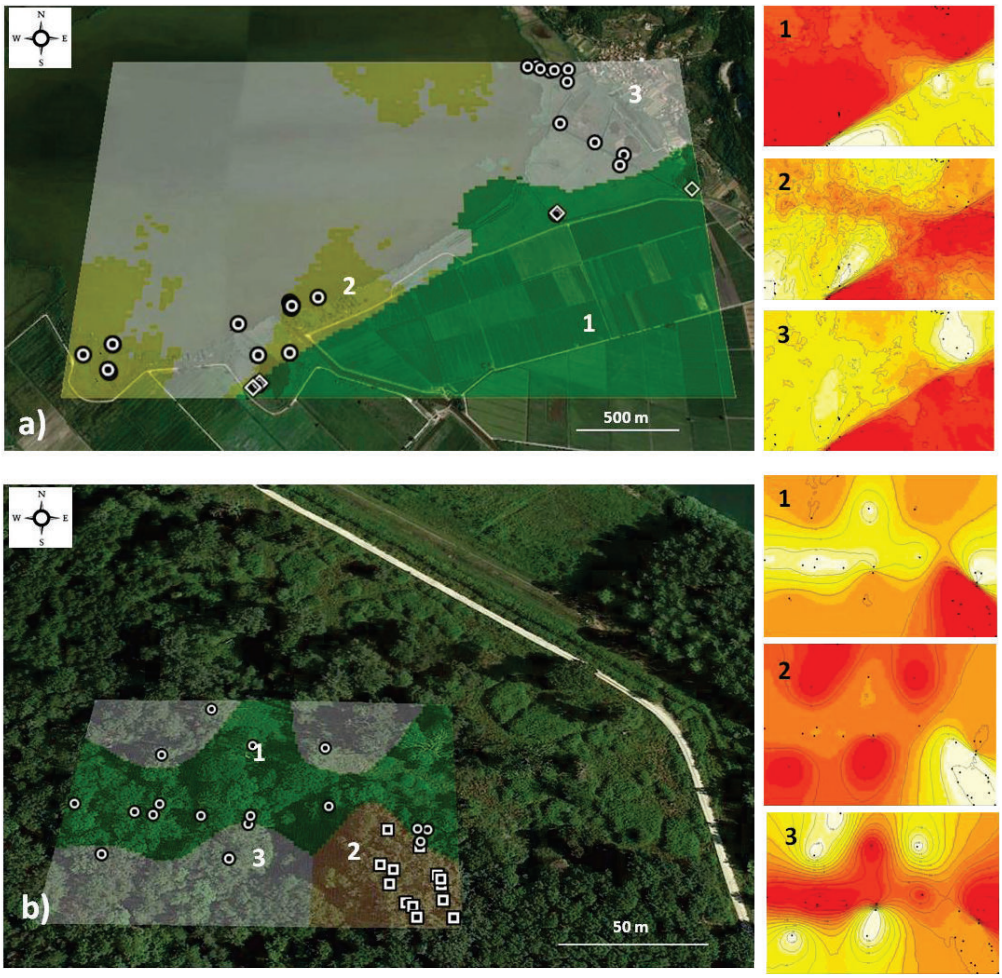

Fig. 2.5 Spatial organization into clusters and maps of posterior probability of each cluster in a) A1 and b) A2. ◇ *P. nigra*, ○ *P. x canescens,* □ *P. alba*.

Crossing between poplar cultivations and wild relatives

During the spring 2010, between the end of March and the beginning of April flowering of male and female poplars was detected, and it was possible to observe the overlap of flowering times between natural and cultivated poplars. In June of the same year seeds were collected directly on the maternal parent plants in both areas. The average rate of germination was 50% in A1 and 90% in A2.

The paternity assignment was carried out by 10 microsatellite loci used in the identification and in the spatial structure diversity analysis of the parent trees.

In A1 it was possible to identify the crossings of the mother trees PM12, PM22 and PM25 (*P. x canescens*) involving trees distant among them more than 2 km. As example, we only reported the crosses involving PM22 tree (Fig. 2.6a). All crossings

involved male individuals belonging to the taxonomic identity *P. x canescens*, while, as expected, none of the crosses involved black poplars present along the shores of the Massaciuccoli lake or in the close plantation. In the A2 the crossings with the *P x canescens* mother plants (P1, P2, P3, P5 and P6) involved male individuals belonging to the *P x canescens* and *P. alba* species. In Fig. 2.6b, crosses involving P3 tree are reported as examples. The poplar gray, called P3 (a female plant), produced progeny with white and gray poplars, but unexpectedly P3 also produced progeny with individuals indicated with the abbreviations T1 and T11 of nearby plantation (Fig. 2.6b).

The analysis of forest structural data (diameter, height and crown width) showed that P3 is a large tree without barriers, due to competition with other crowns, and thus easily received the pollen flow from the plantation. Furthermore, during the pollen diffusion the direction east-northeast to west-southwest of the wind favoured pollen dispersion from T1 and T11 trees ("Triplo") to the P3 tree (Fig. 2.6b).

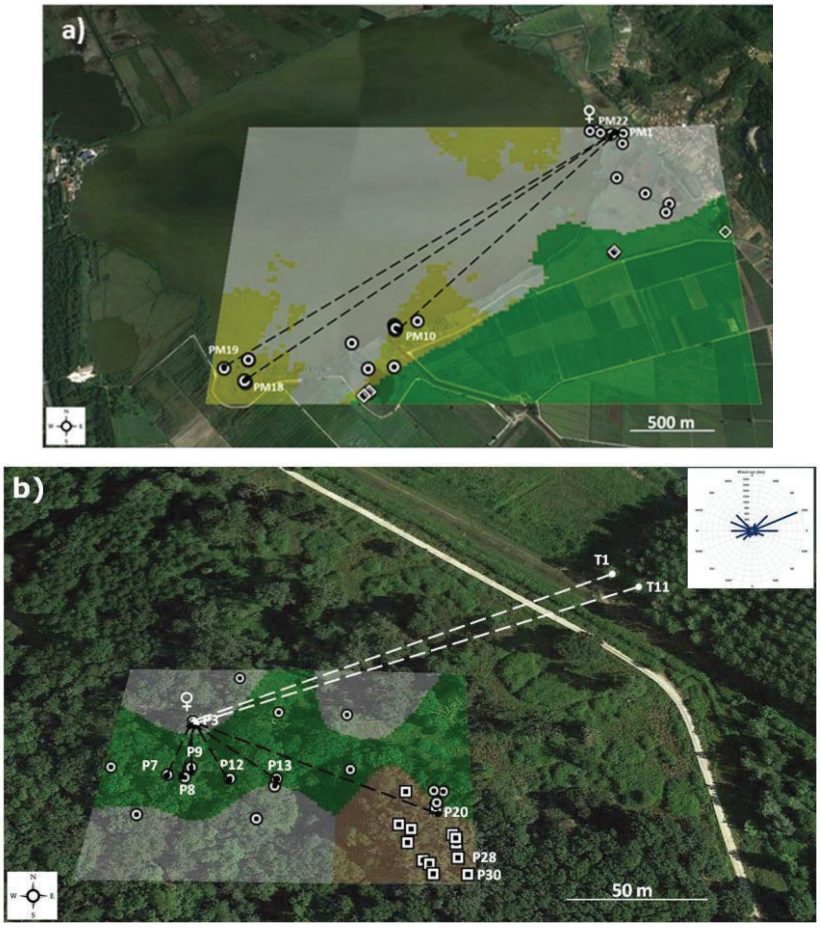

Fig. 2.6 Crosses for PM22 tree in A1 and for P3 tree in A2.

Possible coexistence between poplar cultivations and wild relatives

The agricultural crop types map was used to extract the distribution of poplar plantations in the Park. Then we hypothesized that poplar plantations were composed by *P. x euramericana* trees. Thus a total of 29 poplar plantations covering a total area of 291 hectares were considered for GIS modelling (Fig. 2.7a). Pollen and seed dispersal mediated by wind direction and natural barriers were used as criteria for assessment. In the Migliarino-San Rossore-Massaciuccoli Regional Park naturally originated poplar trees (*P. alba* and *P. x canescens*) can be found in mixed broadleaved forests dominated by hygrophilous species and in the wetlands of Massaciuccoli lake. Therefore the potential distribution of poplar trees in the study area was assumed to be equal to the distribution of hygrophilous forests, meso-hygrophilous forests, and wetlands (Fig. 2.7b), where naturally originated poplar trees (*P. alba* and *P. x canescens*) are present. Gene flow due to pollen flow was modeled taking into consideration data of pollen dispersion, which can reach by wind up to a distance of 2 km as indicated by genetic data in the Massaciuccoli lake (A1). Forest cover and rows of trees were used as natural barriers; artificial barriers were not considered. Data from weather stations showed that wind direction changed during the blooming season. Accordingly we considered the wind in all directions on the basis of a precautionary principle.

Therefore pollen dispersal was modeled using a buffer 2 km large delineated around the edges of poplar plantations, then the buffer was clipped to take into consideration the presence of natural barriers (Fig. 2.7c). In addition, we considered that pollen can penetrate forest cover up to a distance of 50 m as indicated by genetic data in the Serchio river area (A2). Pollen dispersal and the distribution of wild relatives were intersected to map the potential areas where poplar cultivations and wild relatives might occur. Moreover, to assess the persistence and invasiveness of cultivar poplar in the ecosystem and the risk of breeding for the progeny, we considered that the poplar dissemination occur: i) up to a distance of 2 km in open area and in absence of barriers, and ii) within 50 m inside the forest (Fig. 2.7c).

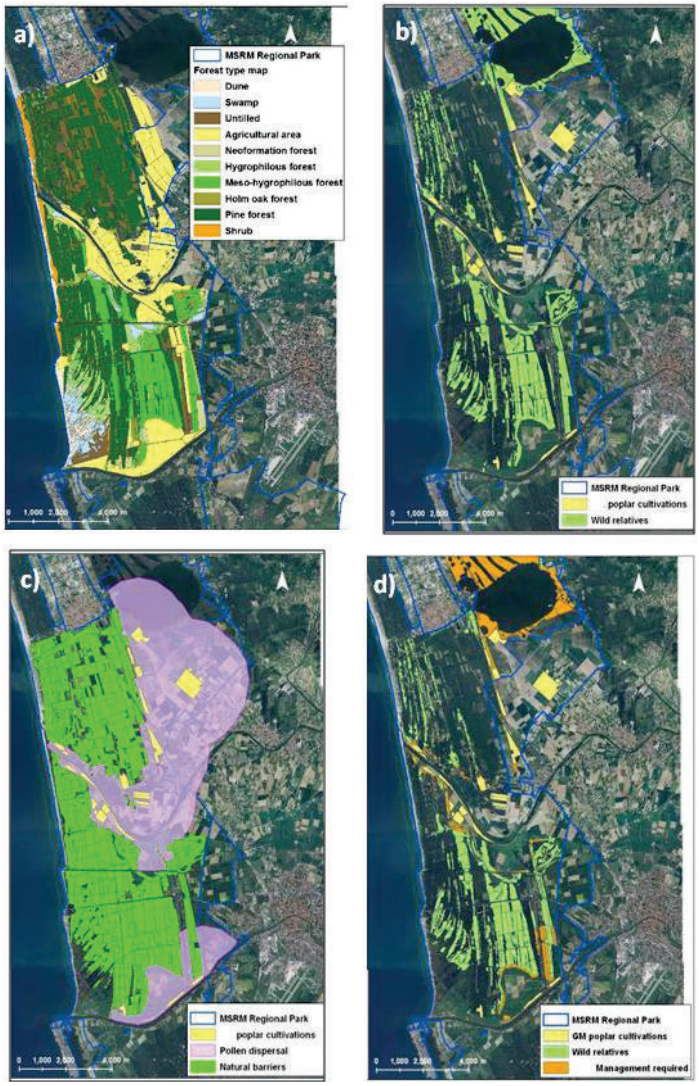

Fig. 2.7 a) Forest type map of the study area; b) Distribution of poplar cultivations (*P. x euramericana*) and of its wild relatives (*P. alba* and *P. x canescens*); c) Distribution of pollen dispersal and of natural barriers; d) areas of risk for crossing between poplar cultivation and wild relatives including their progeny.

Discussion

Several studies verified that breeded poplars can mate naturally with their parent species under field conditions and that hybridization can differ significantly with the involved specific populations (Imbert & Lefèvre 2003; Broeck et al. 2005).

In this study, in order to understand the possible hybridization between natural and cultivated poplars, the analysis of two poplar natural populations in different ecosystems was carried out, investigating the spatial genetic structure and the potential breeding between natural and cultivated populations.

Different *Populus* taxa are difficult to distinguish only on the base of their morphology (Holderegger et al. 2005; Pautasso 2009), especially because of hundreds of hybrids, varieties and cultivars (Eckenwalder 1996; Hamzeh & Dayanandan 2004). In order to assign species to each individuals, both nSSR and *trn*L cpDNA region analysis were carried out. Besides, further clarification made by STRUCTURE analysis, allowed to depict the definitive assignment of species overcoming, for example, the problem of the identical cpDNA haplotypes.

From the spatial structure analysis of genetic diversity by clustering analyses interesting results came up. In A1, the cluster 1, comprising only *P. nigra* individuals (section *Aigeiros*), had the highest F_{ST} values which indicated a strong genetic isolation from the other two clusters (section *Populus*). This result was expected considering that the hybridization among the two sections is excluded. This is in agreement with previous works demonstrating that crosses are possible only among *Aigeiros* and *Tacamahaca* (Zsuffa 1975; Ronald 1982; Eckenwalder 1996; Hamzeh & Dayanandan 2004; Broeck et al. 2005; Smith & Sytsma 1990). Gene flow barriers were absent between other two clusters composed of *P. x canescens* as expected considering that Massaciuccoli Lake is an open environment which favoured the genetic exchange.

In A2 the genetic isolation of the cluster 1, exclusively composed of *P. x canescens* trees, could be explained by the presence of environmental barriers: the analysis of structural diversification indicated the presence of other tree species, acting as barriers to pollen flow, as previously indicated in *P. nigra* by Rathmacher et al. (2010), and in *Populus simonii* L. by Wei et al. (2013).

An unexpected result concerns the crosses occurred between a gray poplar female of the mixed forest stand and two “Triplo” individuals from the neighbour plantation.

Gray poplar could be considered strictly related to the *Populus* section, as both of its parents belong to it, while “Triplo” is a hybrid of *P. nigra* x *P. deltoides*, belonging to the *Aigeiros* section (Eckenwalder 1996).

In literature, numerous studies stated that intersectional crosses are generally incompatible, mostly due to pre-fertilization barriers and hybrid inviability (Stettler et al. 1996; Slavov & Zhelev 2010). Hamzeh and Dyandanandan (2004), starting from previous investigations (Ronald 1982; Smith & Sytsma 1990; Eckenwalder 1996), assessed that in *P. nigra,* cpDNA showed similarity to species of the section *Populus,* but nuclear rDNA was distinct (Smith & Sytsma 1990). Thus, the extant *P. nigra* may derive from crossing between an ancestor species of section *Populus* as the maternal (cpDNA) donor and an ancestor of the section *Aigeiros* as the paternal (rDNA) donor (Smith & Sytsma 1990).

Ronald (1982) observed seed yield and germination in different crossings experiment, using *Populus* section individuals as females. The results showed incompatibility of *P. alba* with the other sections; while a complex hybrid (*P. x canescens* x *P. alba* x *P. grandidentata*) produced seeds in all the experiment. Germination Seeds and production seedlings percentage was up to 78% in case of mating with *P. nigra* L. var. "Italica" and up to 99% when crossing with an intrasectional (*Aigeiros*) hybrid *P. x euramericana*.

Moreover, the distribution ranges are very similar, so that white poplar and black poplar (EUFORGEN 2009) are sympatric species. Even though Ronald (1982) asserted that seasonal flowering barriers together with seedling weakness and inviability have preserved the natural isolation of *Populus* section from the other two ones, it is not unreasonable to assume that there have been opportunities for gene exchange among sympatric species, even between taxa of different sections (Stettler et al. 1996; Hamzeh & Dayanandan 2004).

Another important consideration is the demonstration that habitat loss and fragmentation interfere with mating systems and with patterns of gene flow of populations, altering ecological and genetic processes (Nason et al. 1997; Young & Boyle 2000; Young et al. 2000). Gene flow perturbation may affect population size of species (Lande 1988; Schemske et al. 1994; Burczyk et al. 2004) and leads to lose them by promoting hybridization with common congeners (Ellstrand 1992; Ledig 1992; Ferdy & Austerlitz 2002; Burczyk et al. 2004).

All these observations remark the importance of not underestimating the detected hybridization event: even though the estimated frequency of breeding was 8.8×10^{-3}, it must be considered from a "genetic conservation" point of view since several and potentially severe consequences and threats of hybrids on the genetic diversity (Wolf et al. 2001) can occur.

Indeed, hybridization can play an important role in affecting evolution and conservation biology of native poplar populations, even when occurring infrequently, with or without introgression (Broeck et al. 2005).

The increase of hybridization occurrence is becoming more and more hazardous, especially in forest tree of considerable economic importance, such as *Populus* (Broeck et al. 2005; Farnum et al. 2007), as it undermines the adaptability for the future (Rehfeldt 1999; Kramer & Havens 2009; Vornam et al. 2004) both at species and population level (Rajora and Mosseler 2001).

Native populations are negatively influenced by hybrids by threatening their genetic integrity (i.e., by limiting local adaptation) (Talbot et al. 2012) and by affecting biodiversity at the level of communities and ecosystem processes (Whitham et al. 1999; Talbot et al. 2012).

Furthermore, several studies have reported that genetic erosion in poplar trees (*Populus* spp.), as foundation species, affects coenosis and ecosystem structure and function (Whitham et al. 2006; Bangert et al. 2005; Bangert et al. 2006). It may lead to alter the stability of ecosystem, modifying the composition and the interaction of species (Ledig 1992; Whitham et al. 1999; Talbot et al. 2012).

In particular riparian forest, as threaten habitat, is even more sensitive to any change of the delicate equilibrium of biotic and abiotic elements (Bravard et al. 1986; Clerici et al. 2013; Gundersen et al. 2010; Kramer & Havens 2009).

In addition, widespread crops often touched relative wild species, resulting frequently in hybridization (Hancock et al. 1996; Snow & Moràn-Palma 1997). In the same way, breeded poplar plantations in natural habitats pose a severe potential threat for the ecosystem and for the genetic diversity of native poplars (Broeck et al. 2005; Wolf et al. 2001; Whitam et al. 2006).

Gene flow between crops and wild species relatives is a natural event (Hancock et al. 1996; Ellstrand et al. 1999; Messeguer 2003), but nowadays hybrid poplar plantations may soon include genetically engineered trees (DiFazio 2002; Ellstrand 2003), in which the novel trait has potential and several uncertain effects on community and ecosystem (Whitham et al. 2006). The consequent major concern is the transfer of the transgene through hybridization in wild relatives' gene pool (Broeck et al. 2005; Adams & Burczyk 2000).

If the plantations exhibited lower genetic diversity, even modest gene flow may have negative impacts on natural stands, potentially reducing diversity and adaptability of future generations (Adams & Burczyk 2000; Burczyk et al. 2004). Thus, one of the major task for conservation biologists should be the development of

simple methods for measuring and monitoring gene flow from plantations to natural population (DiFazio et al. 2004), predicting their potential interactions (Wolf et al. 2001).

By now distance and density of receiving populations, pollen and seed production, their dispersal distances, synchrony of flowering (Adams & Burczyk 2000; Bialozyt 2012) are parameters useful to study gene flow (Messeguer 2003), since they have a remarkable effect on the occurrence of outcrossing (Bialozyt 2012). In our study we considered most of these factors, but the most influencing one seemed to be the wind (intensity and direction) and the competition with other trees.

Moreover, the potential threat due to crossing between poplar cultivations and its wild relatives in the surrounding environments was investigated. Based on the information obtained, and using the spatial dataset available for the study area, it seems that pollen could affect important habitats hosting naturally originated poplar population. The assessment of this potential hazard indicates that some management measures are required to mitigate the threat. The results performed with GIS modelling indicate that the area of the habitats exposed to threat of breeding is of 187.9 ha. The area exposed to threat enlarge up to 822.5 ha when the threat for the progeny is considered, representing 23.5% of the total area of the habitats. For forestry habitats the risk concentrates along the edges of forest cover because tree crowns acts as barriers against pollen dispersal. Instead, in the wetlands open area of Massaciuccoli lake, the risk is larger because natural barriers are scarce (Fig. 2.7d).

Acknowledgements

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Supporting Information

Variable and informative sites after aligned DNA sequences of the *trn*L chloroplast region of *Populus* species (Appendix S 1) is available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding author.

Appendix S 1. Variable and informative (indicated by *) sites after aligned DNA sequences of the *trn*L chloroplast region of *Populus* species.

Species	Site positions																												
	20	26	198*	256	257	279*	360	362	365	366	367	368	384	385	386	387	388	389	390	391	392	393	404	405	406	407	479
P. alba	A	-	T	-	-	A	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	-	A
P. x canescens	A	-	T	-	-	A	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	-	A
P. tremula	-	-	T	-	-	A	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	-	-	G
P. deltoides	-	-	G	G	G	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A
P. x euramericana	-	-	G	G	G	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A
P. nigra	A	-	T	-	-	A	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A	-	A
P. trichocarpa	-	-	T	G	G	G	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	A
P. tremuloides	A	G	T	-	-	A	A	-	T	T	A	A	T	T	A	A	T	A	A	T	A	A	A	A	A	-	A

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Chapter 3
Biodiversity in Agroecosystems: crop-to-weed hybridization issues in Brassicaceae

BIODIVERSITY IN AGROECOSYSTEMS: CROP-TO-WEED HYBRIDIZATION ISSUES IN BRASSICACEAE

RUNNING TITLE: INTERSPECIFIC CROSSES IN BRASSICACEAE

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ABSTRACT

Herbaceous vegetation of field margins holds high species richness and may cover different roles by providing diversity and supporting many other species of the agroecosystems. Recently, species of arable weed, as wild mustard (*Sinapis arvensis* L.), are facing a great decline due to the increasing intensification of agricultural practices. Moreover, one of the most dangerous threats to species maintenance, both at demographic and genetic levels, is represented by hybridization. Indeed, in agroecosystems, the mating of crops-wild relatives can profoundly affect the ecology and evolution of species in the surrounding habitats. Conservationists should preserve herbaceous species of field margins, because the transfer of new genes may potentially and negatively affect the genetic integrity of wild populations, which is necessary to the local adaptation to the environment.

Consequently, the aim of this work was to establish the potential hazard of crop-to-weed hybridization in the field margins.

The study site is located in Regional Park of Migliarino-San Rossore-Massaciuccoli (MSRM) (Pisa, Italy), where organic agriculture with *Brassica napus* L. var. *oleifera* D.C. is performed. Both the analysis of the field margins' vegetation and the phenology allowed to designate *S. arvensis* as potential breeder of oilseed rape. 510 pollen donors of *B. napus*, 25 plants of wild mustard as mothers and 781 seedlings were genotyped and the crossing evaluated by nuclear microsatellites markers (nSSRs). A number of seedlings equal to 9×10^{-2} originated by interspecific crosses, suggesting that management measures for the coexistence between crops of oilseed rape and wild mustard must be considered to mitigate the threat of loss of genetic integrity. Besides, intersecting genetic information and topographic maps and thematic layers of the study area, we found the habitats eventually affected by the threat.

Introduction

One of the main threats responsible for the loss of biodiversity is the fragmentation of habitats (Honnay et al. 2005). Habitat fragmentation is the main trend of landscape change in several bioregions of the world, causing a dramatic reduction in biodiversity and strong alteration in plant richness and composition (Fahring 2002, 2003; Hoffmeister et al. 2005).

On a global scale the rate of loss of species diversity is increasing. This is especially true for forest ecosystems, because of high human population pressures (Gilliam 2007). Habitat destruction or alteration through changing in land use (for example forest use or conversion to agriculture) can intensify the loss of native species.

Nature conservation in Europe have focused mainly on designations of nature reserves and parks. However, the juxtaposition of land uses, particularly farmed areas and natural habitat, forms mosaics in the landscape (Marshall 2004). So, protected areas often must cope with human activities, as agriculture. Crop management may impact biodiversity within ecosystems, including agroecosystems, by affecting soil processes, nutrient cycling and trophic interactions (Marshall et al. 2003).

Agroecosystems hold a variety of relations between fields and their margins. Although field margins are not usually ranked as habitat types, they contain a variety of plant communities in different structures (Marshall et al. 2003) and have different roles in agronomic, environmental, nature conservation and recreational use (de Snoo 1999; Marshall 2004).

Recently, herbaceous vegetation of the field margins, as species of arable weed, is facing a great decline (Rich & Woodruff 1996; Gibson et al. 2006) with increasing intensification of agricultural methods (Robinson & Sutherland 2002; Wilson & King 2003). Special attention should be focused by conservationists on herbaceous plants. Even though they do not play a keystone role in natural ecosystems, they hold higher species richness (Gilliam & Roberts 2003; Roberts 2004; Whigham 2004) and higher natural extinction rates than plant species in other strata (such as hardwood tree species) (Levin & Wilson 1976).

Weeds may cover different roles by providing diversity and supporting many other species. First of all, weed community contributes to the net primary productivity (in terms of conversion of light energy into biomass) and as part of an ecosystem interacts with biotic components. Weeds are sometimes superior competitors for soil nutrients, compared with tree seedlings (Lyon & Sharpe 2003), so that the herb layer could affect the composition of the regenerating forest (Gilliam 2007). A certain

number of insects depends on the presence of weeds for part or whole of their life cycle. Birds feed both on seeds and plant, or on invertebrates, which in turn can be weeds' parasites (Marshall et al. 2003).

Preserving field margins, including genetic diversity of species, is pivotal since erosion of diversity may thus result in damage to the ecosystem functionality (Naeem et al. 1994; Tilman et al. 1996; Chapin et al. 1997; Marshall 2004).

One of the most dangerous threats to species maintenance, at demographic and genetic levels, is represented by hybridization, especially if facilitated by human activities.

Hybridization succeeds if populations are sympatric, they share pollination vector, the times of flowering overlap and, not less important, the donor and recipient plants are sexually compatible. Even modest levels of compatibility could produce a few viable offspring, which would be sufficient to establish a population of hybrid plants (Ellstrand 2003). In agroecosystems, the mating between crops and wild relatives has profound effects on the ecology and evolution of species in the surrounding habitats (Ellstrand et al. 1999). The production of viable and fertile interspecific hybrids is one of the most controversial issues in agriculture, and recently crop-to-weed hybridization is more and more pointed out by the potential escape of crop transgenes into natural populations.

The transfer of new genes (genetic pollution) may potentially and negatively affect the genetic integrity of wild populations (Rieseberg 1991). Genetic integrity refers to the maintenance of a natural gene pool, both at species and population level (Vergeer et al. 2008). It is important to notice that preserving the genetic integrity of a species or a population does not mean saving high levels of genetic variation, because low levels are not always a problem (Huenneke 1991). Many species may be highly adapted to their specific local environments. Therefore, these species maintain a relatively narrow range of genotypes (Lande & Shannon 1996).

One of the most widely distributed weeds in Europe is *Sinapis arvensis* L. (wild mustard), an annual cruciferous (Brassicaceae). This weed is well adapted to growing in disturbed habitat where specific mechanisms, as seed dormancy, are very important to survive (Rees & Long 1992; Moyes et al. 2002). Wild mustard resulted relevant in supporting agroecosystem diversity and recent studies assessed the importance of weeds on the base of associated species. *S. arvensis* is considered an arable species important for in-field biodiversity. In fact, thirty-seven species of phytophagous insects, belonging to thirteen families, are associated with wild

mustard and three of them are host specific-species. Moreover, 3-8 species of birds are related to wild mustard for diet (Marshall et al. 2001).

In the Regional Park of Migliarino-San Rossore-Massaciuccoli (Pisa, Italy), the presence of *S. arvensis* was detected within the field margins of a crop field sown with *Brassica napus* L. var. *oleifera* D.C.

The aim of our work was to establish the potential threat associated with the crop-to-weed hybridization in the field margins.

Materials and methods

Study area

In the Regional Park of Migliarino-San Rossore-Massaciuccoli (Pisa, Italy), the SCI/SIR/SPA "Selva Pisana" (Natura2000 Code IT5170002) includes coastal dune habitats and internal system of fossil dunes and interdunes with alternation of pine forests, wide forests with *Quercus robur* L., high bush, freshwater and brackish wetlands.

We selected the study area in the "Culatta" Locality (43°41'39"N, 10°19'43"E), close to the Arno river and within the SCI, where organic agriculture is performed (Fig. 3.1). Most of the species occurring in the "Culatta" site are typical of cultivated field weed communities. According to the classification proposed by Rivas-Martinez et al. (2002), most of them belong to the *Stellarietea mediae* Tuxen, Lohmeyer & Preising ex von Rochow class, including annual grasses communities composed of ruderal, nitrophilous and semi-nitrophilous species. In marginal areas, perennial, sub-nitrophilous and mesophilous species of the *Artemisietea vulgaris* Lohmeyer, Preising & Tuxen ex von Rochow class have been also recorded. On the boundaries of drain ditches and on the hedges bordering the woody vegetation of the neighboring alluvial wood, the vegetation is characterized by perennial, mesophilous herbaceous species, such as *Holcus lanatus* L., *Plantago major* L., *Rumex conglomerates* Murray, *Verbena officinalis* L., *Sporobolus indicus* (L.) R. Br., *Cynodon dactylon* (L.) Pers., included in the *Molinio-Arrhenatheretea* Tüxen 1937 class and in the *Plantaginetalia majoris* Tüxen & Preising in Tüxen 1950 order.

The crop area is bounded by a mixed broadleaved alluvional forest, characterized by the presence of peduncolate oak (*Quercus robur* L.), narrowleafed ash (*Fraxinus oxycarpa* Bieb.), maple (*Acer campestre* L.), common hornbeam (*Carpinus betulus* L.), elm (*Ulmus minor* L.), black alder (*Alnus glutinosa* L.) and poplars (*Populus spp.*). The undergrowth is characterized by the significant presence of elmleaf blackberry (*Rubus*

ulmifolius Schott) and common hawthorn (*Crataegus monogyna* Jacq.); the herbaceous layer by *Carex remota* L. and *Brachypodium sylvaticum* (Huds.) Beauv.

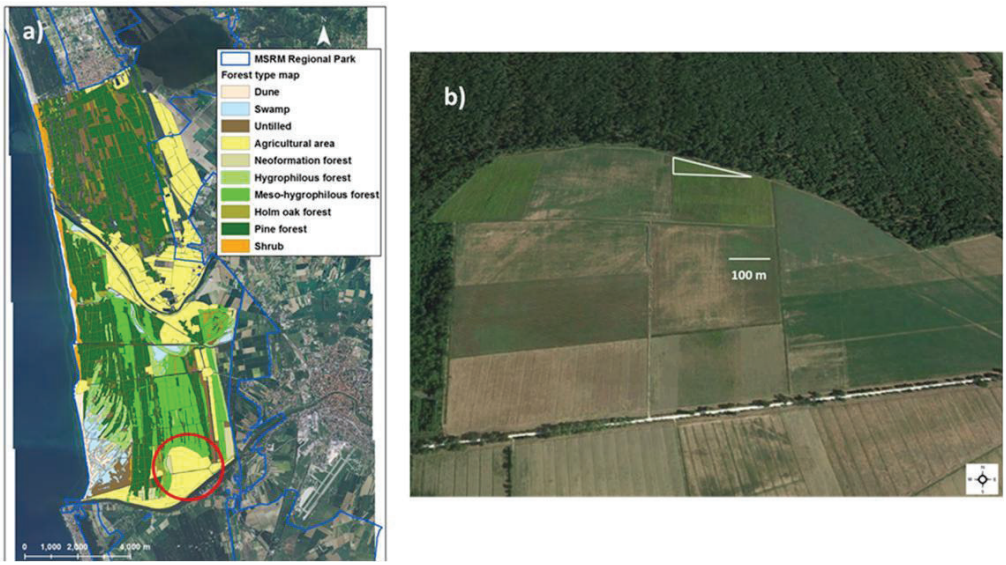

Fig. 3.1 a) Study area location; b) "Culatta" locality: white line defines the border of the triangular plot (2000 m^2 large) with oilseed rape cultivar.

Phenological analysis

We defined the phenology as the study of periodic biological events, as reported in Lieth (1974) and Schwartz (2003). In order to identify the wild plant species susceptible to potential breeding with the cultivated oilseed rape (*Brassica napus* L. var. *oleifera* D.C.), we observed in the study area a group of species belonging to the Brassicaceae family. From March 2010 and throughout the growing season, the phenological phases was monthly examined and monitored. We recorded only generative stages, according to the Dierschke's scale (Dierschke 1989; 1994) (Table 3.1).

Sampling and seed germination

In the winter 2011, in the study area we identified and sowed a triangular plot (2000 m^2 large) with oilseed rape cultivar (Fig. 3.2). In the spring of 2012, we scanned the boundary of the plot, and we found twenty-five wild mustards (Fig. 3.2). For each of them, we registered the x, y coordinates by GPS, and the "Arasystem" traps (Betatech bvba, Gent, Belgium) were installed in order to collect seeds (Fig. 3.3b).

Table 3.1 Phenological generative stages according to Dierschke (1989; 1994)

Woody - Herbaceous (Forbs)		**Herbaceous (Graminoids)**	
Stage	**Description**	**Stage**	**Description**
0	No buds	0	No visible inflorescences
1	Visible buds	1	Recognizable inflorescences, closed
2	Swollen buds	2	Visible inflorescences, not unfolded
3	Just before flowering	3	Inflorescences unfolded
4	Beginning of flowering	4	First flower dusting
5	Flowering at 25%	5	Flower dusting at 25%
6	Flowering at 50%	6	Flower dusting at 50%
7	Full flowering	7	Full flowering
8	First leaf fading	8	First leaf fading
9	Full leaf fading	9	Full leaf fading
10	Development of fruit	10	Development of fruit
11	Seed dispersion	11	Seed dispersion

Fig. 3.2 The triangular plot sowed with cultivar of oilseed rape, where 25 wild mustard individuals were found along the boundary and georeferenced (dots). Yellow dots indicate plants from which seeds had been collected.

At the beginning of the summer of 2012, we carried out the sampling. We considered as potential fathers each individual of oilseed rape, found within 3 m from each wild mustard (potential mothers) (Fig. 3.3a). We sampled and stored at -20 °C the plant tissue from all parents.

We collected and stored at 4 °C all the trapped seeds. In the laboratory, from each trap we randomly selected 100 seeds, and placed them on sheets of filter paper in 7 cm Petri dishes (VWR International PBI srl, Milan, Italy), soaked with water and stored for 1-2 weeks at 4 °C. When seeds appeared swollen or even germinated, we moved

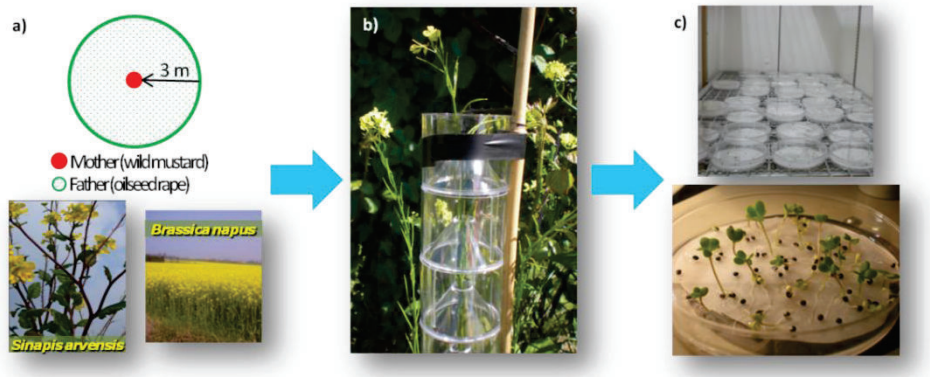

Fig. 3.3 a) Sampling scheme of oilseed rape individuals (potential fathers) within 3 m radius away from wild mustard (mothers). **b)** "Arasystem" (Betatechbvba) traps installed on siliques of wild mustard to collect seeds. **c)** Seeds posed on sheets of filter paper in 7 cm Petri dishes up to germination.

Petri dishes in the germination chambers at 25 °C (Fig. 3.3c). We maintained the underlayer watered and sometimes, in order to facilitate germination, we added KNO_3 solution, according to Warwick et al. (2003). Incubation of seeds took place under 16 h light/8 h dark photoperiod and an irradiance of 35 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Osram Biolux 965).

Molecular data

Total DNA was isolated from plant material (50–100 mg as starting material) using the DNeasy plant kit (QIAGEN, Germany) and following the manufacturer's specifications.

In order to indentify *Brassica napus*, we used microsatellite markers from literature. In total 41 primer pairs (Table 3.2) were retrieved from different papers (Lowe et al. 2002, 2004; Kresovich et al. 1995; Szewc-Mc Fadden et al. 1996, Lagercrantz et al. 1993, Uzunova & Ecke 1999) and tested on different samples of cultivated *Brassica napus* and *Sinapis arvensis*. We amplified by PCR all DNA in a final reaction volume of 25.0 µl consisting of the following: 2.0 µl template DNA (10 ng/µl), 15.4 µl H_2O, 2.0 µl primers (10µM each), 0.5 µl dNTPs (2.5 mM each), 0.4 µl BSA (10 mg/µl), 2.5 µl 10X buffer (EuroClone®, Pero, MI, Italy), 0.2 µl Taq (5U/µl) DNA polymerase (EuroClone®, Pero, MI, Italy). We used a GeneAmp® 9700 PCR System (Life Technologies, CA, USA) for PCR amplification. We used the following temperature profile: 1 min at 94°C, then 30 cycles of 60 s at 94°C, 60 s at 55°C, 60 s at 72°C and finally 5 min at 72°C. We determined the optimal PCR reaction conditions

for each of the primer pairs by testing a range of annealing temperatures (55.0–70.3°C) and different template DNA concentrations (1:5, 1:10, 1:20 and 1:1).

We carried out the sizing of the PCR products on Applied Biosystem® 3500 Genetic Analyzer automatic sequencer (Life Technologies, CA, USA). In order to size the amplified fragments, we used the internal molecular size standard Liz 500 (Life Technologies, CA, USA) and the software Gene Mapper ver. 4.0 (Life Technologies, CA, USA).

Cartographic data

Topographic maps and thematic layers were acquired for spatial analysis using a Geographic Information System (GIS). Tuscany Region supplied the topographic maps at the scale of 1:10,000. The Regional Park provided us with the land use land cover map (D.R.E.AM 2002) at the scale of 1:15,000. A forest type map at the scale of 1:10,000 was obtained by polygon delineation of vegetation maps produced by Tomei and collaborators (2003) and Sani and collaborators (2010). The Regional Park of MSRM supplied a map depicting the distribution of agricultural crop types (e.g., oilseed rape plantation) at the scale of 1:10,000. We also acquired digital aerial images (year 2007) at the nominal scale of 1:10,000 (pixel size = 1 m). All these data were projected in a common coordinate system and registered in a geographic database.

Table 3.2 Brassica primers used in this study.

Primer	Forward	Reverse	Reference
Na10-F06	CTCTTCGGTTCGATCCTCG	TTTTTAACAGGAACGGTGGC	Lowe et al. 2002
Ol09-A06	TGTGTGAAAGCTTGAAACAG	TAGGATTTTTTTGTTCACCG	Lowe et al. 2002
Ni2-C12	ACATTCTTGGATCTTGATTCG	AAAGGTCAAGTCCTTCCTTCG	Lowe et al. 2002
Ni2-F02	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC	Lowe et al. 2002
BN9A	GAGCCATCCCTAGCAAACAAG	CGTGGAAGCAAGTGAGATGAT	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN16A	CGACCGTGGAAGCAAGTGAG	CCATGATTACGCCAAGCTATTTA	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN18A1	TCAATCCCACCAACCAGACAAA	TAAGACAGGTAAGGTTTGGCCC	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN20A	GACAATCAATCCCACCAACCAG	TAAAAGAAGAGTGCCAATCCCAT	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN25A	CACGTGGTATGTTGGTATTGGG	TGATTCTCCTCCGACGCATGC	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN19A	CACAGCTCACACCAAACAAACCTAC	CCCCGGGTTCGAAATCG	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN25C2	AAACCTCCTCAAAAACCCCTAAACG	TCCCCTCTTTCCTCTCTCTCTAGGC	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996
BN26A**	TAAACTTGTCAGACGCCGTTATC	CCCGTAAATCAAGCAAATGG	Kresovich et al. 1995 Szewc-Mc Fadden et al. 1996

BN27B2	CCGGATCCAAGCTTATCGACA	CCGAGAAGGGAAGCTAGAGAGGTC	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
BN38A	TGGTAACTGGTAACCGACGAAAATC	ACGCTGTCTTCAGGTCCCACTC	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
BN40C1	CCCCTCTTTGATTCTCCTCCGA	CGTGGTATGTTGGTATTGGGTCGT	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
BN50F	CGTTGAAGCATCTCTGTATCTCTCC	TTTCTCTCCGCACCAAAACAC	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
BN59A1	TGGCTCGAATCAACGGAC	TTGCACCAACAAGTCACTAAAGTT	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
BN75A*	ACCCCGGGTTCGAAATCG	CAATCCCACCAACCAGACAACC	Kresovich et al. 1995
			Szewc-Mc Fadden et al. 1996
MB1	CAATAATGATGAGATGAAATAAGG	GCAGTAAGGCAGCTAAAAGTGGAT	Lagercrantz et al.1993
MB3*	TATTAGACCATTGCTTACCT	AACCAGCTCTCACATTGATT	Lagercrantz et al.1993
MB4	TGTTTTGATGTTTCCTACTG	GAACCTGTGGCTTTTATTAC	Lagercrantz et al.1993
MR52a	TCGACATGGATTCTACCAAA	GAACTTGCAAGCTGCAATTA	Uzunova & Ecke 1999
MR181	AGATTTGCATGTGGTTTGAC	ATTGCTTANTGATGTTGGGAA	Uzunova & Ecke 1999
MR183	GAAATCATTCAATGCTCTGA	GTAAAAATCCCAATCAATGG	Uzunova & Ecke 1999
Na12-A01*	GCATGCTCTTGATGAACGAA	GCTTCAACCTCTCAATCGCT	Lowe et al. 2004
Na12-02**	AGCCTTGTTGCTTTTCAACG	AGTGAATCGATGATCTCGCC	Lowe et al. 2004
Na12-A07	TCAAAGCCATAAAGCAGGTG	CATCTTCAACACGCATACCG	Lowe et al. 2004
Na12-A08*	AACACTTGCAACTTCATTTTCC	CATTGGTTGGTGAATTGACAG	Lowe et al. 2004

Na12-B05	CAAATATCCGTCATCGGAGC	CCTGCGGGATATTGAAGACC	Lowe et al. 2004
Na12-C08	GCAAACGATTTGTTTACCCG	CGTGTAGGGTGATCTAGATGGG	Lowe et al. 2004
Na12-E05*	CGTATGTTTGTTCCACCTGC	ACTAGCAACCACAACGGACC	Lowe et al. 2004
Na12-E06A	TTGGGTTGACTACTCGGTCC	CCGTTGATTTGGCTAAGACC	Lowe et al. 2004
Na12-G05*	CCGATCATACCTTTTACTCTAGCC	GATGTTCCTCTCGGTGATGC	Lowe et al. 2004
Na14-E11	TCATCCTTCTCACACCAAAATC	CCTCGAAATAGCTCCAACCC	Lowe et al. 2004
Na14-G02	TTCCCTTTATTGAGCAAGCTG	TCCCGGTCGCTAAGATATTG	Lowe et al. 2004
Ni2-A11	AACAAACAAGAGTCGAATACGG	AATGCCCTCTAACTGAGCCC	Lowe et al. 2002 Lowe et al. 2004
Ni3-G04B	ATACTCGGGATAGGTGTGCG	CATGTGGCAATCCTACATTTAC	Lowe et al. 2004
Ni4-D10*	ACATGCGAAAGGGATTTGAC	TGCAAGTGAACTCAAAACAAAAG	Lowe et al. 2004
Ni4-E08*	GATTTTGAGGAAGCGGAGG	CAAAGCACTGAGAGAGAGAGAGAG	Lowe et al. 2004
Ni4-G04	GAGGCGCGTGGACTAACC	TTACACCCCATCCAAACTCC	Lowe et al. 2004
Ni4-H04	CAAGAAAGGGTATTGCGTCG	TGTTTTAGAAATGGTATGCCCC	Lowe et al. 2004

* Amplification occurred in both *Brassica napus* L. var. *oleifera* D.C. and *Sinapis arvensis* L.

**Polymorphism was detected between *Brassica napus* L. var. *oleifera* D.C. and *Sinapis arvensis* L.

Results

Phenological analysis

The phenological analysis aimed at identifying the most liable wild plants to the crossing with the cultivated oilseed rape (*Brassica napus* L. var. *oleifera* D.C.). In the "Culatta" locality, a group of species belonging to the Brassicaceae family was found and continuously observed during the growing season, from March to August, and the observations were repeated for three years (2010, 2011 and 2012).

We identified the following species: *Sinapis arvensis* L., *Cardamine hirsuta* L., *Capsella bursa-pastoris* (L.) Medicus, *Cardamine pratensis* L. and *Alliaria petiolata* (Bieb.) Cavara et Grande.

S. arvensis and *C. bursa-pastoris* were recorded in the fallow lands surroundings the cultivated areas; the mesophilous species *C. pratensis* and *A. petiolata* were collected in the herbaceous layer of the neighbor mixed alluvial forests, while *C. hirsuta* was found in both the environments.

Their phenological data were monthly recorded throughout the growing season, which are summarized in Table 3.3.

The longest flowering period was detected in *S. arvensis*. It bloomed from March to May, overlapping the flowering period observed in *B. napus*. On the base of its flowering period and its wide and close distribution, wild mustard was considered the best potentially candidate for crossing with the cultivated oilseed rape.

Table 3.3 Phenology of Brassicaceae in the "Culatta" Locality: Cropped area (C) and marginal land of the Mixed Wood (MW) (the numbers refer to the phenological generative stages, as reported in Table 3.1).

Species	SITE	March	April	May	June	July	August
Sinapis arvensis L.	C	4-5	6-7	7-10	7-11	11	11
Cardamine hirsuta L.	C/MW	5-6	7-10	10-11	\	\	\
*Capsella bursa-pastoris*L.	C	6-7	10	11	\	\	\
*Cardamine pratensis*L.	MW	0-1	5-6	7-10	10-11	\	\
Alliaria petiolata	MW	0	4-5	6-10	10-11	11	\

Crossing of *S. arvensis* and *B. napus*

Nuclear microsatellites (nSSR) were selected on their transferability among Brassica species, and 10 out 41 primer pairs amplified both *Brassica napus* and *Sinapis arvensis* (Table 3.2). Only two out these 10 nSSR markers permitted to distinguish different alleles between *B. napus* and *S. arvensis* (Na12-A02 and BN26A as reported in Table 3.2). In the field, we collected 510 individuals as potential fathers of oilseed rape around wild mustard. Twenty-five wild mustard individuals were detected along the boundary of the plot, but only 18 of them were considered in the following molecular biology analysis by nSSR, because the remaining 7 individuals did not produce any seeds (Fig. 3.2). We sowed seeds from 18 mothers and we stopped the experiment once the number of seedlings reached 100 per mother. On total, 47% of seeds of seeds germinated, but germination resulted quite variable among mothers ranging from 9% to 100% of germinated seeds (Table 3.4).

The molecular analysis with nSSR (Na12-A02 and BN26A as reported in Table 3.2) indicated that six genotypes generated from four allelic variants were present in oilseed rape individuals, while wild mustard plants showed 11 genotypes from 7 allelic variants.

Thanks to the specific allelic variants for each species, it was possible to determine the origin of seedlings. Most of them (39%) is the product of intraspecific crosses of *S. arvensis.* Nevertheless, the remaining seedlings originated from the cross between wild mustard and oilseed rape (Table 3.4). We compared the data using the Student's t-test ($P< 0.05$ was assumed to be statistically significant), and the mean percentage of intraspecific crosses (39%) resulted significantly different from the mean percentage of interspecific crosses (9×10^{-2}) getting: ($t=4.2062$, $gdl=34$, P-value= 0.0002, $p<0.05$).

Table 3.4 Seed germination (i.e. percentage of germinated seeds), intraspecific and interspecific crosses (i.e. the percentage of seedlings originated respectively from the crosses between wild mustards and the crosses between oilseed rape and wild mustards).

ID mother	Seed germination	Intraspecific crosses	Interspecific crosses
1	50%	44%	6×10^{-2}
2	28%	17%	11×10^{-2}
3	36%	27%	9×10^{-2}
4	9%	6%	3×10^{-2}
5	55%	38%	17×10^{-2}
6	83%	44%	39×10^{-2}
7	26%	20%	6×10^{-2}
8	44%	31%	13×10^{-2}
9	34%	30%	4×10^{-2}
10	35%	24%	11×10^{-2}
11	28%	13%	15×10^{-2}
12	97%	92%	5×10^{-2}
13	21%	20%	1×10^{-2}
14	100%	99%	1×10^{-2}
15	60%	55%	5×10^{-2}
16	23%	23%	0×10^{-2}
17	100%	99%	1×10^{-2}
18	23%	21%	12×10^{-2}
Total	*47%*	*39%*	9×10^{-2}

For spatial analysis we assumed that the distribution of *S. arvensis* (wild relative of oilseed rape) was equal to the distribution of non-cultivated lands obtained from vegetation and agricultural crop type maps. On the basis of field observations we considered also the presence of *S. arvensis* within a buffer 5 m large delineated around the edges of oilseed rape cultivations. Non-cultivated lands, where *S. arvensis* grows, covered 91.6 ha of the protected area. This area was shaped on the base of proximity to oilseed rape cultivations (Fig. 3.4a), on pollen dispersal data and on the presence/absence of natural and artificial barriers (Fig. 3.4b); each map was represented by means of a layer in GIS software. Intersecting these layers, the resulting map showed the area exposed to risk of breeding, whose extension was 58.7 hectares, corresponding to 64.1% of the total area occupied by *S. arvensis* (Fig.3.4c).

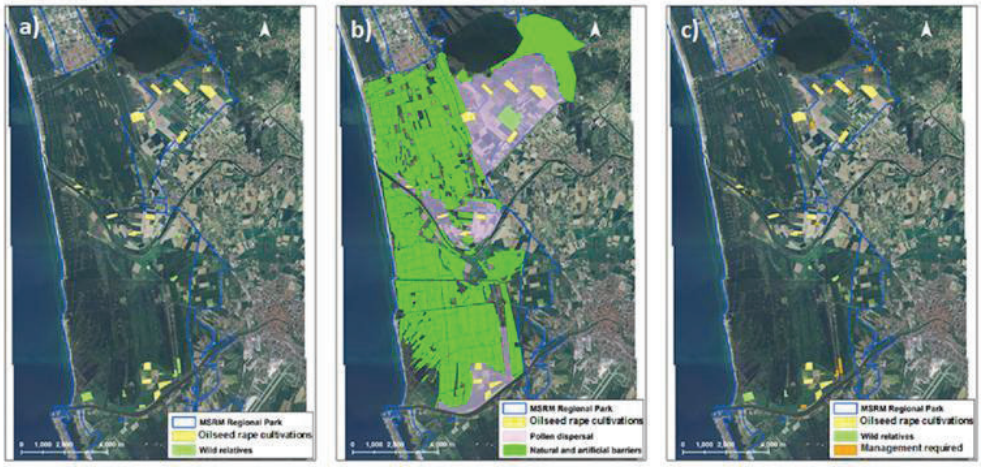

Fig. 3.4 a) Distribution of oilseed rape cultivations and of its wild relative (*Sinapis arvensis*); **b)** distribution of pollen dispersal and of natural and artificial barriers; **c)** area exposed to risk of breeding between oilseed rape and its wild relative (*Sinapis arvensis*).

Discussion

The implication of this work for the conservation management is discussed below, considering that even a few viable offspring would be sufficient to establish a population of hybrid plants (Ellstrand 2003). In this study, preliminary analysis on contemporary occurrence of the studied species, blooming season and pollen flow were contingent to direct molecular analysis and evaluate the possibility that crops and wild relative could hybridize in the field. We found that *Brassica napus* and the weed *Sinapis arvensis* can naturally hybridize at a rate of 9×10^{-2}, though we cannot affirm if the hybrid is fertile, able to survive in the natural environment and able to give a progeny. This is due to the fact that the grown seedlings were destroyed to perform molecular analysis. Nevertheless, it cannot be underestimate that the hybridization event can naturally occur in the environment.

Crop-to-weed hybridization causes genetic erosion of species which in turn may have effects on the biodiversity of neighbouring environments, especially considering that arable weeds, as *S. arvensis*, support species richness in agroecosystem (Marshall 2004).

Biodiversity is fundamental both to agroecosystem health and to progress in agricultural production (Thrupp 2004). In agroecosystem, the long-term success of species is guaranteed by sufficient level of genetic variation; otherwise, the condition

of any agroecosystem consistently deteriorates and eventually the production declines (Jana 2005).

The most harmful genetic consequences of interspecific hybridization are the loss of genetic diversity and the loss of locally adapted populations (Reisenberg 1991; Ellstrand & Elam 1993). In both cases, the changes in the autochthonous species' gene pool can lead to their extinction (Abbott 1992). Moreover, the hybrids themselves can threat native species by affecting resources or components of a community (e.g., pollinators, herbivores, pathogens) (Vilà et al. 2000).

In our study we investigated the possibility of hybridization: some wild mustards did not produce offspring, while most of them showed quite high level of crossing with conspecific. The rate of interspecific hybridization with oilseed rape was significatively low but demonstrated that they are sexually compatible in the field.

To our knowledge, the sexual compatibility between *B. napus* and the weed *S. arvensis*, is still controversial. Several works, developed both in controlled and field conditions, gave not consistent results about their hybridization. Most of the investigations were focused on testing if the introgression of genetically modified traits from oilseed rape to wild mustard was possible and relevant for the environment. Raybould and Gray (1993) stated the sexual incompatibility, since hybrids were achieved only by embryo rescue. Chèvre and collaborators (1996), and Lefol and collaborators (1996) found that hybrids could form but only with *B. napus* as the maternal parent. Lefol and collaborators (1996) demonstrated that hybrids can be obtained by hand pollination assisted with in vitro ovary culture; they found 1.2% hybrid yield per flower considering oilseed rape as maternal parent, while only 0.1% when wild mustard was the mother. Moreover, they observed no spontaneous crosses. Warwick and collaborators (2003) conducted an experiment on introgression of modified traits by screening seedlings for glyphosate resistance. They reported that the hybridization frequency between *B. napus* and *S. arvensis* is less than 2×10^{-5}, when the first is considered the paternal parent while the latter the maternal one. These results must be carefully considered, since Lefol and collaborators (1996), and Warwick and collaborators (2003) did not use molecular analysis to detect hybridization; since their scope was to verify if the modified trait was transferred from oilseed rape to wild mustard, they tested the resistance by spraying seedlings and recording the survival. Finally, Moyes and collaborators (2002) used molecular markers for analysis; they obtained hybrids at a very low rate in controlled experiments performed in glasshouses, even when *S. arvensis* was the maternal parent, but no crossing was detected under field conditions.

Hu and collaborators (2002) demonstrated that somatic hybrids between *S. arvensis* and *B. napus* can be obtained by fusing mesophyll protoplasts, reaching 1.4% of plant regeneration efficiency.

Finally, Daniels and collaborators (2005) found evidence of possible hybridization between *Sinapis arvensis* and *Brassica napus.* In the follow on years of the experimental oilseed rape crop, they tested several plants of wild mustard which were present around the margins of the crop. Glufosinate ammonium was sprayed on leaves for testing the herbicide resistance, and just one single individual showed no reaction. This plant belonged to a very large population of the self-incompatible *S. arvensis*, even though they highlighted that the most likely production of hybrid occurs where a single plant is subjected to a massive overload of "unrelated" pollen.

In our study, the main risk of an even low rate of hybridization is that a number of *B. napus* genes can be transferred to and thus change the *S. arvensis* genome (Moyes et al. 2002). The evolutionary consequence of interspecific hybridization essentially depends on the relative fitness of hybrids (Arnold & Hodges 1995).

If the hybrid is not favoured, immigration of disadvantageous alleles reduces local fitness and can lead a population up to extinction (Ellstrand & Elam 1993, Ellstrand 1999).

If hybrids show vigor, they could grow faster and have higher fitness than parents, achieving a higher competitive ability (Arnold & Hodges 1995); even the sterile ones can be superior competitors when vegetative proliferation occurs (Vilà et al. 2000). The higher fitness could result in invasiveness of hybrids, which would alter the composition and relative abundance of plant species (Ammann et al. 2000). Several cascade effects can thus occur; for example, frugivores may change food choice and as a consequence, seed dispersal within the plant community could be affected (Vilà et al. 2000). In our case of study, possible direct and indirect effects can influence the survivorship of invertebrate taxa, such as the Heteroptera, Auchenorrhynca and Coleoptera, which are related to *S. arvensis* (http://www.brc.ac.uk/dbif/homepage.aspx)and are important in the diet of birds (Marshall 2004).

With the available data we cannot affirm that those few hybrids, we found, would eventually survive and spread, but several characteristics of their parents suggest that a hypothetical hybrid population could be invasive.

This idea is also supported by the studies of Hu and collaborators (2002), who found a relative high fertility of the hybrid plants obtained by protoplast fusion; they

not only suggested that an additional trait can be accepted in the oilseed rape genome, but they also observed increase of fertility in successive generations.

Moreover, weed characteristics (Schlink 1994; Ammann et al. 2000), which enhances the probability of increased weediness of hybrids, are present both in oilseed rape and in wild mustard. Both of them show i) a wide period of the flowering time, as demonstrate in our study by phenological analysis; ii) an extensive seeds and pollen dispersal by wind and by unspecialized pollinators; iii) seed persistence and high level of tolerance to disturbed environments (Ammann et al. 2000).

The studied species are characterized both by wind and entomophilous pollination. In our analysis of pollen dispersion by wind, most of pollen granules drastically diminished within 5 m, but a quite high concentration is still available up to 30 m far away from the border of the crop area (data not shown).

This means that a hybrid population could establish even outside the crop, in the natural environment where it would be uncontrollable.

In particular, the entomophilous pollination enhances the possibility to easily overcome natural barriers, usually present in agroecosystems.

Additional investigations are trying to quantify the contribution made by wind and insects in the dispersal of oilseed rape pollen (Cresswell et al. 2004; Ramsay 2005; Hoyle et al. 2007). To date, Adegas and collaborators (1992) report that bees (*Apis mellifera* L.) are the most frequent visitors and also the most efficient pollinators on oilseed rape (Devos et al. 2009); moreover, when abundant, they deliver pollen to flowers more rapidly than any other type of pollination (Hayter & Cresswell 2006).

Another fundamental issue, related with the risk of spreading of hybrid population in agroecosystem, is seed dormancy and persistence (Lutman et al. 2003). In oilseed rape, volunteers arise from seeds lost in the field after crop harvest or by natural processes, such as fungal attack or invertebrate predation. Their seeds have essentially no primary dormancy, but if conditions inhibitory to germination (e.g. water stress, low temperatures, oxygen stress) together with darkness occur, secondary dormancy develops (Pekrun et al. 1997a; Lopez-Granados & Lutman 1998). This happens when ploughing, just after harvest, buries seeds which probably become secondarily dormant. Numerous studies show that seeds can survive for several years after the removal of the crop, up to 10 years (Pessel et al. 2001; Simard et al. 2002; Squire et al. 1999). Moreover, it is very important to underline that seed persistence is affected by environmental and agronomic practices but also by the genetics of the cultivars concerned (Pekrun et al. 1997b; Momoh et al. 2002). So, the risk and cascade effects on agroecosystem biodiversity, above mentioned, related to hybridization are

not blown over within only one season of cultivation. This aspect was not the aim of our study, but additional analysis would be useful and desirable to obtain a complete data set monitoring hybridization risk.

Further investigations to understanding the ecology of agroecosystems should have an integrated approach, combining disciplines across agriculture and ecology and across different spatial scales (Le Coeur et al. 2002). In order to provide conservationists with an operational tool, molecular data are not enough by themselves. We argue that GIS analysis is an important tool in natural resources management. In our case of study, the GIS analysis contextualized the result to the environment, by considering information about land use, vegetation, pollen dispersion, natural and artificial barriers. In this way, it was possible to define and easily visualize on maps the areas within which the risk of hybridization is present and where the maximum attention should be focused in case of introducing crops, even more if genetically modified.

Most of the total area occupied by *S. arvensis* is exposed to risk of breeding, but it is important to notice that the scarce presence of natural and artificial barriers increased the possibility of contact of the potential breeding species.

Ultimately, the correct management of field margins is desirable, since it can favour both adjacent cropping and wildlife (Marshall 2004).

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Conclusions

The PhD project contributed with its results to implement a Quick Monitor Index (QMI) as a tool to assess environmental impacts of transgenic crops, within the frame of the European funded LIFE+ Nature DEMETRA project (LIFE08 NAT/IT/000342). The development of the monitoring instrument for genetically modified plants (GMPs) needed the collection of several kind of data on the biological, physical and climatic parameters of the study areas. Finally, the QMI resulted suitable to predict the potential hazard of cropping GMPs on ecosystems, providing crucial information to define the operating methods for environmental monitoring; moreover, it might be used in the future as a forecasting model, especially because it can be conveniently adjusted to the different geographic situations.

The PhD research collected useful data and obtained relevant information to the development of QMI, with reference to the assessment of the influence that even non-GM crops might have on the surrounding environment. Indeed, gene flow was studied since it represents the most direct way for crops to influence the environment, by changing the genetic diversity of potential wild relatives.

In particular, the DEMETRA project expected also to highlight that the potential environmental hazards linked to GM trees differ from those associated with transgenic crop plants at both spatial and temporal scales, firstly because trees are long-lived perennials, unlike annual crop plants.

For this reason, the main aim of the PhD was to examine the potential wild-to-crop hybridization, considering two cases of study, in order to highlight the differences among woody and herbaceous crops.

In both cases, the crossing between crop and wild relatives was detected: gray poplars of the mixed forest stand can hybridize with “Triplo” individuals from the neighbour plantation and few seedlings of *S. arvensis* originated by interspecific crosses with oilseed rape. As already mentioned, these results are still controversial in the scientific community, but we argue that the specific environmental conditions favoured the crossings.

The other relevant outcome concerns the differences between herbaceous and woody crops, deduced mainly by esamining genetic data. The influence of crops on the environment resulted mainly dependent on the distance at which the pollen can disperse.

For woody crops, the maximum distance at which hybridization was detected, was taken in account. The results highlighted that in case of open environment, the range

of pollen dispersion of poplar reaches 2 km of distance, while within the forest the pollen can spread to a distance of 50 m.

On the other hand, for herbaceous crops, the influence on the surrounding environment is reduced to 1 km, the maximum distance reachable by an insect pollinator, considering that oilseed rape is also characterized by entomophilous pollination.

All the results contributed to the development of the QMI, as generated data were useful for establishing the level of hazard due to cropping GMPs by means of observations in the field and following the case-by-case approach.

Curriculum Studiorum

In 2008, the candidate graduated at the University of Parma in MSc "*Ecology*". In 2011, she attended a Post-Master Course in "*Sustainable development and management of environmental systems*" at the University of Bologna.

Her research areas of interest are: *Management and Protection of Environmental Systems; Population Genetics; Ecology; Climate Change; Sustainable Development; Environmental Impact Assessment.*

Publications (2012/2014)

Papers ISI

1. Paffetti D., Travaglini D., Buonamici A., **Labriola M.**, Bottalico F., Fiorentini S., Materassi A., Nocentini S., Vettori C. (2014). Potential threat due to crossing between poplar cultivations and wild relatives in Mediterranean environment. *Manuscript in preparation to submit to the journal "Conservation Biology"*
2. Buonamici A., **Labriola M.**, Paffetti D., Travaglini D., Bottalico F., Tomaselli V., Materassi A., Vettori C. (2014). Biodiversity in agroecosystems: crop-to-weed hybridization issues in Brassicaceae. *Manuscript in preparation to submit to the journal "Conservation Biology"*

Chapters in books

1. Paffetti D., Buonamici A., Travaglini D., **Labriola M.**, Bottalico F., Fiorentini S., Materassi A., Vettori C. (2013). Chaper 2: Pollen flow and breeding evaluation 2.2 - Breeding Evaluation. In: Vettori C., Travaglini D., Bartalucci L., Lazzarotto L., Paffetti D., Chelazzi L., Perfetti A.. DEvelopment of a quick Monitoring index as a tool to assess Environmental impacts of TRAnsgenic crops (DEMETRA), pp. 27-38 Tipografia Francesconi, Lucca, ISBN:9788890365386.
2. Paffetti D., **Labriola M.**, Buonamici A., Lisa C., Adoni F., Vettori C. (2014). Diversità Genetica. In: Gabbrielli A., Giannini R., Mancini M., Marchi E., Orlandini S., Paffetti D., Togni M., Travaglini D.. La valorizzazione della biomassa legnosa dei boschi del Chianti. *In Press.*
3. Paffetti D., **Labriola M.**, Buonamici A., Lisa C., Adoni F., Vettori C. (2014). Certificazione genetica del legname. In: Gabbrielli A., Giannini R., Mancini M., Marchi E., Orlandini S., Paffetti D., Togni M., Travaglini D.. La valorizzazione della biomassa legnosa dei boschi del Chianti. *In Press.*

Posters

International Congress

1. Paffetti D., Buonamici A., Travaglini D., **Labriola M.**, Bottalico F., Fiorentini S., Lisa C., Materassi A., Fasano G., Chelazzi L., Tomaselli V., Vettori C. (2014). Potential impact of poplar plantations cultivated in proximity of protected areas. 13th International Symposium on the Biosafety of Genetically Modified organisms (ISBGMO13). Cape Town (South Africa). November 9th-13th, 2014.
2. Salbitano F., Paffetti D., Giovannini G., **Labriola M.** (2014). Genetic diversity of *Quercus ilex* L. as a tool for retracing the dynamics of the Mediterranean forest ecosystems. XXIV IUFRO World Congress 2014. Salt Lake City (UT, United States). October 5th-11th, 2014. International Forestry Review ISSN 2053-7778. Vol. 16(5), 2014.
3. **Labriola M.**, Buonamici A., Paffetti D., Travaglini D., Bottalico F., Fiorentini S., Materassi A., Fasano G., Vettori C. (2013). The breeding among autochthonous and cultivated populations in Mediterranean environment: one step for the risk assessment. VIII Encuentro de Latinoamericano del Caribe de Biotecnologìa – REDBIO2013. Mar del Plata (Argentina). November 18th-22nd, 2013.
4. Buonamici A., Paffetti D., Biricolti S., Travaglini D., Balducci E., Tomaselli V., **Labriola M.**, Bottalico F., Materassi A., Fasano G., Vettori C. (2013). Pollen flow of *Brassica napus* cultivar and possible breeding with *Sinapis arvensis*. In: IUFRO Tree Biotechnology 2013 Conference. Asheville (NC, USA). May 26th-June 1st, 2013.
5. Paffetti D., Buonamici A., Travaglini D., **Labriola M.**, Bottalico F., Fiorentini S., Lisa C., Materassi A., Fasano G., Chelazzi L., Tomaselli V., Vettori C. (2013). Spatial genetic structure in poplar stand and evaluation of potential breeding between natural and cultivated populations in Mediterranean environment. In: IUFRO Tree Biotechnology 2013 Conference. Asheville (NC, USA). May 26th-June 1st, 2013.
6. Buonamici A., **Labriola M.**, Paffetti D., Tomaselli V., Travaglini D., Bottalico F., Balducci E., Materassi A., Fasano G., Vettori C. (2012). Evaluation of a Possible Breeding between *Brassica napus* cultivar and *Sinapis arvensis* in the Field. 6th International Symposium on Brassica and 18th Crucifer genetics workshop – ISHS. Catania (Italy). November 12th-16th, 2012.
7. Buonamici A., Paffetti D., Travaglini D., Bottalico F., Fiorentini S., Lisa C., **Labriola M.**, Materassi A., Fasano G., Chelazzi L., Tomaselli V., Vettori C. (2012). Characterization Of Genetic Diversity In Poplar Stands and Evaluation Of Potential Breeding Between Natural and Cultivated Populations In Mediterranean Environment. P2.29. 12th International Symposium on Biosafety of Genetically Modified Organisms 2012. St. Louis (Missouri, USA). September 16th-20th, 2012.

National Congress

8. Buonamici A., Paffetti D., Biricolti S., Travaglini D., Balducci E., Tomaselli V., **Labriola M.**, Bottalico F., Materassi A., Fasano G., Vettori C. (2013). Pollen flow of *Brassica napus* cultivar and possible breeding with *Sinapis arvensis*. In: Proceedings

of the 57th Italian Society of Agricultural Genetics Annual Congress, Poster Communication Abstract – 9.04. ISBN: 978-88-904570-3-6. Foggia (Italy). September 16th-19th, 2013.

9. Buonamici A., Paffetti D., Travaglini D., Biricolti S., Bottalico F., Chelazzi L., Cimò F., Colombini I., Fiorentini S., **Labriola M.**, Tomaselli V., Fasano G., Materassi A., Giannini R., Vettori C. (2012). Impatto sulla biodiversità di possibili coltivazioni di pioppo transgenico in aree protette. Poster section. IX Convegno Nazionale Biodiversità. Istituto Agronomico Mediterraneo di Bari. Valenzano (Bari, Italy). 5-7 Settembre 2012.

Oral presentation

International Congress

1. Buonamici A., Paffetti D., Biricolti S., Travaglini D., Balducci E., Tomaselli V., **Labriola M.**, Bottalico F., Materassi A., Fasano G., Vettori C. (2014). *Brassica napus* culture and potential hybridization with *Sinapis arvensis*. 13th International Symposium on the Biosafety of Genetically Modified organisms (ISBGMO13). Cape Town (South Africa). November 9th-13th, 2014.
2. Paffetti D., Travaglini D., Buonamici A., **Labriola M.**, Bottalico F., Fiorentini S., Materassi A., Vettori C. (2014). Potential hazard due to breeding between poplar cultivations and wild relatives in Mediterranean environment. Forest and Sustainable Development Symposium. Brasov (Romania). October 24th-25th, 2014.
3. Paffetti D., Travaglini D., Buonamici A., **Labriola M.**, Bottalico F., Fiorentini S., Materassi A., Nocentini S., Vettori C. (2014). Crossing between breeding poplar cultivations and wild relatives in Mediterranean environment. IUFRO Forest Tree Breeding Conference. Prague (Czech Republic). August 25th-29th, 2014. ISBN 978-80-213-2471-8.
4. Buonamici A., Paffetti D., Travaglini D., **Labriola M.**, Bottalico F., Fiorentini S., Lisa C., Materassi A., Fasano G., Chelazzi L., Tomaselli V., Vettori C. (2013). Spatial genetic structure in poplar stand and evaluation of potential breeding between natural and cultivated populations in Mediterranean environment. BIT's 2nd Annual World Congress of Biodiversity, Ecology and Environment. Nanjing (China). April 24th-27th, 2013.

National Congress

5. Paffetti D., Buonamici A., Travaglini D., **Labriola M.**, Bottalico F., Fiorentini S., Materassi A., Vettori C. (2013). Potential hazard due to breeding between poplar cultivations and wild relatives in Mediterranean environment. In: Proceedings of the 57th Italian Society of Agricultural Genetics Annual Congress, Oral Communication Abstract – 5.05. ISBN: 978-88-904570-3-6. Foggia (Italy). September 16th-19th, 2013.
6. Vettori C., **Labriola M.** (2012) "Il progetto Life+ DEMETRA, un caso di studio: Caratterizzazione della diversità genetica di popolamenti di pioppo e valutazione

del potenziale breeding tra popolamenti naturali e coltivati." Workshop finale LIFE+ MANGMP- ITA : "Uso degli OGM e salvaguardia delle aree protette: dai progetti LIFE+ un contributo alle conoscenze degli ambienti italiani". Roma (Italy). 12 Dicembre 2012.

Participation to

International Congress

1. COST ACTION FP0905 Biosafety of forest transgenic trees: improving the scientific basis for safe tree development and implementation of EU policy directives – FINAL CONFERENCE. Rome (Italy). March 4th-5th, 2014.
2. 12th International Symposium on Biosafety of Genetically Modified Organisms 2012. St. Louis (Missouri, USA). September 16th-20th, 2012.
3. 6th International Symposium on Brassica and 18th Crucifer genetics workshop - ISHS. Catania (Italy). November 12th-16th, 2012.

National Congress

4. Workshop finale LIFE08 NAT/IT/000342: "DEvelopment of a quick Monitoring index as a tool to assess the Environmental impact of TRAnsgenic crops". Firenze (Italy). 20 Giugno 2013.
5. IX Convegno Nazionale Biodiversità. Istituto Agronomico Mediterraneo di Bari. Valenzano (Bari, Italy). 5-7 Settembre 2012.
6. Workshop finale LIFE+ MANGMP- ITA : "Uso degli OGM e salvaguardia delle aree protette: dai progetti LIFE+ un contributo alle conoscenze degli ambienti italiani". Roma (Italy). 12 Dicembre 2012.

International Courses

7. AMIGA Summer School on Environmental Risk Assessment (ERA) of GM crops. (2-5 September 2014). Carlow, Ireland.
8. Training school "*Plant genome modification and the regulatory framework for GM plants in Europe*". Supported by EU COST Action FP0905 "Biosafety of Forest Transgenic Trees: improving the scientific basis for safe tree development and implementation of EU policy directives" (April 29th- May 1st, 2013). Tromsø, Norway.

Projects

9. Progetto ex 60%. Titolo: "Caratterizzazione genetica di *Quercus ilex* come strumento di studio del dinamismo della vegetazione mediterranea". Ente Finanziatore: Ateneo Fiorentino. Ruolo: Partecipante dell'unità operativa.
10. Progetto: ex 60%. Titolo: "Valutazione della diversità neutrale ed adattativa di popolamenti di pioppo in relazione allo sviluppo di un indice di monitoraggio per Piante Geneticamente Modificate (PMG)". Ente Finanziatore: Ateneo Fiorentino. Ruolo: Partecipante dell'unità operativa.

11. Progetto LIFE+Environmental. Titolo: “DEMETRA: Development of a quick in monitoring index as a tool to assess environmental impacts of transgenic crops”. Ente Finanziatore: Institute of Biosciences and Bioresources - CNR. Ruolo: Borsista.
12. Progetto FP7-INFRASTRUCTURES-2011-1. Titolo: “TREES4FUTURE Grant Agreement n.284181. Designing Trees for the future”. Ente Finanziatore: Institute of Biosciences and Bioresources - CNR. Ruolo: Assegnista.
13. Progetto: CISIA. Titolo: “Conoscenze integrate per la sostenibilità e l’innovazione del *Made in Italy* Agroalimentare”. Ente Finanziatore: Institute of Biosciences and Bioresources - CNR. Ruolo: Assegnista.

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