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LOCAL ADAPTATION AND GENE FLOW IN SERPENTINE AND LIMESTONE POPULATIONS OF *DIANTHUS SYLVESTRIS*.

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Abstract

Patchy distribution and stressful conditions of environment can induce the emergence of locally adapted phenotypes. Evolutionary theory supports that local adaptation is drown by strength of divergent selection to favor the genotype that better performs in a specific habitat. Nevertheless, adaptation could occur also via phenotypic plasticity that allows individuals to rapidly change their phenotypic response to environment and this ability may even slow down the effect of adaptive genetic divergence.

Plants from serpentine represent a typical model for studying local adaptation to soil type as selection in this environment is very intense and leads to the evolution of locally adapted populations, a phenomenon known as "serpentine syndrome". *Dianthus sylvestris* Wulfen (Caryophyllaceae) is frequently found both on serpentine and limestone bedrocks along Apennine chain.

Here we investigated populations of *D. sylvestris* in North-Center of Italy in order to clarify if phenotypic variation among populations of *D. sylvestris*, on both serpentine and limestone soils, could be defined as an example of local adaptation or is due to strong phenotypic plasticity. We used a molecular approach based on EST SSR marker to infer on genetic diversity and populations structure. Moreover, to verify if serpentine populations are locally adapteted we use an ecologiac approach based on transplanting field experiment and morphological and physiological measurements.

Population genetic analyses showed a high percentage of polymorphic loci (ranging from 71% to 100%) and the distribution of allele frequencies showed no significant differences among populations from the two soil types. Similarly, allele richness was comparable among populations of serpentine and limestone. Both ANOVA and the low values of differentiation among populations (mean F_{st}= 0.119, among populations) confirmed the

low overall genetic differentiation. Bayesian (STRUCTURE) and multivariate approach (PCoA) ruled out that populations from limestone and serpentine soils cluster in two genetically differentiated groups, even if according to Mantel test, subdivision was on geographic distribution more than on edaphic base. Thus, no evident genetic differentiation among *D. sylvestris* populations from serpentine and limestone was found with neutral markers.

To determine the contribution of selective factors and/or phenotypic plasticity to local adaptation of *D. sylvestris* to serpentine, from populations already examined in the genetic analysis, we estimated the metal content in plant aerial parts, collected data on morphological traits, and performed field reciprocal transplantations. High metal content (Ni, Cr) in plants aerial part confirmed, as in previous studies, the bioaccumulation of heavy metals in *D. sylvestris* plants from serpentine soils. In these plants, several morphological traits were found statistically decreased when compared to plants from limestone so highlighting that serpentine is a less permissive habitat than limestone. However, most of the morphological differences disappeared in transplanted individuals suggesting a large contribution of phenotypic plasticity in determining the observed morphological divergences. Nevertheless, in transplanted plants from serpentine soil to limestone, a two-way ANOVA resulted in a significant difference in biomass with an effect of the original soil on the transplanting soil. Significant differences were also found in flowering time, as plants from serpentine, when transplanted on limestone, flowered before than resident limestone plants. These differences, persisting independently from the original soil type, should have genetic bases. Thus genetic differentiation of populations of *D. sylvestris* is occurred at least in a few selected loci determining different affinity for the two habitats. This divergence is maintained among populations from different soil types even in the face of extensive gene flow as observed at neutral loci.

Introduction

Species abundance, distribution and diversification are influenced by environmental factors, and understanding "how" is central questions in both ecology and evolution (Schluter, 2009). Environment may be defined as the surrounding of a living organism, thus environmental factors are all the external forces, biotic or abiotic, that affect the life of an organism. Broadly, environmental factors are classified as: biotic factors, climatic factors (precipitations, temperature, humidity or wind), physiographic factors (latitude, longitude or altitude), and edaphic factors, including physical, chemical and biological characteristics of soil. Each of these factors doesn't act individually, but interacts with others creating different types of ecosystem that influence the existence and success of an organism. For this reason, natural landscapes are highly heterogeneous resulting in selection pressures that differ between ecosystems.

In a certain range of environmental condition organisms perform better and this is referred as the range of the optimum. When some important features of environmental conditions mutate, species changes in response. If these variations are within the limits of tolerance species remain constant in spite of changing external habitat: this tendency is known as homeostasis, the property of (a mendelian) population to equilibrate its genetic composition and to resist sudden changes (Lerner, 1954). Moreover, when the environmental factors change beyond a certain level they may affect the performance and fitness of organisms. Environmental changes could be beneficial, but most will be stressful (Fisher, 1958). Organisms can react to stressful changes trough three general and non-exclusive mechanisms (Larcher et al., 1973):

- avoid or reduce the stress by using dormancy or different behaviour, for example changing habitats or temporal activity patterns;

- evolve resistance increasing stress tolerance, reducing sensitivity or enhancing plasticity;
- activate recovery mechanisms as regeneration of damaged tissues or cellular stress responses.

While animals can use all three strategies, plants cannot run away from stresses and are more likely to emphasize dormancy, stress-resistance or stress-recovery mechanisms (Huey et al., 2002). For the same reason, plants should tolerate a broader range of environmental conditions showing greater phenotypic plasticity and experiencing stronger selection in nature due to their sessile growth, (Bradshaw, 1972 Thus, in plant species phenotypic variation may occur and could be the result of both phenotypic plasticity, i.e. ability of a genotype to modify the phenotype without genetic changes (Schlichting, 1986; Ghalambor et al., 2007),), and local adaptation, i.e. evolution of traits adapted to a specific habitat due to divergent selection pressures (Linhart & Grant, 1996; Silvertown & Charlesworth, 2001; Kawecki & Ebert, 2004) whereas low gene flow occurred among populations (Lenormand 2002; McKay & Latta 2002; Raesaenen & Hendry 2008).

Phenotypic plasticity

Phenotypic plasticity is an intrinsic response to environment changes and it is defined as the ability of a genotype to alter its phenotype in response to changes in habitat conditions (Bradshaw, 1965). Nowadays, this term is broadly used to describe all phenotypic responses such as acclimation or acclimatization, as well as learning (Kelly et al., 2012), encompassing all types of environmentally induced changes (morphological, physiological, behavioural or phonological) that may or may not be permanent throughout an individual's lifespan.

In 1965, Bradshaw suggested that plasticity may lead to two forms of modifications,

morphological and physiological, with different mechanisms, resource costs and ecological implications; he postulated that morphological plasticity is essentially meristematic in character and involves replacement of existing tissues by new plant parts with different characteristics, while physiological plasticity occurs in differentiated tissues and it is usually associated with a change in properties brought about by reversible subcellular rearrangements. Moreover, first kind of plasticity appears to present a highly cost solution than the second one, in which the response can be much rapid, occurring in existing cells. Further, Grime, reviewing Bradshaw's concept of plasticity, supposed that pattern due to plasticity cannot evolve independently of habitat and it is impossible to consider them regardless the selection mechanisms that operate in parallel on other (Grime, 1977; Grime et al., 1986). Grime suggested that the two forms of plasticity have consistent associations with distinct sets of traits, coinciding with particular habitats and ecologies. From this point of view, Grime hypothesized three "adaptive" response strategies for plant in changing environment:

- competitive strategy, occurred in environments characterized by low disturbance and low stress;
- ruderal strategy, occurred in environments with low stress and high disturbance;
- stress-tolerant strategy evident under regimes of low disturbance and high stress.

He arranged them along the classical r–K life history continuum with the ruderal strategy being the most r-selected, the stress-tolerant being the most K-selected, and the competitive strategy occupying the mid-point between these extremes.

Following this theory, assuming an equilibrium model among competition, stress and disturbance it could be possible to predict life history and growth characteristics of plants. These characteristics cover a wide range of modifications from the morphology of shoots, leaf forms, leaf and plant longevity, as well as reproductive phenology and reproductive

allocation. Indeed, for example, it is possible to predict that plants using competitive strategy have high expansion for both aerial part and roots complex, while ruderal selected plants could be small and with limited lateral spread. Ruderals were most likely to be annual herbs, while long-lived trees were most likely to be stress-tolerant (Grime, 1977). Thus, merging with Bradshaw concept of phenotypic plasticity, competitive plant of resource-rich productive habitats could show morphological plasticity via rapid root and shoot meristematic growth. Indeed, in this situation, activities of the plants themselves generate a very dynamic spatial mosaic of resources above and below ground and it's possible a continuous replacement of those leaves and roots that have become trapped in the depleted zones. In contrast, in stress tolerant plants is most relevant the physiological response, while ruderal plants could respond morphogenetically as well as developmentally to stress by diversion of available resources to reproduction. These scenarios can lead to important predictions regarding matches between coarse or fine-grained resource distributions and expected plastic responses.

A good example of phenotypic plasticity is the different growth of plants in shaded vs. sunny patches (Bradshaw, 1965), or morphological defence structures, such as spines, expressed by many aquatic organisms in the presence of predators (Tollrian, & Dodson, 1999).

The 'reaction norm' is the best way to describe the phenotype distribution of a genotype across heterogeneous environment conditions (Via et al., 1995): that is the line or curve obtained plotting in a two dimensional axis all phenotypic value for any specific trait of a genotype against the environmental value.

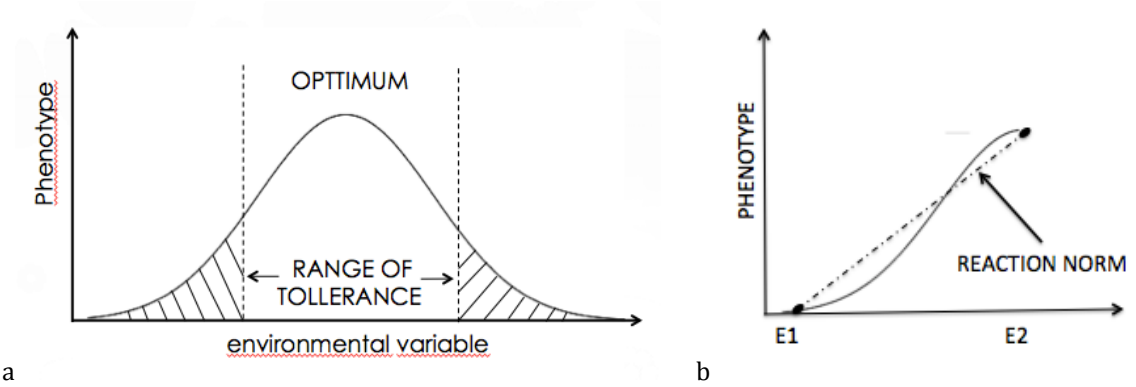


Figure 1: Examples of norm of reactions.

Figure a shows all possible phenotype values depending on changes in environmental variable; in this case norm of reaction is represented by the Gaussian curve.

Figure b shows phenotype values in two different environments: in this case norm of reaction is represented by straight line joining between two phenotypic states.

This line or curve shows how each state of environment changing result in phenotypic expression. Phenotypic plasticity can be visualized as a change in the slope of the reaction norm between ancestral and derived populations or species (Doughty, 1995; Gotthard & Nylin, 1995), while selection can act directly on the shape of the reaction norm (Harshman et al., 1991; Scheiner & Lyman, 1991; Scheiner, 2002). Thus, plasticity is what makes the appearance of an environmentally induced novel phenotype possible, and a process of selection on the expression of such phenotype in a new environment may end up genetically fixing it by altering the shape of the reaction norm (Pigliucci et al., 2006). Such change occurs in nature between species subjected to different selection pressures (Cook & Johnson, 1968; Ghalambor & Martin, 2002) and this has the potential to explain a variety of evolutionary ecological processes (Van Kleunen & Fischer, 2002; Pigliucci et al., 2006).

In the early 1960's, Bradshaw pointed out that phenotypic plasticity, like other traits, is under genetic control and therefore is subject to evolutionary mechanisms such as natural selection or drift (Bradshaw, 1965). However, still now, the genetic bases of phenotypic plasticity are not completely clear. However, three models were suggested to describe the genetic basis of this phenomenon: overdominance, pleiotropy and epistasis.

Overdominance model considers plasticity as a function of homozigosity, assessing for an inverse relationship between plasticity and genic heterozygosity. On the other hand, pleiotropy and epistasis models expect plasticity as a function of differential expression of gene. In the first case, the same gene or pattern have different expression in different environment, in the second one, the set of gene determine the magnitude of response to environment effects interact with gene that determine the average expression of the character.

Evidences supporting the overdominance model are really few and controversial: some studies have found no relationship between plasticity and heterozygosis (Scheiner & Lyman; 1991), and only few studies underline the inverse relationship (Gottlieb, 1977; Schlichtin, 1984). There are two main reasons for which authors hypothesized this relationship: a first hypothesis assess that phenotypic plasticity should increase as the amount of heterozygosity decreases, due to the increase in developmental instability caused by deleterious homozygous recessive genes (Bradshaw, 1965). However, the relationship between phenotypic plasticity and developmental instability is far from clear (Pederson, 1968). A second hypothesis states an inverse relationship because they represent alternative methods of dealing with environmental heterogeneity (Marshall & Jain, 1968): a population with well-developed plastic responses has no needed for genetic variation and vice versa (Schlichting, 1986). Nevertheless, one population could express together plasticity and heterozygosity allowing a population to respond to a variable environment by becoming both more plastic and genetically variable (Scheiner & Goodnight, 1984). In contrast, numerous studies support other two models. Heat shock, genes which express heat shock proteins produced only at high temperature, are example of pleiotropy model, while the change in expression of regulatory gene as the one for cold tolerance in Arabidopsis is an example for epistasis.

Genetic basis of plasticity implies a relationship with gene flow, the key force responsible for a marked change in allele frequencies and in the addition of new genetic variants to the established gene pool of species or populations. Phenotypic plasticity, in contrast with local adaptation, could increase if high gene flow occurs among populations in selective environments: gene flow between populations could increase the likelihood that plasticity and not specialization will evolve (Scheiner, 1998; Sultan & Spencer, 2002). With high migration, rate the specialists encountering more frequently environment where they are not "well adapted" (Scheiner, 1998; Sultan & Spencer, 2002). On the other hand, plasticity by itself can promote migration, as plastic individuals are more likely rather than specialists to survive in novel environments with different selection pressures (Price et al., 2003). However, gene flow may limit this relationship immigrating maladapted specialist whereas the more plastic populations live in the more heterogeneous localities (Alpert & Simms, 2002; Crispo, 2008). High gene flow might also result in selection for phenotypic, because gene flow increases the likelihood that a migrating individual will disperse to an environment if it is not adapted to (DeWitt and Scheiner 2004).

Despite the abundant examples (Bradshaw, 1965; Gotthard & Nylin, 1995), phenotypic plasticity is not always current in nature (Delasalle, & Blum, 1994; Pigliucci, 1997), and the degree of plasticity often varies between populations (Donohue et al., 2000; Van Buskirk & Arioli, 2005). These evidences indicate that phenotypic plasticity is the result of balance between modifications, to better perform in a changing habitat, and trade off. Though, in evolutionary literature, cost of plasticity and cost of phenotype are merged, referring to trade-off of phenotypic plasticity, it needs to distinguish between them (Callahan et al., 2008). Cost of plasticity are defined as the fitness decrement paid by a more plastic genotype relative a less plastic one (DeWitt et al., 1998), limiting the evolution of plasticity (Pigliucci, 2001 Cost of phenotype concerns to the fitness trade off in allocating resource to

one trait, instead another one and the efforts of receive information on the environment (Callahan et al., 2008). In addition is important to note that while phenotypic costs are genotype specific but environment dependent, plasticity costs are genotype specific but global, that mean existing in all environment (Murrem et al., 2015). DeWitt, in 1998, supposed that plasticity has not only costs, which could lead to reduce fitness when a trait is produced via plasticity rather than constitutively, but also limits, relate to the inability to produce the optimal trait value. From this point of view, he considers costs of plasticity:

- maintenance costs of the sensory and regulatory mechanisms that produce plasticity,
- costs of inducible phenotypes against costs paid by fixed genotypes to produce the same phenotype,
- information acquisition costs obtained during environmental sampling, and genetic costs such as linkage of plasticity genes with genes conferring low fitness (De Witt 1998).

On the other hand, limits include:

- information reliability
- limits associated with imperfect correlations between the cue that triggers plasticity and the true state of the environment,
- lag time limits where there is a delay in sensing and responding to environmental information,
- developmental range limits if plastic development is incapable of producing extreme phenotypes that are possible through fixed development,
- epiphenotype problem, where add-on phenotypes may be less effective than developing the phenotype during early ontogeny (De Witt, 1998).

When plasticity is costly or limited, it is expected that genetic adaptation is favoured

instead of plastic response. Moreover, if population are located in homogeneous environment and the migration rate is low, plasticity could be lost due to neutral process as drift (Crispo 2006). It clears that with phenotypic plasticity, the environment plays a dual role in evolution: it creates both phenotypic variation and selects among that variation. Plasticity allows colonizing novel environments increasing the potential for future adaptive genetic divergence (Price et al., 2003; Crispo, 2007; Ghalambor et al., 2007).

Plasticity, as response to environmental variations, must be adaptive regarding the environment disturbances, but fitness could be not likely to be enhanced. This happens because even if some traits may be plastic, others may be under natural selection, thus constraints and trade-off may result in a maladaptive response.

Since evolution is generally defined as a change in gene frequencies, the variants associated with environmental conditions and plasticity are frequently classified as "nongenetic" in nature, and therefore unimportant for evolution (West-Eberhard, 1989), but starting with Bradshaw, some authors proposed that plasticity might have a heritable genetic component. This issue, however, has been for long the centre of numerous controversies (Via et al., 1995), and the principal debate was whether selection can act directly on plasticity or plasticity is indirectly selected through selection in other trait (Scheiner & Lyman, 1989; Schlichting & Pigliucci, 1993; Via, 1993). However assuming this link, if plastic response enhances local adaptation, plasticity would increase. Thus, plasticity can be an important factor in the evolution of diversification, and the effects may be either positive or negative, relating on the nuances of the specific system (Crispo, 2006).

Local adaptation

Local adaptation is another possible result of interaction between a genotype and its environment. A key prerequisite for the emergence of local adaptation is the existence of a

spatially heterogeneous environment. This generates a heterogeneous selective pressure that, in contrast with phenotypic plasticity, does not aim towards a global optimum but is determined by the balance between gene flow and local selection acting on a genotype, leading in change of allele frequencies (Levene, 1953; Nagylaki, 1980; Gavrilets & Gibson 2002; Whitlock & Gomulkiewicz, 2005; Yeaman & Otto, 2011; Blanquart et al., 2012).). The strict criterion to assess the presence of locally adapted populations is that population genotypes must have higher fitness in its native site than any other populations introduced to that site (Kawecki & Ebert, 2004). However, as described above, local adaptation can occur rapidly by the plastic response allowing an entire population, or group individuals, to adapt simultaneously.

While the role of selection in local adaptation could be easy to understand, gene flow plays a controversial role. Restricted gene flow is considered a key condition to have local adaptation because such situation makes the more favourable condition for the maintenance of polymorphism. Local adaptation can, in fact, occur if the direction of selection changes for an allele among habitats (antagonistic pleiotropy) leads no advantage for one genotype in all habitats, and resulting in trade-off of adaptation in different environments (Lenormand, 2002). However, when an allele with antagonistic environmental effects is maintained at a migration–selection equilibrium, gene flow changes allele frequencies in a direction opposite to natural selection, and each population is sub optimally adapted (Lenormand, 2002); thus, if migration rate is large compared with selection the polymorphism, that show antagonistic pleiotropy, is lost.

On the other hand, gene flow can increase local adaptation, by increasing variation in genetic pool, when selection pressures change rapidly. A population that has low levels of genetic variation for ecologically relevant traits would have a reduced ability to adapt to adverse environmental conditions because genetic variation is a prerequisite for adaptive

evolution by natural selection (Slatkin, 1987; Hoffmann & Blows, 1994; Gomulkiewicz et al., 1999; Barton, 2001; Lenormand, 2002; Blows & Hoffmann, 2005; Kellermann et al., 2009). Indeed, as immigration can also increase standing genetic variation within a population, these migrants can enhance the selection response in peripheral populations thereby creating a situation where resident species are under pressure to adapt to the changing environment (Colautti et al., 2010). In the case where gene flow can have a facilitating rescue effect on adaptation, it is possible that its negative effects (accumulation of deleterious mutations under stressful conditions) are masked by the genetic variation and beneficial mutations provided by the same dispersers. Thus, it helps to maintain adaptive potential (Lande, 1995; Holt & Gomulkiewicz, 1997; Gomulkiewicz et al., 1999; Holt, 2003; Garant et al., 2006; Holt et al., 2011).

The complex role of gene flow is illustrated by a wide array of empirical findings. Evidence for its homogenising effect is provided by the inverse relationship often documented between levels of gene flow and phenotypic divergence (Hendry & Taylor, 2004), and by studies that have experimentally reduced gene flow and documented subsequent divergence (Nosil, 2009). The positive effects of gene flow are generally few appreciated, although several studies document adaptive divergence despite naturally high gene flow (Hoekstra et al., 2004) or an increase in hybrid fitness when divergent parents are crossed (Bijlsma et al., 2010). Thus, it's clear that local adaptation may be maximal for intermediate levels of gene flow (Gandon, 2002; Blanquart & Gandon, 2011). Otherwise, the existence of a pattern of local adaptation despite gene flow certifies the strength of natural selection imposed by particular environmental factors.

According to theory, the ability of a population to evolve to in local conditions in the face of gene flow depends on the genetic basis of the traits involved (Haldane, 1930; Bulmer, 1972; Yeaman & Otto, 2011). Using a theoretical approach, Yeaman shows that local

adaptation occurs much more readily with alleles of large effect, that show greater differentiation of allele frequencies under divergent selection (Hedrick et al., 1976). Furthermore, it is less likely to lost alleles with strong effects by drift (Crow & Kimura, 1970). Therefore, loci with large effects on fitness should disproportionally contribute to local adaptation (Macnair, 1991). This is the case in the classic examples of local adaptation of plants to sites contaminated with heavy metals (Macnair 1987, 1991). However, such adaptation is possible also from a polygenic response (LeCorre & Kramer, 2012), that is, the response due to alleles of small effect, and the genetic divergence among populations caused by subtle shifts in frequency of a large number of loci. Many relevant theories for adaptation point out on evolution of ecological specialization, assuming the trade off in fitness across habitats is mediated by a quantitative trait or traits. The evolution of local adaptation for quantitative traits, typically controlled by multiple loci, is not yet well understood. Polygenic models with many loci often make strong assumptions, such as the assumption that the alleles at all loci have equal effects on the phenotype and that these effects are additive within and across loci.

Local adaptation can be influenced also by genetic drift: small populations may not be well adapted to their native environment because drift can reduce additive genetic variance and make it difficult for advantageous alleles to reach high frequency (Whitlock 2003) and lead to the random fixation of a reduced number of genotypes (Yeaman & Otto 2011; Blanquart et al. 2012). In addition, genetic load due to the chance fixation of deleterious alleles leads to low fitness or extinction (Lynch and Gabriel 1987; Lande 1994; Whitlock et al. 2000).

To explore the adaptive significance of phenotypic variation and test whether populations show different fitness across different habitats associated with traits of interest, are typically used reciprocal transplant experiments in the field or common garden experiment, thus plant groups are transplanted into their home site and away sites.

Reciprocal transplanting experiment.

Reciprocal transplant experiments in the field are the main strategy used to detect evidence of local adaptation and test fitness traits of two or more plant groups transplanted into their home site and away sites. This experimental approach has been conducted on closely related plant populations from serpentine and non-serpentine soils.

Fitness can be estimated with floral, vegetative, and survival measurements. Ideally, seed number or weight is best measures of fitness, but in long-lived species fitness is often estimated from growth measurements (e.g., plant height) because larger plants probably produce more seeds (Wright and Stanton, 2011). Flowering time can be also considered an important measure because differences in the maturation of reproductive structures can lead to changes in pollination, and reproductive success (Levin, 2006). However, Wright and Stanton (2007), found no significant difference in various estimates of fitness such as emergence date, cotyledon size, date of first flower, petal width, calyx length, corolla length, or petal colour intensity between plants grown in serpentine and non-serpentine soils, concluding that measured traits in their study were not driving local adaptation.

Average above ground biomass of non-serpentine plants growing in serpentine soil increased as planting density increased, but no significant biomass increase was demonstrated in serpentine plants growing in non-serpentine soil. Dense planting when competition occurs may also negatively affect plants. Sambatti and Rice (2006) found that when competition of Helianthus exilis A. Gray (Asteraceae) was prevented local adaptation occurred. Mortality was generally higher with competition.

Demonstrating local adaptation require a significant interaction between the effects of population origin and transplant habitat as well as evidence for local genotype to have higher fitness in their habitat than foreign genotype; this implies satisfy local vs. away criterion as descript in Kawecki and Ebert (20004). In addition, it can be test home vs.

away criterion (Kawecki & Ebert 2004), that assesses if local genotype has on average a higher relative fitness in its own habitat rather than in another habitat. Fitness can be estimated with floral, vegetative, and survival measurements. Kawecki and Ebert (2204) argued that an overall local vs foreign pattern would be better support for local adaptation even with unsatisfied home vs away criteria. This because intrinsic effect of habitat quality may bias the interpretation of divergent selection: an adapted population in local site might have higher fitness in non-local one, if the non-local habitat is richer than the local one.

Relationship between local adaptation and phenotypic plasticity.

Environmental heterogeneity favours the evolution of adaptive phenotypic plasticity. In the absence of costs of and constraints on plasticity, a genotype that in each habitat produces the locally optimal phenotype will become fixed in all demes. Adaptive phenotypic plasticity would lead to adaptive phenotypic differentiation, but without underlying genetic differentiation (Kawecki & Ebert, 2004). Moderate levels of phenotypic plasticity are the ideal condition in allowing populations survival in a new environment and in bringing populations toward an adaptive peak, and high levels of plasticity may increase the probability of population persistence reducing the likelihood of genetic variation, because the plastic response itself places the population close to a peak (Price et al., 2003). If phenotypic plasticity may drive population to have high fitness in new environment, it's not immediately clear why directional selection should act on population and thus why genetic divergence occurs. Following De Witt and some other authors, genetic divergence is expected when there is a cost to plasticity (DeWitt et al., 1998; Ancel, 1999, 2000; Sultan & Spencer, 2002); this implies that if an environment become constant, it means no chancing disturbance or stress, there is no selection to maintain plasticity and this would be lost.

This led one population to differentiate from each other with a loss of plasticity and the evolution of specialization (Price et al., 2003). The costs of plasticity may contribute to genetic differentiation but are unlikely to be the only, or even major, cause. Another possibility for the way by which plastic traits may become genetically based lies in the process known as genetic assimilation (Waddington, 1961). Genetic assimilation implies the conversion to a fixed genetic trait of an initially totally environmentally induced phenotypic threshold response (Waddington, 1942, 1953); the environmentally induced response doesn't need to be adaptive. After genetic assimilation, the phenotype is no longer plastic: phenotypic plasticity, in this case becomes only an intermediate stage to a new genetically fixed and phenotypically invariant state (De Jong, 2005). On the other hand, West-Eberhard (2003) proposes that adaptive evolution involves four stages and there is not assimilation but genetic accommodation, gene frequency changes due to selection on variation in the regulation form or side effects of the novel trait in the subpopulation of individuals that express the trait. West-Eberhard (2003) proposes that adaptive innovation begins with reorganization of an already highly adapted genotype, in which negative effects are improved by adaptive developmental plasticity. Gene frequency change follows, as a response to the developmental change. In this framework, most adaptive evolution is accommodation of developmental-phenotypic change. Genes are followers, not necessarily leaders, in phenotypic evolution (West-Eberhard 2003).

The idea that plastic traits in general could become genetically fixed was raised by Baldwin (1896), Morgan (1896) and others (Simpson, 1953; Wcislo, 1989). Bradshaw (1965) recognized that phenotypic plasticity could itself be under genetic control and would therefore be subject to selective pressures. He and others (Thoday, 1953; Levins, 1963; Marshall & Jain, 1968; Jain, 1979) have postulated that selection for phenotypic flexibility and genetic variation would be antagonistic, that there would be selection for a population

to be either phenotypically flexible or genetically variable. Several studies comparing congeneric species (Cumming, 1959; Marshall and Jain, 1968; Jain, 1979), have found evidence that one of the species is more genetically variable and the other more phenotypically plastic. One study (Grant, 1974) has found differences in genetic variation and phenotypic plasticity among adjacent populations of a single species.

Molecular approach: EST SSR, link between genetic and phenotypic variation.

The genomes of all eukaryotes contain iterations of 1- to 6- bp nucleotide motifs. This class of DNA sequences is known as microsatellites (Litt and Luty 1989) or simple sequences repeats and are abundant and randomly distributed across the genome (SSRs; Tautz et al. 1986, Li et al. 2002). These markers can be frequently used as highly variable and multi-allelic PCR-based genetic markers (Brown et al. 1996) and are usually considered as evolutionarily neutral DNA sequences.

Recently, SSRs have been identified in genes and expressed sequence tags (Li et al. 2004). ESTs are single-pass sequence segments of expressed genes (Adams et al. 1991). They derive from cDNA libraries made from multiple tissues under various treatments and used to identify as many genes as possible in an organism.

Expressed Tag Sequence (EST) libraries has provided a way to mine for microsatellites and SNPs directly linked to genes. Existing and expanding EST resources thus present an opportunity to develop, relatively quickly and inexpensively, gene-associated microsatellite markers. These EST-Simple Sequence Repeats (EST-SSRs) are generally more conserved than traditional microsatellite markers and are often transferable among species within genera and even sometimes between genera (Bodénès et al. 2012; 14Ellis and Burke 2007). EST-SSRs are not only used to examine within and between population genetic diversity and structure, but can also be used to link phenotypic traits with potentially underlying

genes. Furthermore, if these markers are genetically mapped, genomic regions of interest such as those under selection or involved in reproductive isolation can be identified and compared between species (Bodénès et al. 2012). The use of EST-SSRs is of great interest for genetic studies because they link genetic variation to potential adaptive traits. High gene flow might prevent local adaptation unless genomic areas involved in that adaptation are under strong selection (Via, 2012). Markers that show higher differentiation than expected under neutrality (outlier loci) between species or populations with different environmental niches could point towards a gene involved in local adaptation. Using EST-SSRs to identify outlier loci, which potentially represent or are linked to candidate genes, provides a targeted search method for markers that have putative functions related to environmental adaptations in species that do not have a sequenced genome. For example, a study looking at populations of sunflowers (Helianthus annuus) with differences in adaptations to drought and salt conditions using EST-SSRs (some with putative functions in drought and salt tolerance) found that a substantial proportion of the outliers detected are linked to genes with putative abiotic stress response functions (Kane and Rieseberg 2007). Currently, studies using EST-SSRs to detect outliers are limited, but are growing in plant species (Kane and Rieseberg 2007; Lind-Riehl et al. 2014; Scotti-Saintagne et al. 2004; Sullivan et al. 2013). These outlier loci are candidates for further investigation through sequencing and genetic mapping to examine the molecular basis for differentiation and confirm potential involvement in local adaptation. Studies to associate observed nucleotide diversity with phenotypic variation in larger populations along environmental gradients are still trying to confirm the involvement of these candidate genes in local adaptation.

The edaphic factor and serpentine's challenge.

Climate broadly defines major biomes (tropical rainforests, temperate deciduous forests,

deserts, tundra), but edaphic factor is what enriches diversity within these zones (Rajakaruna 2008). According with many ecologists, within a climate region, soil is the ecological determinant of plant distribution (Cain, 1944; Mason, 1946; Kruckeberg, 2002). Thus, if climate limits the flora, geological characteristics largely define habitat diversity.

Edaphic factors pertain to the substratum upon which the plant grows and from which it derives its mineral nutrients and much of its water supply. The soil formation is a complex process resulting from solid rock, or from mineral material deposited by a glacier, wind or water. This process initiated with weathering that may be mechanical, chemical or biological. Mechanical weathering results from physical disintegration or degradation of rock into smaller fragments without changing the chemical composition of the rock and includes breakup of rock caused by the freezing and thawing of water, abrasion, and roots penetrations. Chemical weathering results from broken down by chemical action resulting in a change in the composition of a rock. The main agents of chemical weathering are oxygen, rainwater, carbon and dioxide. Chemical substances produced by plants, which break down to weather rocks, cause biological weathering. After weathering processes, soil development and formations is mainly influenced by five factors, climate, living organisms, parent material, topography and time, which follows weathering, (Jenny, 1940). Thus kind of soils can be considered as result of a particular combination of its forming factors: for a given combination of factors there is only one soil type, and if all but one factors remain unchanged, variation in soil body can be attributed to that factor (Rajakaruna & Boyd, 2008). The vertical layered structure of soil is the soil profile and apparent layers of soil are called horizons. Each horizon has characteristic set of features related to colour, thickness, structure, consistency, porosity, chemistry and composition that affects plant uptake of nutrients and water.

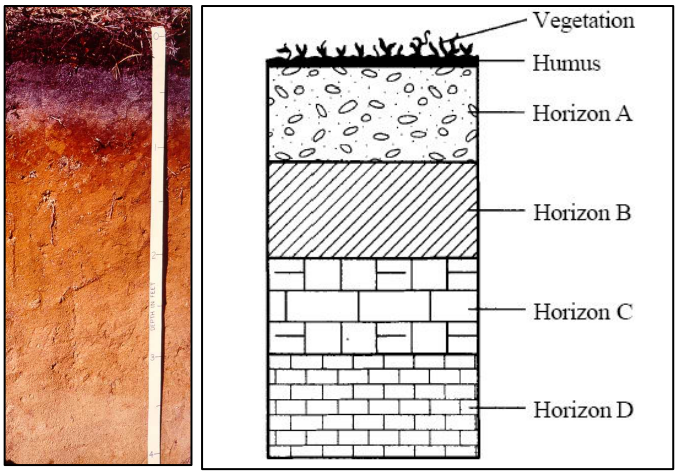


Figure 2: Soil profile example.

All these features create heterogeneity and opportunities for differentiation of species. Soils with extreme features may be strong selective force shaping plant evolution. Vegetation in unusual soils **may be composed** by species found only in that soil, or that evolve populations locally adapted or at least populations that experience strong phenotypic variation. There is an exhaustive literature about unique soils, which impose a challenge to plant growth. Gypsum soils, a substrate formed by the evaporation of saline water, were examined for their distinctive flora (Turner and Powell 1979). This soil presents high sulphate, high concentration of Ca, in contrast with low content of Mg. Sulphate may induce nutrient deficiencies due to ion competition at the root surface (Marschner 2012), while Ca/Mg ratio may limit availability of some macro- and micronutrients, due to precipitation and complexation with calcium ions and limits uptake of K+ and Mg2+ due to similarity in size and charge (Marschner 2012). Additionally, there is an inverse relationship between increasing of gypsum concentration in soils and decreasing of cation exchange capacity, further limiting nutrient availability (Escudero et al. 2014, Castillejo et al. 2011).

Acid soils have high contents of H+ ions and low contents of essential plant nutrients, primarily P and Ca. Those soils are also often characterized by high contents of toxic forms

of Al, Fe and Mn, and by deficits caused by leaching or decreased availability of P, Ca, Mg and some other micronutrients, especially Mo, Zn and B (Narro et al., 2001; Sumner, 2004; Welcker et al., 2005; Kovaåeviã et al., 2006; Jovanoviã et al., 2006; Đaloviã et al., 2007). Acidity restrains root growth and, consequently, the uptake of water and mineral nutrients. Plant community associated to limestone is another example of vegetation in stressful habitat; this substrate resulting from precipitation and litification of calcium carbonate (Lloyd and Mitchell 1973; Lousley 1950; Shimizu 1962, 1963) and many of the earliest observation in plant-soil interaction were made on limestone landscape.

a  b 

Figure 3: Stressful landscape. a) salt soil; b) copper mine.

Because of their characteristic composition, these habits **are toxic** for most plant species, and the patchy distribution results in an ecological discontinuity with potentially strong selection over short distance.

One of the most remarkable stressful habitat in which there is a unique plant communities is a serpentine soil derived from ultramafic and related rocks, where the biomass production depends on one or few limiting factors (Grime, 1979; Brooks, 1987; Baker et al., 1992). While the term “ultramafic” is technically more correct in a broad sense, the term “serpentine” is widely used to refer to all soils of ultramafic origin and their associated plant communities, regardless of specific rock type origin of the soil (Alexander et al., 2007). Serpentine more accurately refers to a group of hydrous magnesium phyllosilicate

minerals, including antigorite, chrysotile and lizardite (Brooks 1987; Kruckeberg 1984). These minerals are derive from the hydration of the ferromagnesian minerals of ultramafic rocks, at low temperatures and pressures, in conditions favourable for each mineral's formation. These low temperatures are usually less than 500°C, and fluid pH in excess of 10, and low CO2. Ultramafic rocks belong to lithological sequence of ophiolites composed, from bottom to top, by peridotite (modified mantle), gabbro, and basalt, originally formed in the oceanic crust at different depths and brought to the surface by tectonic movements. Most ophiolite is subducted, sinking back into the mantle, but some is incorporated into continental crust (Coleman and Jove 1992; Wyllie 1979b). The metamorphism associated with tectonic movements alters the original lithology: ultramafic peridotites and pyroxenites become serpentinites. Serpentine is worldwide patchy distributed concentrated along continental margins and in regions of orogenesis. Sometimes, the limit between serpentine and non-serpentine habitats is strikingly sharp, evidencing a sharp ecological boundary (Brady et al. 2005, Brooks 1987). Serpentine rocks are often rich in (of) chromium, cobalt and nickel, and have relatively low concentrations of silicon, phosphorus, potassium and calcium (Brooks, 1987; Proctor, 1999; Roberts and Proctor, 1992).

Serpentine ecosystems are diffused worldwide, including North and Tropical America, Northwest Europe, Central and Southern Europe, Continental Asia, Japan, Africa, the Malay Archipelago, New Caledonia, Australia and New Zealand (Brooks, 1987). Although ultramafics are widespread, they still only occupy less than 1% of the earth's land surface (Baker, Proctor and Reeves, 1991). These soils are usually regarded as infertile, and are prone to drought, even in areas of high rainfall. (Batianoff and Specht, 1992, Roberts and Proctor, 1992).

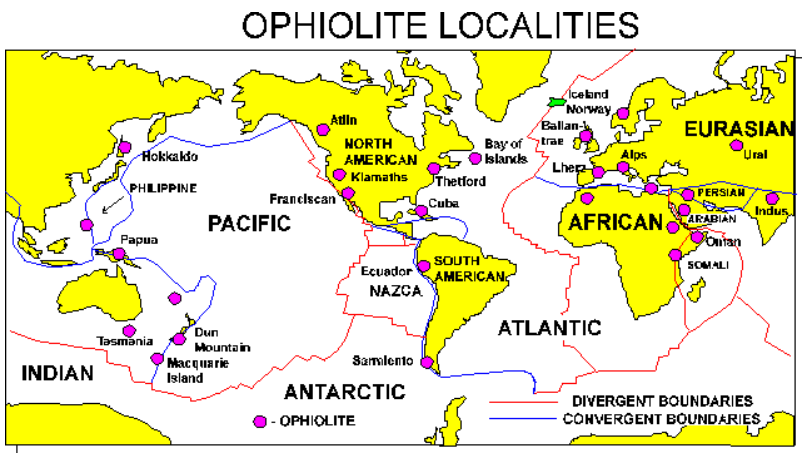


Figure 4: World map of ophiolitic site.

These soils usually contain very high Mg (18–24%), Fe (6–9%) and heavy metals, particularly Ni, Co, Cr and Mn, but very low Ca (1–4%) and Al (1–2%), high pH, low nutrient and water –holding capacity (Nagy and Proctor 1997, Chiarucci et al. 2003).

The high level of magnesium is important to maintain a high pH and to be generally a part of chemical interactions with nickel and calcium (Roberts and Proctor, 1992). Thus, plants growing on these soils are undergone to low Ca2+/Mg2+ ratio causing inhibition of Ca2+ uptake, toxic effects of large concentrations of the heavy metals, particularly Ni (Chardot et al., 2007), low available Fe, because of high pH values and competition with Ni and Co (Kataeva et al., 2004).). Interactions between the magnesium, nickel and calcium are known to be significant to plant growth explaining the unusual flora and fauna of serpentine areas (Proctor, 1999). Low phosphorus and potassium concentrations in soils are a direct result of their very low concentrations in ultramafic rocks; the primary macronutrient deficit appears to vary globally (Kazakou et al., 2008) California serpentine is typically deficient in N, whereas K is the primary deficiency in Europe (Kay et al., 2011)

In addition, lowered plant, fungal and bacterial activity inhibits the biological processes that result in the insufficient nitrogen for dense vegetative growth (Cooke, 1994).

Rocks, usually on open steep slopes, exposed to high light and heat conditions characterize these sites, and implying drought due to soil shallowness and dark colour with consequent

high temperature, sandy texture and erodibility (Oberhuber et al. 1997, Brooks 1987, and Verger 1987).

Biotic factors may also contribute to serpentine adaptation. For example, plants on serpentine experience reduced competition from invasive species (Kruckeberg, 1984; Harrison, 1999; Gram et al., 2004; Going et al., 2009). Nickel hyper-accumulation may confer a defence against herbivores (Martens and Boyd, 1994), and symbioses with serpentine-tolerant ectomycorrhizal communities may facilitate adaptation to edaphic stressors on serpentine (Schechter and Bruns, 2008; Urban et al., 2008; Gonçalves et al., 2009; Moser et al., 2009). Still now, it is quite impossible to define the main limiting factor in plant growth, also because as suggested from, relative strength of each factor differs from site to site (Proctor & Nagy, 1991).

Considering all those criticism of serpentine ecosystem, it's simply understood that edaphic factor is a multifaceted problem, involving chemical, physical, and biotic components. Arguably, the most influential factor on plant life is the chemical one (Kruckeberg, 1985): broadly, the most discussed is the presence of heavy metal, especially Ni because of toxicity on non-adapted species (Lee, 1992; Chardot et al., 2007; Robinson et al., 1996), but, in contrast, many authors suggest that its effect is negligible, particularly if nutrients are sufficiently available (Chiarucci et al., 2001).

Some others claim that excessive Mg content represent an important physiological challenge, especially in cold and wet environments because it cause a strong base leaching (Proctor & Woodell, 1971): huge amount of Mg inhibits the uptake of nutrients for the antagonistic behaviour with Ca (Marschner, 2002; Brady et al., 2005).

As a result of this challenge, the vegetation types have characteristic features. Firstly, is characteristically open and of low stature, and secondly there is a high proportion of endemic or disjointedly distributed species (Brooks, 1987). In California, 176 of the 1,410

plant species (12.5%) endemic to the state are classified as serpentine endemics. Given that, less than one percent of the land area in California is of ultramafic parentage, this level of endemism is noteworthy (Macnair & Gardner, 1998).

Although some variation occurs between sites, Whittaker (1954) identified three collective traits distinctive for serpentine soil: poor plant productivity, high rates of endemism, and vegetation types distinct from those of neighbouring areas. Based on these features, plant species adapted to "serpentine soils" tend to have traits considered adaptive to stressful soils edaphic conditions that lead to distinct morphologies from the ones closely related but colonizing "different" substrata or from members of the same species from non-serpentine sites (Kruckeberg, 1967; Brady, 2005). Referred to as serpentinomorphoses, such adaptations include xeromorphic foliage, including increased glaucousness due to waxes that cover the leaves and the stems of the seedlings and reduce transpiration, pubescence, succulence, and/or anthocyanic pigmentation due both to the lack of nutrients such as nitrogen and phosphorus and to the strong concentration of nickel in the soil that stimulates production of reddish pigments and induces a greater lignification of tissues, reduced stature, including dwarfism and prostrate habitat, and increased root:shoot biomass ratios, presumably to facilitate uptake of water and nutrients (Brooks, 1987), also in wet environments (Kruckeberg, 1984, 2002; Cooke, 1994; Brady, 2005). Physiological adaptations include the hyperaccumulation of heavy metals, selective ion uptake, preferential accumulation of essential elements such as calcium, lower biomass production, and slower photosynthetic and growth rates (Boyer, 1982; Kruckeberg, 1985; Alexander et al., 1989; Cooke, 1994; O'Dell et al., 2006). Other species down-regulate lateral root growth in high Mg soils, allocating more resources to deep-growing roots important in dry conditions. All these features are usually described as "serpentine syndrome" (Jenny, 1980).

Adapted population are undergone to strong trade off according to the specific adaptations and peculiarities of the physiology of different plant lineages, as well as with the biotic and abiotic conditions in the surrounding environment. The cost, and thus the magnitude of trade-offs, should vary (Elmendorf & Moore, 2007). For example, a small, drought-adapted stature and deep roots are advantageous traits but may reduce the growth rate and competitive ability on serpentine. Indeed, Kruckeberg (1954) showed that non-serpentine plants on "normal" soil competitively exclude serpentine endemics. More recent ecological studies have also shown that serpentine plants are poor competitors on higher nutrient soils (Rice, 1989; Huenneke et al., 1990; Jurjavcic et al., 2002). There is some suggestion that plants adapted to serpentine have intrinsically lowered growth rates even when grown on more fertile soil (Sambatti & Rice, 2007), which is expected from plants adapted to stressful environments (Grime, 1977). In contrast, no evidence has been found for a cost to metal tolerance or tolerance of low Ca:Mg ratios in serpentine plants (Brady et al., 2005), even if some authors suggests that tolerance mechanisms such as efficient metal sequestration lead to metal deficiency when metal-tolerant plants grow on nonmetalliferous soil (Baker & Walker, 1990; Harper et al., 1997, 1998). However, several studies using genetic lines of *Mimulus guttatus* selected for contrasting metal tolerance failed to demonstrate any correlation between high tolerance and reduced fitness (Macnair & Watkins, 1983; Harper et al., 1997, 1998).

Serpentine plants show a gradient of tolerance and restriction to serpentine, from widely tolerant to narrowly endemic. Following Kruckeberg classification (1951, 1954), plants found on serpentine could be classified in endemic species, indicator, or bodenvag. Endemics were restricted wholly to serpentine soil, indicators were typically found on serpentine but also occurred occasionally off serpentine, and bodenvag species appeared indifferent to the soil but often showed differences in tolerance to serpentine at a

population level (Kruckeberg, 1985; Safford et al., 2005).

Species able to grow both on serpentine and other soils can be separate in ecotype, thus those growing on serpentine show distinct characters not seen in the those occurring on non serpentine (Kruckeberg, 1967, 1984, 1992, 1995). The term "ecotype," was originally proposed in 1922 to define "the product arising as a result of the genotypic response of an ecospecies or species to a particular habitat" (Turesson, 1922). Nowadays, an ecotype is referred to genotypes (or population) within a species resulting from adaptation to local environmental conditions that confer a selective advantage. The distinction between phenotypic plasticity and local adaptation of an ecotype is based primarily upon genetic analysis and transplantation experiments (Nahum et al., 2008). In reciprocal transplants of *Pinus sabiana* in California, it was found that seedlings from non-serpentine sources grew equally well on serpentine (Griffin, 1965), underlining no patterns of ecotypic variation under genetic differentiation. Conversely, all non- serpentine forms in the *Streptanthus glandulosus* complex were shown in reciprocal transplant experiments to be serpentine-intolerant, evidence of genetically-based ecotypic differentiation likely resulting from the isolation of forms on and off serpentine substrates (Kruckeberg, 1951).

The unique characteristics of serpentine make it an excellent system for examining some of the most fundamental questions about speciation. Serpentine soils can contribute to speciation in two primary ways. First, adaptation to serpentine soils can contribute indirectly to pre- or postzygotic reproductive barriers that genetically isolate serpentine populations from non-serpentine relatives. In fact, due to strong tread off in adaptation, migrants between habitats have reduced fitness and assortative mating increase between individual similarly adapted leading to isolation in face of gene flow. In this way local adaptation contribute to prezygotic isolation between serpentine and non-serpentine lineage. Thus hybrids may be relatively unfit, not for real barriers to reproduction but

because they are poorly adapted to habitats. In addiction, if adaptation to serpentine involves catastrophic selection leading to genomic reorganization in a small founder population (Lewis, 1962), then the process of serpentine adaptation could also confer postzygotic reproductive isolation. Second, the patchy distribution of serpentine can contribute to the geographic isolation of populations. From this point of view, serpentine adaptation might lead to speciation both in allopatry and sympatry, in fact peculiar serpentine conditions could act as strong selective agents picking tolerant genotypes out of mainly non-tolerant colonizing gene pools. This disruptive-selection process often results in ecotypic differentiation (Kruckeberg, 1951, 1967, Rajakaruna et al., 2003), and if reproductive barriers are achieved, the process could proceed to sympatric in situ formation of serpentine endemic (Kruckeberg, 1986; Macnair & Gardner, 1998; Rajakaruna, 2004).

Serpentine soil in Italy

Serpentine soils are widespread around the world, associated with tectonic blocks and intrusion of ultramafic rock, and their sedimentary and regionally metamorphosed derivatives (Coleman, 1977; Coleman and Jove, 1992).

In Italy, serpentine 'islands' outcrops occur in metamorphic sequences scattered around the west-central Alps Ligurian-Piemontese and Tuscan-Emilian Apennines in different biogeographic sectors (Abbate et al., 1984) until the Tiber Valley, in southern Tuscany and the Tuscan archipelago; disjoint nuclei emerge in Calabrian Apennines.


Figure 5: Italian landscapes of serpentine soil.

The greatest concentration occurs in the Tyrrhenian hills of the Pisa, Siena and Livorno provinces, while others are located more in land, such as those in the Arezzo and Firenze provinces (Selvi, 2005). In Emilia Romagna were described ultramaphic outcrop especially near the border with eastern Liguria and in valleys of rivers Toro and Trebbia and torrent Ceno, Nure and Aveto. A lot of interest was also about vegetation and soil of Monte Prinzera, in Parma province (Venturelli et al., 1997; Lombini et al 1998).

The flora of these outcrops is today sufficiently known thanks to a series of studies carried out in single areas, in particular the upper Tiber valley (Pichi & Sermolli, 1948), Monte Ferrato (Arrigoni, 1974), Cecina valley (Selvi & Bettini, 2004) and Monti Rognosi. Descriptive studies were published on the vegetation of the garigue plant communities growing over all outcrops (Chiarucci et al., 1995), on grasslands of the Upper Tiber Valley (Viciani et al., 2002) and on Juniperus scrub communities (Chiarucci et al., 1998); at Murlo site in Siena province vegetation dynamics was also investigated by Chiarucci (1994). The garigue are characterized by the presence of serpentinofite suffruticose as *Stachys recta* ssp *serpentini*, *Thymus acicularis* subsp. *ophioliticus*, *Alyssum bertolonii*, *Armeria denticulata*, *Minuartia laricifolia* ssp. *Ophiolitica* (Gonnelli et al.). In addiction, were found *Stipa etrusca*, *Stipa tirsa*, *Plantago holosteum*, *Trinia glauca*, *Genista januensis*, *Festuca robustifolia*, *Festuca inops*, *Dianthus sylvestris* ssp. *longicaulis*, *Silene paradoxa*, *Sedum rupestre*. Isolated individuals of *Fraxinus ornus* e *Juniperus oxycedrus ssp. oxycedrus* also

enriched vegetation. On crevices of rocks, especially in the northern side, appear *Asplenium cuneifolium, Notholaena marantae* with *Ceterach officinarum Asplenium trichomanes L. subsp. quadrivalens, Polypodium interjectum* and more rare *Asplenium nigrum adiantum*. The grasslands are widespread areas of bounded surfaces and consisted primarily of *Bromus erectus, Danthonia Alpine, Carex humilis* and characterized by the presence of species of great phytogeographical and conservation interest as *Stipa Tirzah, Etruscan Stipa, Festuca robustifolia, Chrysopogon gryllus.* In these plant communities is described a new association *Festuca robustifoliae-Caricetum humilis Viciani, Fogg* (Gabellini et Rocchini 2002). In shrubberies, which are located in areas whose soil is deep, there are contact surfaces dominated by *Juniperus oxycedrus ssp. oxycedrus* and *erica scoparia.*

Pedological studies (Angelone et al. 1991, 1993) and vegetation analysis (Chiarucci et al 1998, 1998, 2001), suggest that, in contrast to previous studies, metal fraction available to plants rather than the total metal concentrations was the most limiting factor in ultramafic soil, according to other authors that made similar observations in well-studied serpentine sites. For example, Kruckeberg (1992) did not find any evidence that cobalt, chromium, iron and nickel affect plant growth in the ultramafic soils of western North America, and in New Zealand, Lee (1992) observed that only in some southern ultramafics nickel toxicity is likely to reduce plant growth. In addition, Proctor and Nagy's (1992) review suggested that many assumptions about the role of nickel in causing the unusual serpentine vegetation are unfounded. However, exchangeable fraction of metals is higher in soil under more developed and structured communities, both in natural and anthropogenic habitats; for example in Italian soil soluble fraction of chromium is generally too small to affect vegetation (Pandolfini & Pancaro, 1992; Chiarucci et al., 1998c, 2001). However, exchangeable fraction of metals is higher in soil under more developed and structured communities, both in natural and anthropogenic habitats; for example in Italian soil soluble

fraction of chromium is generally too small to affect vegetation (Pandolfini & Pancaro, 1992; Chiarucci et al., 1998c, 1998d, 2001). In addition, Chiarucci (1998) found out that garigue are located in soils with lowest concentrations of potentially heavy toxic metal and in site with a wide range of phisycal condition (Chiarucci et al 1998). Thus, according to this, one of the most important limiting factors for the vegetation of Tuscan ultramafic soils appears to be drought stress due to topographical position. Water stress and soil nutritional deficiencies constantly limit vegetation development. In both Mediterranean and inland sites, the annual solar radiation **is** significantly higher in juniper scrub communities, a relatively undisturbed vegetation type where the serpentine endemics grow, than in sites with a proper woodland vegetation (Chiarucci et al., 1998c, 1998d).

Study species.

Dianthus sylvestris Wulfen is a group of species evergreen herbaceous biennial or perennial belonging to the Caryophyllaceae family. Within the genus Dianthus, the *D. sylvestris* Wulfen group can be considered, as one of the most complex and it is still not severely investigated. This group is represented by *Dianthus arrosti C. Presl, D. siculus C. Presl, D. graminifolius C. Presl, D. cyathophorus Moris, D. gasparrinii Guss., D. longicaulis Ten., D. virgatus Pasquale, D. tarentinus Lacaita, D. morisianus Vals., D. japygicus Bianco & Brullo, D. sardous Bacch., Brullo, Casti & Giusso, D. busambrae Soldano & F. Conti, D. brachycalyx Huet sp. nov., D. oliastrae sp. nov., D. insularis sp. nov., D. genargenteus sp. nov. And D. ichnusae sp. nov.* Besides, two new subspecies are recognized within *D. ichnusae* (subsp. ichnusae and subsp. toddei)(Bacchetta et al, 2010): all those sub species are characterized by flowers far and isolated just dented petals and short apex scales. This group of species, known until 1732 is still waiting for an exhaustive classification.

Figure 6: Examples of phenotypic variation in flower morphology of Dianthus sylvestris group.

The generic name derives from the greek 'Theos' (God) and 'Anthos' (flower) and therefore means “flower of God”; the specific name, from the Latin 'sylva' (forest), could be misleading because the species does not grow in woods. This plant presents stems closely united or arising from a single woody root. The stem can be ascending or erect, long up to 50 cm, rarely up to 60 cm, glabrous, sparsely branched, swollen nodes, sometimes reddened towards the apex.

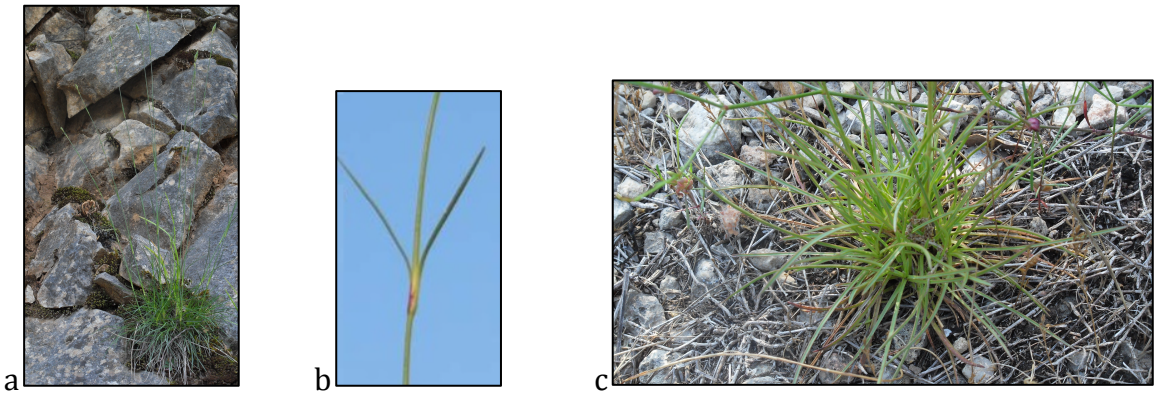


Figure 7: a:*D.sylvetrsis* growing on limestone rocks; b: detail on internode and stem leaves; c: basal rosette.

The basal leaves are usually linear, up to 25 cm, while stem leaves are smaller, but proportionately more extended, opposite to sheathe the stem and joined at the base; sometimes they occur as semi lamine often bent upwards. The margin is both membranous

(this characteristic is most noticeable at the base), slightly rough-toothed, or even in full, with an acute apex.

The flowers are mostly solitary at height of the stem, delicately smell, sometimes almost odorless. Epicalyces consists scales roughly orbicular and acute, with more or less noticeable beak, long approximately ¼ pipe calicino.

Figure 8: Details of flower in *D. sylvestris.*

The cup is gamosepal, cylindrical, with streaks inconspicuous, equipped with five triangular teeth with apex ranging from dull to sharp. The corolla has a diameter that can be up to about 2.5 cm, formed by five petals completely glabrous, pink color, generally tending to whiten toward the nail, to-truncated apex rounded and irregularly notched. The androecium consists of 10 stamens, while the gynoecium of two carpels, a unilocular ovary and two long stigmas; pollination is entomogamy. The mating system of these plants is gynomonoecious-gynodioecious with commonly mixed individuals (Shykoff et al., 1997) and several flowers often open per plant, allowing for geitonogamy. As commonly find for many other gynodioecious species (Delph, 1996; Shykoff et al., 2003), pistillate flowers of *D. sylvestris* are smaller than the perfect ones (Collin et al., 2002), with differences in

outcrossing rates at both plant and flower level. Most flowers of *D. sylvestris* receive pollen from more than one donor except pistillate flowers from mixed plants. Pollinators of this species are usually two species of Lepidoptera, one diurnal, *Macroglossum stellatarum* L. (Lepidoptera: Sphingidae) and one nocturnal, *Hadena compta* Schiff. (Lepidoptera: Noctuidae). Lepidoptera present a coiled proboscis that allows carryover of small amounts of pollen (Wiklund et al., 1979), so several pollinator visits may contribute to pollination (Pettersson, 1991), leading to multiple paternity of seeds from single fruits. On the other hand, outcrossed seeds from pistillate flowers on mixed plants appeared to be sired by a single pollen donor, suggesting fewer visits of these flowers. The flowering period is from May to August. The fruit is a cylindrical capsule 4 provided with teeth apical welded together, and that separate only at maturity letting out the seeds. Seed set does not differ between pistillate flowers from mixed and female plants (Collin et al., 2002).

D. sylvestris is mainly distributed on mountain around Mediterranean Sea; it's common in all Italian regions and more frequent in Alps and Apennines.

It grows in dry meadows and rocky areas, with optimum on limestone substrates, from sea level to alpine zone, but as described above, it's also documented in garigue vegetation associated to serpentine flora. For this ability to growth both on limestone than on serpentine, *D. sylvestris* could be considered as a bondveg species, even if is still not clear if population growing on different sites are locally adapted.

Other Dianthus species were described on contaminated soil for tolerance to heavy metal or adaptation to serpentine soil. For example, Chen and Lee (1997) found that *D. sylvestris chinensis* grown in a Cd-contaminated site in northern Taiwan for 5 weeks, the Cd concentration in plant shoots increased of 73.7-fold (from 1.56 mg kg^{-1} (before planting) to 115 mg kg^{-1}), and that total Cd uptake in the shoot of plant can reach the threshold (100 mg Cd kg^{-1}) of a Cd hyperaccumulator (Baker et al., 2000).

Moreover, *D. carthusianorum* is described as one of dominating plant in waste heap of southern Poland, characterized by a water deficit, intensive insolation, and elevated levels of heavy metals in the soil (on average: zinc, 4000 mg kg^{-1}; lead, 1650 mg kg^{-1}; cadmium, 170 mg kg^{-1} (Godzik, 1984) and also on Poland serpentine soil (Leszek & Kasowska, 2009). Different studies comparing metallicous and non-metallicous population show evidence for adaptive divergence in plant growing in waste heap. In particular differences in accumulation and morphological and physiological traits were found with a clear molecular marker signature. In fact, *D. chartosianorum* in contaminated soils shows lower biomass of aerial parts, shorter and narrow leaves with more water in their tissues, and fewer leaves for plants. They present, also, shortened and less numerous shoots that reduce the transpiration surface of plants by 25%, and a very dense toots hair. The described differences point to the adaptation of waste-heap plants not only to high heavy metal concentration but also to xerothermic conditions. In addiction, it was also shown that the smaller size of the aerial parts of the waste heap plants was accompanied by early entry in reproductive stage, increasing the fertility of these plants; these are all signs of "r" strategy that increases the change of survival (Wierzbicka & Rostanski, 2002).

Figure 9: basal rosette of D.sylvetsris in Murlo (Si), serpentine site.

D. sylvestris is also documented in the book of Shows in which was described European flora of all heavy metal sites in Europe. Moreover, *D. sylvestris* is not considered in relation

with serpentine soil but in relation with calcareous metalliferous soil on Alps where population evolve tolerance to zinc and lead.

a  b 

Figure 10: Detail of basal rosette of D. sylvestris in two different limestone sites: a, Capraia (Si); b Gerfalco (Gr).

For all these reasons *D. sylvestris* become a suitable non-model species in which investigate whether and how the edaphic factor affect genetic or morphological variation between populations growing on and off serpentine soils.

Objectives and outline of the thesis

Since *D. sylvestris* populations usually grow on limestone hills, in dry meadows and rocky, scrubland, rock faces and cliffs, but their presence is also documented on serpentine outcrops, it is possible to assume that serpentine populations has evolved some form of tolerance to edaphic criticality which could prove an example of local adaptation. For this reasons the following research project wishes to investigate differences phenotypic and genotypic between populations of *D. sylvestris* living on serpentine and limestone soils to verify if serpentine *D.sylvestris* could be defined locally adapted or this species has strong phenotypic plasticity.

Therefore, it will analyse the amount of gene flow to evaluate the levels of polymorphism and the differentiation of populations to define genetic structure and examined genotype-habitat interaction in term of fitness differences.

In detail, expected questions are:

- What are the gene flow dynamics governing the exchange of migrants between populations from serpentine and limestone soilsWhat are the levels of genetic variability within each population and among populations? ? Are there barriers that obstacle gene flow?

Since divergent selection is the driving force of local adaptation, but the result depends on its interaction with gene flow, estimates quantitative gene flow may provide important insights into the hypothesis test of local adaptation. The gene flow is usually estimated in an indirect way based on the degree of differentiation markers loci presumably neutral. Thus, it will be estimated the degree of variability between populations and within populations. In the presence of local adaptation, gene flow between populations of different soils is reduced, and this means that Fst index has high values comparing populations of different soils than populations belonging to the same soil type. In fact, the reduction of gene flow, and therefore the exchange of migrants between populations, should encourage crosses between individuals of the same population, leading to a consequent increase in the frequency of favourite alleles rather than disadvantaged alleles. Alternatively, if the populations have a high genetic continuity, the base of the tolerance to soil edaphic criticality serpentine may be phenotypic plasticity, then it should find no significant differences in the architecture of different genetic populations, or these differences were attributable only to stochastic processes.

- What are the differences in the accumulation of heavy metals in serpentine and limestone populations compared with concentrations of metals in the soil?

The plants that live on metalliferous soils have physiological mechanisms that make them capable of tolerate metal toxicity. These mechanisms typically do not prevent the

absorption of metal, rather than acting on the internal detoxification. Furthermore, the plants adopt two main strategies, accumulation and exclusion. In the first case, the metals are translocated from root to aerial part and they can be concentrated in both parts of the plant. On the contrary, in the exclusion, the absorption and accumulation are predominantly in roots, thus concentrations of metals in the leaves can be very low despite the high concentrations in soil (Baker, 1981). Moreover, in the light of Procter & Nagy studies (1992), according to which the flora control factors ultramafic may differ from site to site, it will be also analysed the composition of the soil to test any differences between different sites of a same type of soil, and to ascertain the differences between soils limestone and serpentine.

- What is the relationship with habitat of local populations compared to non-local populations? Are the differences in traits among populations due to genetic differences or plasticity?

In the case of local adaptation, given the same site, the fitness of the local populations in the origin site should be strongly higher than fitness of non-native populations and each population needs to have a higher fitness in the home site than in other sites. In statistical terms, the average fitness of the study population should be systematically higher for combinations of sympatric type rather than those of allopatric type. To test if in *D.sylvestris* populations occur these conditions it will be set up transplanting experiments and measured some fitness parameters..

Materials & Methods

In this study were investigated 10 serpentine populations distributed in main serpentine out crop in Tuscany and Emilia Romagna and 10 population of limestone site distributed in the same regions.

Distance among site ranges between serpentine site 6 Km (SAN-MUR) and 198 Km (PRI-STE); among limestone site it ranges between 10 Km (VEN-SCA) and 303 Km (TAN-VEN). The smallest distance between a serpentine and limestone site was 5,7 Km (SAN-IES) and the longer one was 337 Km (PRI –TAN).

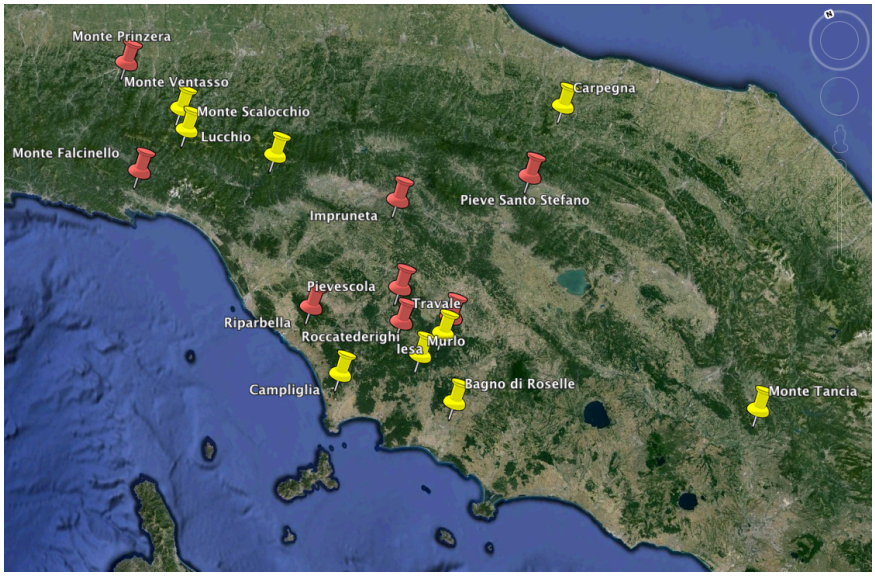


Figure 11: Map of distributions populations sampled in serpentine and limestone sites.

A total of 198 serpentine individuals of *D. sylvestris* were harvested in Pievescola (PIE), Impruneta (IMP), Travale (TRA), Podere il Santo (SAN), Roccatederighi (ROC), Monte Prinzera (PRI), Riparbella (RIP), Falcinello (FAL), Pieve Santo Stefano (STE), Murlo (MUR). In each site fresh and young leaves from an average of 20 individuals per population (larger population SAN, 26 accession, smaller one FAL 10 accession) were collected and dried by silical gel. These sites represent the main distribution area of serpentine soil in Italy and are well documented in an exhaustive literature (Vergnano Gambi, 1992, Mengoni

et al., 2000, 2006; Pandolfini & Pancaro, 1992). **In nearly areas, but on limestone sites,** fresh leaves were collected from 206 individuals 10 non serpentine **sites**: Cornate di Gerfalco (COR), Iesa(IES), Monte Tancia (TAN), Bagno di Roselle (BAG), Lucchio(LUC), Monte Ventasso (VEN), Scalocchio (SCA), Carpegna (CAR), Capraia (CAP), Campiglia Marttima (CAL). On average 20 individuals for populations were collected (larger population LUC, 26 accessions, smaller population SCA, 8 accessions). Plants were collected randomly in each site and for each population GPS coordinates were taken in the central point of the sampling sites.

Table 1: List of the localities of the studied populations of *D. sylvestris*.

SOIL	**SITE**	**GPS COORDINATES**	POPULATION TAG	N° of SAMPLES
SERPENTINE	Pievescola	43°19'N; 11°06'E	PIE	19
	Impruneta	43°40'N; 11.16'E	IMP	19
	Travale	43°11' N; 11°02'E	TRA	24
	Podere il Santo	43°05'N; 11°18'E	SAN	26
	Roccatederighi	43° 02'N, 11° 04'E	ROC	20
	Monte Prinzera	44°38'N; 10°04'E	PRI	23
	Riparbella	43°22'N, 10°36'E	RIP	25
	M.te Falcinello	44°08'N; 09°57'E	FAL	10
	Pieve S. Stefano	43°34'N; 12°02'E	STE	12
	Murlo	43° 08N 11°18'E	MUR	20
LIMESTONE	Cornate di Gerfalco	43°09' N; 10°58' E	COR	19
	Val di Farma, Iesa,	43°05'N; 11°14'E	IES	21
	Monte Tancia	42°18' N; 12°44'E	TAN	20
	Bagno di Roselle	42°48'N; 11°09' E	BAG	22
	Val di Lima, Lucchio	44° 02'N, 10°42'E	LUC	26
	Monte Ventasso	44°22'N; 10°17' E	VEN	25
	Monte Scalocchio	44°16'N; 10°16'E	SCA	8
	Carpegna	43°47' N; 12°22'E	CAR	23
	Capraia	43°11'N; 11°16'E	CAP	20
	Campiglia Marittima	43° 05N, 10°36'E	CAL	24

In order to assess morphologic variations in natural population and to find out evidence of local adaptation or validate the phenotypic plasticity of serpentine plant genome, a subsample of populations investigated with molecular marker was used for transplanting experiment and morphological and chemical analysis of field-collected individuals. For

each ecotype of *D.sylvestris*, serpentine and limestone, were selected eight populations, four from serpentine sites, Pievescola (Pie), Murlo (Mur), Riparbella (Rip) and Roccatederighi (Roc) and four non-serpentine populations, Campiglia Marittima (Cal), Lucchio (Luc), Cornate di Gerfalco (Cor) and Castello di Capraia (Cap).

DNA extraction and EST SSR Genotyping

Total DNA was extracted according to Doyle & Doyle (1987) starting from ≈ 100 mg of silica gel-dried leaf material. The extracted DNA was quantified with a spectrophotometric method (Nanodrop). Further; integrity of DNA was checked by electrophoresis in agarose gel (0.8% w/v) in TEB buffer (1 mM EDTA, 40 mM Tris-HCl) containing 1% v/v ethidium bromide.

A pattern of 48 primer pairs for Simple Sequence Repeats (SSR) were developed on EST sequences of *Dianthus superbus;* these sequences was part of SiESTa,, a database of EST sequences of different species of *Silene* where *D. superbus* was used as outgroup. Primer pairs were tested on few individuals from different populations of *D.sylvestris* to verify the successful amplification and variability. The 14 primer pairs that successfully amplified were optimized and amplified on the panel of 406 DNA samples for genotyping.

Each locus was amplified independently in a reaction volume of 10 µL, containing 25 µM of each dNTP, 1× PCR buffer with MgCl2 included, 0.02 µM of forward (fluorescently labelled) and reverse (non labeled) primers, 0.5 units of Taq DNA polymerase, and approximately 5–10ng template DNA. The PCR program for all primer was 30'' at 94°C, 1' at 50 (or 55)°C and 1' 72°C repeated for 35 cycles, after 3' 94°C for initial denaturation and followed by 7' minute of final extension. For each individual, PCR products, obtained with primers labelled with different fluorochromes, were pooled in pairs and loaded on Applied Biosystems 3130 Genetic Analyzer using the GeneScan LIZ 500 as the internal size

standard (Applied Biosystems, Carlsbad, CA). Fragment lengths were scored in Genemapper 4.0 software (Applied Biosystems) and manually assigned. Ambiguous peaks were considered as missing data, in order to decrease genotyping errors due to stuttering and large allele dropout (Dewoody et al., 2006).

Descriptive Statistics

For each locus were calculated observed number of alleles, allele frequency, and polymorphism information content (PIC) following Botstein et al. (1980) using Powermarker 3.25 software (Liu and Muse 2005). Since some populations had few individuals, and small samples usually contain less alleles than large ones (Kalinowski 2004), unbiased measures of allelic richness (Ar) corrected for differences in sample size, were estimated by rarefaction method implemented in FSTAT (Kalinowski 2005). Errors of scoring were detected for all loci in Microchecker, and locus-by-population frequencies of null alleles were estimated with GENEPOP (Rousset 2008) choosing the default estimation method of maximum likelihood based on the EM algorithm (Dempster et al. 1977). Linkage disequilibrium (LD) for each pair of loci in each population and across them within the species was checked using the log-likelihood-ratio statistic in GENEPOP 4.1.4. The Markov chain method was applied with 500 batches and 10000 iterations per batch. Deviations from Hardy–Weinberg equilibrium (HWE) were also verified in GENEPOP both within and across sites, using the probability-test (Haldane 1954; Guo and Thompson 1992; Weir 1996) and the score test (U test; Raymond and Rousset 1995), the latter allowing testing both for heterozygote deficiency and for heterozygote excess. The Markov chain settings in the HWE tests were the same as in the LD analysis for loci with more than five alleles. It was used sequential Bonferroni correction for multiple tests for all analyses.

Population Structure

An analysis of molecular variance (AMOVA) was used to assess the proportion of genetic variance among populations of different soil origin. The AMOVA was computed in Arlequin v. 3.1, and the significance tests were based on 10000 permutations. ANOVA was also computed independently within serpentine and limestone populations groups. Weir and Cockerham's (1984) estimators of the level of inbreeding within population (f) and within the whole set of sample (F) were obtained using Genetix. These parameters are analogue of Fis and Fit index of Wright (1951), but should be unaffected by sampling scheme (Weir and Cockerham's 1984). The significance of f and F were assessed by a permutation test.

Genetic differentiation among populations were estimated using different statistical index, as there are different opinion concerning alternative method and index describing population divergence,: **θ** (weir & cockerham, 1984), Gst (Nei, 1973) and D_{st} (Jost 2008). **θ** should provide estimation of variability, and represent the proportion of genetic diversity due to allele frequency differences among populations or the correlations between alleles within populations relative to the entire populations (holsinger & weir, 2009); Gst is also similar to **θ**, but is based on heterozygosity. These statistical indexes were implemented in Fstat and permutations were used to assess significance. However, fixation indexes like these can underestimate differentiation with highly polymorphic markers like microsatellites (Hedrick 2005; Jost 2008; Meirmans 2006). This limitation is overcame by Dest, which measures the fraction of allelic variation among population, enabling to separate whole genetic diversity into independent within- and between population components (Jost 2008, 2009); Dest is an estimator of actual differentiation corrected for small sample size, based on the effective number of alleles.

The combined use of fixation and differentiation-based measures is often recommended for a more exhaustive assessment of population structures (Meirmans and Hedrick 2011).

In general, these index range from 0, no, differentiation to 1, complete differentiation. Usually, an $\boldsymbol{\theta}$ of 0,00 to 0, 05 indicates low level of differentiation, 0,05 to 0,15 indicate moderate level and $\boldsymbol{\theta}$ > 0,15 indicate high levels (Holsinger and Weir, 2009; Hartl and Clark 1997).

Differentiation between pairs of sites was performed in ARLEQUIN calculating a global estimate across loci of the fixation index $\boldsymbol{\theta}$ (Weir and Cockerham 1984), and its statistical significance was evaluated with 1000 random permutations. Pairwise comparison was also estimated for Nei's G_{st} (Nei 1973) and D_{est} with the R package DEMEtics (Gerlach et al. 2010). Confidence intervals (CIs) and associated P values of this comparison were evaluated through 1000 bootstrap replicates with a Bonferroni correction for multiple testing.

Principal coordinate analysis (PCoA) was performed on pairwise Dest matrix, as an ordination method, to reveal variance between serpentine and non-serpentine populations; the analysis was implemented in GeneAlex.

A Mantel (Mantel 1967) test was performed to determine the correlation between matrices of genetic and geographic distances, in order to test isolation by distance. The analysis was performed with 10,000 randomizations in Genepop. Genetic linearized D_{est} was used as distance matrix, while geographic distance was linearized with natural logarithm (Hutchinson and Templeton 1999). The analyses were carried out both on a global scale and on dividing populations in two clusters relating to edaphic origin. Significance of correlations was estimated with 1000 random permutations. Geographic distances were calculated with distance matrix generator as straight-line distances in kilometres between pairs of populations described by GPS coordinates.

A non-spatial Bayesian analysis of genetic structure was implemented to determine populations based on genetic clusters and levels of admixture using STRUCTURE 2.3.3

(Pritchard et al. 2000). The model assumed admixture, correlated frequencies, and no prior population information. The following parameter settings were applied: 5 independent replicates each for a number of populations (K) ranging from K = 1 to 15, a burning period of 20000 iterations, 200000 subsequent Markov Chain Monte Carlo (MCMC) repetitions. The most likely number of populations was estimated with the ΔK statistic of Evanno et al. (2005) using STRUCTURE HARVESTER software (Earl and VonHoldt 2011). Structure analysis was also run within serpentine populations group and limestone group with same parameters analysis.

Characterization of soils

At each study site three-soil sample were collected as well as soil pH were determined.

In each study, site three soil samples were collected from 1 to 15 cm depth, as field replicates, to quantify metal content. Samples were dried (at 75°C, to complete dryness), sieved and the <2 mm fraction was retained for analysis. Soil pH was measured by potentiometry. Soil/deionized water suspension (1:2,5 p:v) was agitated for 20 minutes on a oscillating plate allowing to settle overnight. To estimate Pb, Cu, Ni, Cr and Cd total content, the oven-dried (75 °C) soil samples were grounded into a fine powder by an agate pocket (Fritsch pulverisette) and 250 mg of each sample were mineralised with the addition of 2 ml of HF (40%) and 4 ml of HNO3 (65%) in a micro-wave oven (Milestone mls 1200, Microwave Laboratory Systems). The available fraction of the same elements was extracted from 25 g of dried soil with 50 mL of a diethylenetriamine-pentaacetic acid and triethanolamine solution (0.005 M DTPA + 0.01 M CaCl2 + 0.1 M TEA, pH 7.3) at room temperature in continuous agitation for 2 h, according to the official Italian methods for soil analysis (Violante, 2000). The elemental concentration (both total and available) was measured by atomic absorption spectrophotometry via graphite furnace (SpectrAA20

Varian) using standard solutions (STD Analyticals Carlo Erba) diluted in the same acid matrix as for extraction.

In order to find out the evidence of heterogenic distribution of total and bioavailable metal content in soil site, data were analysed with descriptive statistics. Anova's analysis was used to investigate significant differences among soil site, while Mann Withney's test was to compare serpentine and limestone sample. Principal component analysis using both data of total content and bioavailable portion were used as ordination analysis among site, while correlation analysis was used in order to find our significant correlation among total and bioavailable metal in soil sample.

Morphological variation.

Quantitative variation among populations of *D. sylvestris* growing in their natural sites was also investigated measuring several morphological (the number of leaves per plant, average leaf length and width, length of inflorescence) traits. The biometric measurements were carried out in 20 randomly selected individuals of each population in the field. In particular number of stems, number of flowers, height of plant (included stems) and height of rosette were harvested in field while for the measurement of length of stem leaves, number of internodes and distance between them, three shoots for each plant were collected and results were given as mean value. The length of inflorescence was measured in plants grown in their natural environments in the early August 2013. Because of xeromorphic appearance of basal leaves, to measure length and width of rosette, ten leaves for each plant were collected in the field and then were blocked on a white paper sheet with a reference for measure. Then paper was scanned and images were processing with image j . The width of leaves was measured at the maximum width of the blades. Results were given as an average leaf length or width per plant.

The significance of differences in morphological parameters, between analysed populations was analysed using one-way ANOVA with the Bonferroni at the 0.05 probability level for post hoc test. Principal component analysis (PCA) was performed on the morphological and physiological data to reveal phonotypical variance between serpentine and limestone populations grown in their natural habitats. All the data were analysed using SPSS (StatSoft, Inc. 2004) and Past.

Transplanting experiment

At each study sites, mature seeds were collected as mother plants from 20 distinct individuals in order to obtain plants of all populations grown under uniform environmental conditions for further analysis. Transplanting experiment was established in the field. Only four sites were chosen, 2 serpentine (Pievescola and Murlo), two in limestone environment (Castello di Capraia and Cornate di Gerfalco). These sites were easily accessible to establish transplant experiment without human disturbance and are the nearest and similar sites to minimize the geographic variable. The inter-population distances range from a minimum of c. 7 km (CP-CM) to a maximum of 133 km (MA-GB).

Seeds derived from field collected mother plants in spring summer 2013, from each serpentine and non-serpentine populations were germinated on potting soil in green house. The seeds germinated were cultivated on a double autoclaved commercial garden soil in the vegetative room under controlled temperature (24/18 °C, day/night), 16 h photoperiod and relative humidity of 60–70%. After four months from the germination seedlings were marked with iron labels, to find out easily in the field, and transplanted as small rosettes with bare roots.

Four blocks of transplanting plant were established in each of four chosen sites. Each block was a rectangle (1m x 1,2m) and a planting guide was used to make holes in a 20 × 20 grid

where seedling were planting. Twenty individual of one serpentine population and 20 of non-serpentine one were planted in each block disposed alternatively. The couple of populations planting together in a plot were the same in the four sites. The couple of populations planting together were chosen according to their geographic distance. In each transplanting site there were 160 plant, organized in four plot and each plot had 40 plants belonging two populations, one from serpentine and one from limestone. Before planting out, resident vegetation was mown to limit competition, and roots of seedlings were relieved of compost balls. Seedlings were watered once just after planting out.

Transplants were scored for survival in spring (5 months after transplanting) and then monthly thereafter. Vegetative growth was assessed measuring rosette area in the more vegetative state as well as scoring flowering time and height of plant. Presence or lack of flowering (1–0) and, in flowering plants, the number of flowers, was collected as reproductive parameters.

At the end of the experiment, one of the serpentine gardens, Pievescola, was excluded from analysis because it was lost within a month of transplanting: after many seedlings were washed away by heavy rains and is therefore not discussed further.

Data in transplanting experiments were collected until the end of august 2014, nearly all plants had flowered or died. All plants that did not flower but were still alive were included in the survival analysis. Differences in survival between serpentine, non-serpentine were analysed using X^2 test.

Statistical analysis on fitness component, height of rosette and rosette area, were implemented converting fitness of population from different site to relative fitness. Relative fitness was obtained by dividing the magnitude of the fitness components of each population at a field site by the mean fitness of local population in that site.

Relative fitness component were analysed by nested MANOVA after logarithmic

transformation (using SPSS). The factors tested were transplanted soil (serpentine and limestone) and soil of origin (SERP and LIME). Rosette diameter was also analysed separately, using one-way analysis of variance (ANOVA).

Local adaptation was explored at two levels: environment and site within environment. To demonstrate local adaptation, it was used the 'local vs foreign' criterion (Kawecki & Ebert, 2004): local adaptation is showed if the local ecotype (or population) outperforms the foreign ecotype (or population) in its home environment (or site). Data were also analysed using the 'home vs away' criterion, which compares fitness of populations across sites. Each should show higher fitness in its own site (at home) than in others (away). This last criterion has the disadvantage of confounding the effect of divergent selection with intrinsic differences in habitat quality, but is still informative.

Plant metal content analysis

In addition, in order to evaluate the metal content of transplanting plant in serpentine soil, 12 individual randomly chosen from the serpentine and limestone populations, were planted on serpentine under controlled condition. For this purpose, a box with a mixture of serpentine soil was established in the green house of biology department of university Federico II of Naples, in the same period of the transplanting in the field. Plants were completely scarified to assess the content of Pb, Cu, Ni, Cr and in different part of plant after one-year. Plant were washed with deionized water and dissected in three parts: roots, stems, and leaves. Samples were oven-dried at 75°C to complete dryness and pulverized in an agate mortar. A homogenized sample (about 250 mg) was dissolved in a Teflon beaker with a mixture of HNO3 : HF (2: 1) and the residue recovered to constant volume with deionized water (Angelone et al., 1993; Dinelli and Lombini, 1996). The elemental concentration (both total and available) was measured by atomic absorption

spectrophotometry via graphite furnace (SpectrAA20 Varian) using standard solutions (STD Analyticals Carlo Erba) diluted in the same acid matrix as for extraction.

Basing on result of common garden plant analysis, rosette's leaves of plant harvested in natural site were also analysed following the same protocol. However, a pool of leaves from different individual for each site was used as reference for all population due to small size of the majority of plant,

Result

Descriptive statistics

All markers used were polymorphic, with at least three alleles. The PIC value in both groups of populations fell in the highly informative category only for eight loci, while other loci showed moderately informative or low informative values (Tab1). The average number of alleles per locus was 11 across all populations. In the serpentine populations it was found a high rate of polymorphism in the locus SSR28 (0.9209) while lower degree in SSR15 (0.0051). In limestone populations a similar range of PIC with highest value for locus SSR28 (0.909) and lowest value for SSR16 (0.0097) was observed. Private alleles of each population were compared and it was found that serpentine pops present 14 private alleles compared to the 19 found in the limestone ones, while both groups share 124 alleles.

Table 2: Diversity indicators for EST SSR loci.

MAF: major allele frequencies, GNo: genotype number, ANo: number of alleles, AR: allele richness (based on minimum population size 8), Ht: total gene diversity, Hs: diversity within populations, PIC: polymorphism content index

Marker	MAF	GNo	ANo	Ne	Ht	Hs	PIC
SSR28	0.114	108	21	7.144	0.9176	0.716	0.911
SSR19	0.657	10	5	1.763	0.4648	0.4	0.375
SSR25	0.221	40	14	4.105	0.8138	0.715	0.787
SSR15	0.991	3	3	1.017	0.0174	0.002	0.017
SSR33	0.828	9	6	1.388	0.2934	0.091	0.265
SSR10	0.392	39	15	2.960	0.7595	0.576	0.728
SSR7	0.195	63	19	4.162	0.8725	0.340	0.859
SSR41	0.413	48	14	3.161	0.7695	0.61	0.746
SSR16	0.998	4	4	1.014	0.0124	0.012	0.012
SSR31	0.629	14	6	1.956	0.513	0.461	0.440
SSR22	0.819	16	11	1.448	0.3217	0.235	0.312
SSR6	0.230	94	20	5.588	0.8846	0.539	0.875
SSR20	0.538	22	10	2.119	0.6135	0.255	0.555
SSR12	1	15	9	1.885	0.5026	0.2	0.450
Mean	0.549	35	11	2.838	0.554	0.368	0.524

The allelic number decreased testing for allele richness. In particular the mean value in serpentine populations was 3.22, while 3.18 was the mean value in limestone populations. On the other hand, testing for allele richness within the edaphic group the mean value for

serpentine increase (4.55). However, as pattern of variation among all populations did not change, the result was likely due to the reduced size of minimum population (smallest population: 8 samples).

Table 3: tables of allele richness (AR). Table a shows results of AR, based on smallest number 8 individuals. on all sampling populations. Table b shows results of AR, based on smallest population size 8 individuals., within limestone populations Table c shows results of AR, based on smallest population size 10 individuals, within serpentine populations.

	PIE	IMP	TRA	SAN	ROC	PRI	RIP	FAL	STE	MUR	COR	IES	BAG	TAN	LUC	VEN	SCA	CAR	CAP	CAL	MEAN
SSR28	6.01	6.07	6.45	7.10	6.16	6.84	5.88	7.84	6.23	6.32	6.28	6.76	7.36	5.94	6.57	6.25	5.50	5.53	5.67	5.19	7.11
SSR19	1.98	2.37	1.99	1.99	2.39	2.60	1.78	2.00	1.99	1.97	1.91	2.24	2.25	2.46	2.60	2.19	2.00	2.22	1.70	1.96	2.19
SSR25	5.74	3.56	5.27	4.74	4.21	4.46	4.58	3.52	4.59	4.54	4.58	3.68	4.51	4.81	4.47	3.73	2.99	3.32	4.83	5.57	4.91
SSR15	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.46	1.00	1.00	1.00	1.00	1.00	1.59	1.38	1.00	1.00	1.00	1.00	1.09
SSR33	1.00	1.72	1.79	2.20	1.25	1.63	2.06	1.82	1.71	2.17	2.00	2.33	1.46	1.99	1.97	1.38	1.99	2.11	1.89	1.93	2.07
SSR10	4.96	4.47	3.23	4.71	4.77	4.14	3.18	2.40	2.96	3.83	3.67	3.55	4.18	5.33	3.28	3.48	1.96	3.23	4.11	2.67	4.51
SSR7	4.99	5.47	5.12	5.47	5.06	4.89	4.11	4.63	3.30	4.93	4.88	6.48	4.94	6.24	5.08	3.61	3.80	3.00	3.63	1.65	6.11
SSR41	4.43	3.95	4.52	2.95	5.75	3.13	4.12	3.03	3.92	3.99	4.14	3.39	5.43	4.39	4.56	3.88	3.24	3.51	2.62	4.05	4.92
SSR16	1.00	1.00	1.00	1.00	1.00	1.00	1.36	1.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.39	1.00	1.00	1.06
SSR31	1.97	2.22	2.57	2.34	2.50	2.80	2.18	3.35	2.32	2.26	2.00	2.99	2.58	1.97	2.95	2.46	2.00	2.97	2.84	2.18	2.63
SSR22	1.94	1.91	1.00	2.36	2.22	2.45	1.86	1.50	2.65	1.94	1.00	2.28	3.01	2.50	2.37	2.19	1.00	2.71	1.00	1.59	2.53
SSR6	5.94	5.55	5.23	5.90	4.39	6.18	6.48	6.67	6.70	5.03	4.96	6.55	7.21	4.49	4.16	5.71	5.48	4.32	4.71	4.45	6.56
SSR20	2.33	3.59	3.81	3.29	2.59	2.00	2.16	2.85	2.65	1.89	2.94	2.10	3.09	3.99	3.32	2.88	2.63	2.23	1.59	3.08	3.27
SSR12	1.29	2.07	2.85	2.72	2.83	2.55	3.27	3.00	2.73	1.91	1.80	2.00	2.97	2.62	3.40	1.87	3.00	2.42	2.00	1.63	2.76
MEAN	3.18	3.21	3.27	3.41	3.29	3.26	3.14	3.22	3.16	3.05	3.01	3.31	3.64	3.48	3.38	3.00	2.69	2.85	2.76	2.71	3.69

a

	COR	IES	BAG	TAN	LUC	VEN	SCA	CAR	CAP	CAL	MEAN
SSR28	6.21	6.76	7.36	5.94	6.57	6.25	5.50	5.53	5.67	5.19	7.08
SSR19	1.91	2.24	2.25	2.46	2.60	2.19	2.00	2.22	1.70	1.96	2.23
SSR25	4.58	3.68	4.51	4.81	4.47	3.73	2.99	3.32	4.83	5.57	4.90
SSR15	1.00	1.00	1.00	1.00	1.59	1.38	1.00	1.00	1.00	1.00	1.14
SSR33	2.00	2.33	1.46	1.99	1.97	1.38	1.99	2.11	1.89	1.93	2.24
SSR10	3.67	3.55	4.18	5.33	3.28	3.48	1.96	3.23	4.11	2.67	4.51
SSR7	4.88	6.48	4.94	6.24	5.08	3.61	3.80	3.00	3.63	1.65	6.23
SSR41	4.14	3.39	5.43	4.39	4.56	3.88	3.24	3.51	2.62	4.05	4.76
SSR16	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.39	1.00	1.00	1.05
SSR31	2.00	2.99	2.58	1.97	2.95	2.46	2.00	2.97	2.84	2.18	2.76
SSR22	1.00	2.28	3.01	2.50	2.37	2.19	1.00	2.71	1.00	1.59	2.64
SSR6	4.96	6.55	7.21	4.49	4.16	5.71	5.48	4.32	4.71	4.45	6.45
SSR20	2.94	2.10	3.09	3.99	3.32	2.88	2.63	2.23	1.59	3.08	3.47
SSR12	1.80	2.00	2.97	2.62	3.40	1.87	3.00	2.42	2.00	1.63	2.81
MEAN	3.01	3.31	3.64	3.48	3.38	3.00	2.69	2.85	2.76	2.71	3.73

b

	PIE	IMP	TRA	SAN	ROC	PRI	RIP	FAL	STE	MUR	MEAN
SSR28	8.23	8.18	8.55	9.80	8.17	9.32	8.15	11.3	8.64	8.70	9.80
SSR19	2.00	2.72	2.00	2.00	2.70	3.03	1.95	2.00	2.00	2.00	2.29
SSR25	7.33	4.19	6.40	5.59	5.60	5.34	5.59	3.99	5.49	5.33	5.94
SSR15	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.82	1.00	1.05
SSR33	1.00	1.93	1.96	2.36	1.45	2.07	2.37	2.00	1.97	2.47	2.12
SSR10	6.48	5.43	3.72	5.86	5.81	4.95	3.74	2.90	3.00	4.54	5.49
SSR7	6.70	7.33	6.48	6.98	6.28	6.54	5.10	5.00	3.75	6.21	7.60
SSR41	5.50	5.16	5.84	3.73	7.30	4.61	5.24	3.89	5.20	4.71	6.50
SSR16	1.00	1.00	1.00	1.00	1.00	1.00	1.60	1.90	1.00	1.00	1.14
SSR31	2.00	2.47	2.99	2.60	2.90	2.97	2.36	3.90	2.75	2.47	2.85
SSR22	2.00	1.99	1.00	2.69	2.45	2.86	2.34	1.90	3.49	2.60	3.21
SSR6	7.63	7.11	6.33	7.97	5.12	8.40	8.78	8.69	9.53	6.61	8.81
SSR20	2.92	4.20	4.33	3.88	2.84	2.00	2.66	3.00	2.95	1.99	3.66
SSR12	1.53	2.60	3.27	2.94	3.29	2.91	4.00	4.60	2.99	1.99	3.20
MEAN	3.95	3.95	3.92	4.17	3.99	4.07	3.92	4.01	3.90	3.69	4.55

c

The average observed heterozygosity among serpentine populations across all loci was 0.379 (range from 0.333, in IMP population, to 0.417 in population SAN) and 0.349 in limestone populations (range from 0.190, in SCA, to 0.424, in TAN): these values indicated moderate levels of polymorphism. On the other hands expected heterozygosity ranging from 0.446 (in PIE) to 0.547 (in SAN) for serpentine populations, and from 0.393 (in CAL) to 0.547 (in TAN) for limestone populations. Expected heterozigosity was higher than observed and this could be attributed to high level of inbreeding or high selection pressure.

Table 4: Population's heterozigosity.

Ho: observed heterozigosity, He: expected heterozigosity, Hue: unbiased expected heterozigosity.

	Ho	He	Hue
COR	0.365	0.471	0.484
IES	0.372	0.494	0.506
TAN	0.424	0.547	0.562
BAG	0.401	0.537	0.549
LUC	0.322	0.521	0.531
VEN	0.326	0.46	0.469
SCA	0.19	0.432	0.463
CAR	0.383	0.476	0.487
CAP	0.358	0.417	0.428
CAL	0.349	0.393	0.402
Mean	0.349	0.475	0.488

	Ho	He	Hue
PIE	0.39	0.446	0.458
IMP	0.333	0.474	0.487
TRA	0.382	0.504	0.515
SAN	0.417	0.547	0.558
ROC	0.375	0.519	0.533
PRI	0.34	0.489	0.5
RIP	0.369	0.461	0.471
FAL	0.4	0.447	0.471
STE	0.387	0.477	0.497
MUR	0.399	0.465	0.477
Mean	0.379	0.483	0.497

Significant deviations from Hardy Weinberg equilibrium were detected across all loci in all populations due to heterozygote deficiency ($P<0.05$). However, after correction for multiple comparisons (sequential Bonferroni correction), all population remain out of HWE only for few loci due to heterozygote deficiency.

Microchecker revealed the possibility of null alleles at SSR7, and hence many further analyses were performed both with and without this locus. Evidence of linkage disequilibrium was detected between pair of loci ($P<0.05$) underlining non-random assortment among the 14 loci.

Population structure

AMOVA showed off 10.75%, 23.20%, and 66.04% of variation among populations, among individual within population, and within individuals, respectively. Implementing AMOVA within each edaphic group emerged that serpentine populations are less differentiated than limestone one (Fig 12).

Figure 12: AMOVA calculated in each edaphic group.

Looking at average genetic differentiation between individuals within their sampling locations, was observed that *f* index was positive and high (0.260); in addition, F, that quantifies genetic correlation within individuals in the total population was 0.339.

Table 5: *f* values for each populations across loci (a- limestone group, b- serpentine group).

a

	f	IC	
COR	0.252	0.139	0.316
IES	0.263	0.115	0.345
TAN	0.250	0.121	0.329
BAG	0.274	0.175	0.327
LUC	0.398	0.287	0.468
VEN	0.311	0.185	0.388
SCA	0.608	0.368	0.667
CAR	0.217	0.107	0.280
CAP	0.168	0.058	0.288
CAL	0.134	0.014	0.191
Mean	0.288		

b

	f	IC	
PIE	0.152	0.041	0.204
IMP	0.322	0.221	0.365
TRA	0.262	0.162	0.315
SAN	0.257	0.157	0.311
ROC	0.301	0.176	0.381
PRI	0.326	0.214	0.392
RIP	0.220	0.125	0.274
FAL	0.158	-0.040	0.216
STE	0.229	0.080	0.277
MUR	0.168	0.047	0.226
Mean	0.240		

Values of *f* index for each population were positive and ranging from 0.134 to 0.698 in limestone populations and 0.52 to 0.326 in serpentine ones. The greater value in limestone population Sca was probably due to the reduced sample size. However this effect was not evident in serpentine group considering that smaller *f* value is for Pie populations that was

not the smallest one. Differentiation among populations was calculated with θ, G_{st}, D_{est}; these index were calculate both among all populations than among populations within each edaphic group. Both θ and G_{st} showed moderate values of differentiation among populations (θ= 0.107, G_{st} = 0.096). On the other hands, D_{est} was twice higher (0.202), underlining quite strong differentiations among populations. These indices, calculated among populations within groups, were lower in serpentine group rather than in limestone groups (tab 6), underlining stronger population structure within limestone group rather than serpentine group, confirming AMOVA results.

Table 6: Mean values of differentiation indices (θ, D_{est}, G_{st}) in pairwise comparison

	θ	D_{est}	G_{st}
Among serpentine pops	0.08	0.15	0.07
Among limestone pops	0.14	0.24	0.14
Serpentine VS limestone	0.12	0.19	0.11

Pairwise Fst values among all 20 populations range between 0.03 and 0.27, indicating low to moderate levels of genetic differentiation (all comparisons were significant after Bonferroni correction). Pairwise D_{est} range from 0.06 and 0.36 underlined the same pattern of differentiation as for F_{st}.

Table 7: Matrix of θ pairwise comparison among populations

	PIE	IMP	TRA	SAN	ROC	PRI	RIP	FAL	STE	MUR	COR	IES	TAN	BAG	LUC	VEN	SCA	CAR	CAP
IMP	0,11																		
TRA	0,05	0,06																	
SAN	0,08	0,10	0,05																
ROC	0,08	0,07	0,05	0,07															
PRI	0,07	0,08	0,06	0,06	0,07														
RIP	0,06	0,09	0,05	0,07	0,10	0,10													
FAL	0,12	0,13	0,07	0,11	0,12	0,12	0,11												
STE	0,05	0,08	0,04	0,05	0,06	0,08	0,04	0,11											
MUR	0,07	0,12	0,04	0,07	0,08	0,09	0,10	0,13	0,10										
COR	0,08	0,10	0,05	0,06	0,11	0,10	0,07	0,12	0,08	0,08									
IES	0,10	0,14	0,07	0,06	0,07	0,08	0,12	0,16	0,11	0,03	0,10								
TAN	0,15	0,09	0,10	0,10	0,04	0,11	0,16	0,16	0,10	0,15	0,15	0,14							
BAG	0,10	0,07	0,04	0,06	0,07	0,11	0,11	0,11	0,07	0,10	0,08	0,10	0,09						
LUC	0,10	0,08	0,05	0,08	0,08	0,08	0,09	0,08	0,06	0,13	0,11	0,13	0,11	0,08					
VEN	0,11	0,12	0,10	0,10	0,14	0,07	0,13	0,11	0,12	0,15	0,14	0,14	0,17	0,13	0,10				
SCA	0,10	0,11	0,07	0,09	0,15	0,08	0,09	0,12	0,09	0,15	0,11	0,15	0,19	0,13	0,10	0,07			
CAR	0,17	0,14	0,13	0,13	0,12	0,12	0,16	0,18	0,12	0,20	0,19	0,20	0,11	0,16	0,11	0,15	0,16		
CAP	0,09	0,18	0,09	0,10	0,12	0,12	0,13	0,19	0,14	0,04	0,12	0,06	0,21	0,15	0,15	0,19	0,18	0,26	
CAL	0,14	0,18	0,11	0,15	0,15	0,16	0,19	0,20	0,19	0,10	0,15	0,11	0,21	0,15	0,17	0,21	0,23	0,27	0,13

Since D_{est} index was more informative, D_{est} pairwise matrix were used to implement Principal Coordinate Analysis and Mantel test.

PCoA showed that the first two principal components accounted for more than 54% of total variance (28,69% and 25.01%, respectively). However, no clear repartition of populations with respect to edaphic groups could be detected and it's more evident a clustering based on geographic provenance (fig. 14).

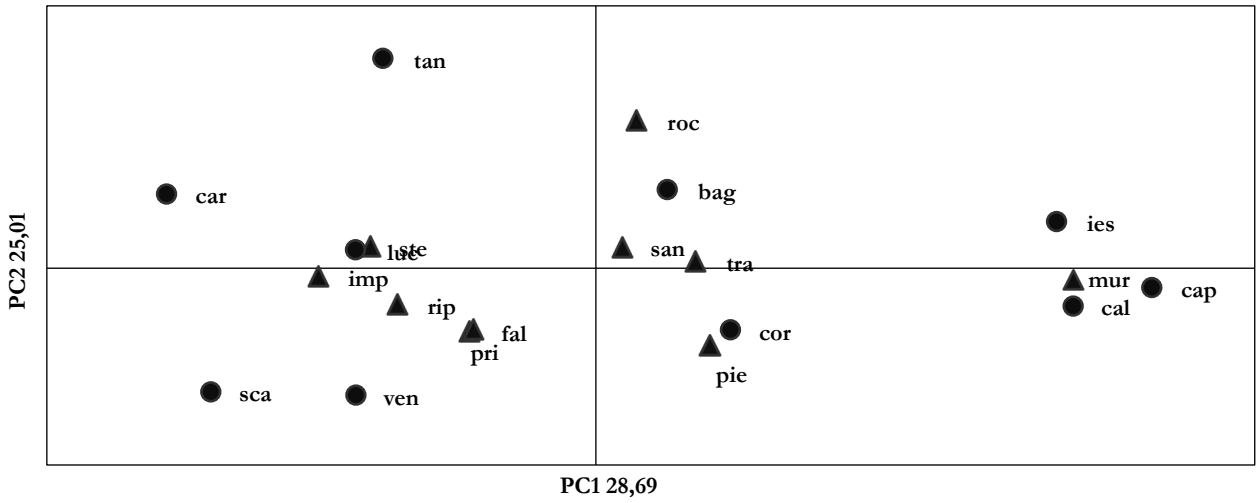


Figure 14: PCoA based on Dst pairwise distance matrix

This result was confirmed by Mantel test for isolation by distance that showed significant correlation between genetic and geographical distances of populations (r^2 0,206; p <0.001) (fig 15).

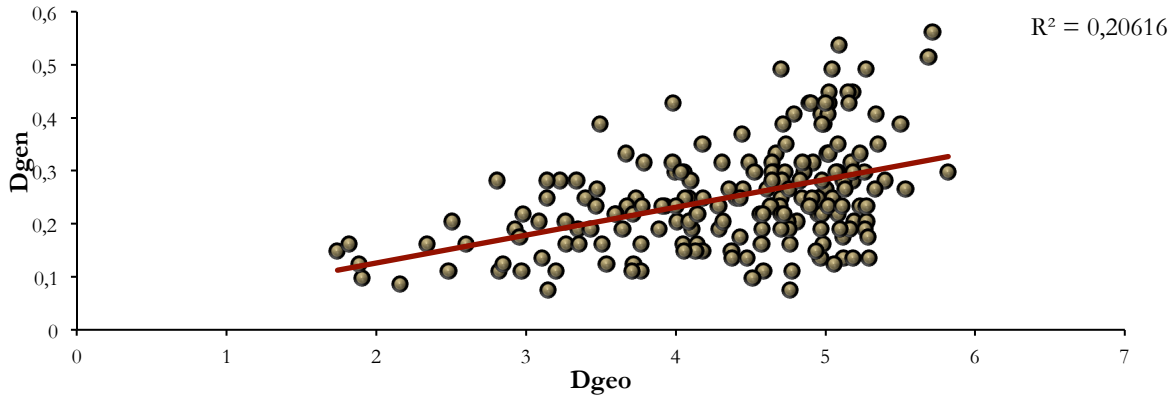


Figure 15: Mantel test to test correlation between genetic distance matrix (linearized Dst) and geographic matrix (natural logarithm of pairwise distance in Km).

Mantel test implemented within edaphic groups was not significant, however correlation between genetic and geographic distance were greater in limestone groups than in serpentine one.

On the other hand, the results of the non-spatial STUCTURE analysis didn't show a clear difference between the serpentine and limestone populations according to their edaphic origins. As reported by results, were indicated 2 possible most likely values of K (Evanno et al., 2005). However each groups includes both serpentine and limestone populations. In particular, PIE, TRA, SAN, ROC, RIP, MUR, COR, IES; BAG, CAP, CAL, which was both serpentine and limestone populations, belonged to first cluster; however, they were distributed in a small spatial scale in Southern West of Tuscany; on the other hand all others populations belonged to second cluster were distributed along Apennine chain. This underlined a more likely classification of populations in relation to their geographic distribution (Fig).

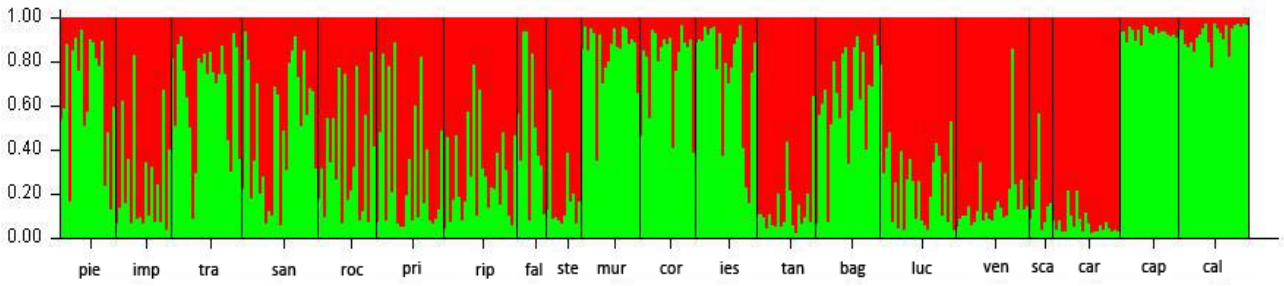


Figure 16: Bayesian analyses output.

Gene flow was also estimated using method that utilizes the standardized genetic variance among populations (Fst) (Wright, 1965).

Table 8: Estimated gene flow expressed by number of migrants (Nm) among populations.

	PIE	IMP	TRA	SAN	ROC	PRI	RIP	FAL	STE	MUR	COR	IES	BAG	TAN	LUC	VEN	SCA	CAR	CAP
IMP	2.02																		
TRA	4.75	3.92																	
SAN	2.88	2.25	4.75																
ROC	2.88	3.32	4.75	3.32															
PRI	3.32	2.88	3.92	3.92	3.32														
RIP	3.92	2.53	4.75	3.32	2.25	2.25													
FAL	1.83	1.67	3.32	2.02	1.83	1.83	2.02												
STE	4.75	2.88	6	4.75	3.92	2.88	6	2.02											
MUR	3.32	1.83	6	3.32	2.88	2.53	2.25	1.67	2.25										
COR	2.88	2.25	4.75	3.92	2.02	2.25	3.32	1.83	2.88	2.88									
IES	2.25	1.54	3.32	3.92	3.32	2.88	1.83	1.31	2.02	8.08	2.25								
TAN	1.42	2.53	2.25	2.25	6	2.02	1.31	1.31	2.25	1.42	1.42	1.54							
BAG	2.25	3.32	6	3.92	3.32	2.02	2.02	2.02	3.32	2.25	2.88	2.25	2.53						
LUC	2.25	2.88	4.75	2.88	2.88	2.88	2.53	2.88	3.92	1.67	2.02	1.67	2.02	2.88					
VEN	2.02	1.83	2.25	2.25	1.54	3.32	1.67	2.02	1.83	1.42	1.54	1.54	1.22	1.67	2.25				
SCA	2.25	2.02	3.32	2.53	1.42	2.88	2.53	1.83	2.53	1.42	2.02	1.42	1.07	1.67	2.25	3.32			
CAR	1.22	1.54	1.67	1.67	1.83	1.83	1.31	1.14	1.83	1	1.07	1	2.02	1.31	2.02	1.42	1.31		
CAP	2.53	1.14	2.53	2.25	1.83	1.83	1.67	1.07	1.54	6	1.83	3.92	0.94	1.42	1.42	1.07	1.14	0.71	
CAL	1.54	1.14	2.02	1.42	1.42	1.31	1.07	1	1.07	2.25	1.42	2.02	0.94	1.42	1.22	0.94	0.84	0.68	1.67

The mean number among populations was 2.37, confirming previous results of differentiation among populations but in face of gene flow. In addition, gene flow within serpentine populations showed a higher value (3.22) than within limestone populations (1.67).

Soil analysis.

Chemical analyses of metal concentrations in the two types of soil highlighted that both serpentine and limestone were alkaline with pH higher than 7.5 for all site analysed but with different elemental compositions. Serpentine soil samples showed very high concentrations of their characterising heavy metals, Ni, 295.75 μg g^{-1} ± 39.71 and Cr 174.18 75 μg g^{-1} ± 45.19 (as mean value overall sites). On the other hand, limestone sites showed lower content of Ni (2.88 μg g^{-1} ± 0.86) and Cr (1.86 μg g^{-1} ± 0.753), but higher concentration of Cd (1.13 μg g^{-1} ± 45.19). High concentration of Cd in limestone was primary due to samples collected in Gerfalco and Campiglia Marittima, that showed also total content of lead significantly higher than serpentine sites (ANOVA, $p < 0.05$); these abnormal values were, maybe, attribute to an anthropic contamination due to the presence

of steel mine in these areas (for concentrations of heavy metals analysed in sites tab. 9). Same differences pattern were show also for bioavailable content (data not showed). Correlation analysis showed a positive linear relationship between total content of metals and their respective bioavailable portions (Rho Sperman Ni_tot / Ni_disp = 0.782, Cr_tot / Cr_disp = 0.444, Pb_tot / Pb_disp = 0.824, Cu_tot / Cu_disp = 0.784 and Cd_tot / Cd_disp = 0.787). All associations were significant with a p value less than 0.01.

Table 9: Metal concentration (µg g^{-1} d.w.) in soils (mean ±SD)

	Pb_tot	Cu_tot	Ni_tot	Cr_tot	Cd_tot
Pie	6.25 ± 5.33	10.76± 2.23	184.62± 104.55	184.65± 122.66	0.01± 0.015
Rip	0.35± 0.60	9.08± 0.34	300.27± 32.44	49.00± 8.19	0
Roc	0.51± 0.45	16.52± 1.77	383.21± 208.78	263.41± 243.30	0.17± 0.28
Mur	9.34± 0.61	19.34± 11.53	325.70± 20.50	212.40± 43.55	0.02± 0.02
Cor	121.56± 124.42	36.88± 13.27	3.96± 2.53	4.85± 3.92	1.14± 0.60
Cal	176.22± 168.48	90.91± 87.37	1.73± 0.67	0	3.31± 0.97
Luc	0.02± 0.02	23.52± 7.22	7.89± 1.40	2.53± 0.41	0.04± 0.05
Cap	1.44± 1.86	4.84± 1.09	0.44± 0.70	0.69± 0.39	0.03± 0.03

Table 10: Speraman correlection between total (tot) concentration and available fraction of heavy metals in soils. (* p<0,05, ** p<0,01)

	Pb_{tot}	Cu_{tot}	Ni_{tot}	Cr_{tot}	Cd_{tot}
$Pb_{available}$	0.824**				
$Cu_{available}$		0.784**			
$Ni_{available}$			0.782**		
$Cr_{available}$				0.444*	
$Cd_{available}$					0.787**

Morphological variation.

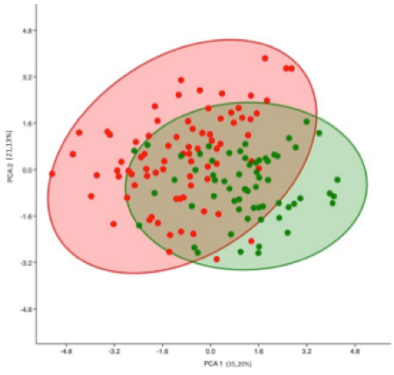
All population investigated in morphological traits differed in terms of general appearance of plants. Plants collected on serpentine soil were significantly smaller with leaves shorted and narrow. The traits that showed higher significant difference among serpentine and limestone populations were: internodes distance, number of internodes, length and diameter of rosette leaves, length of stem leaves, rosette and whole plant height. One-way ANOVA comparison among populations showed a significant difference in variance among

populations for all these traits. However, post-hoc test did not underline differences between edaphic groups. The only traits really discriminant between them was the length of stem leaves that was shortly for all the serpentine plant populations in contrast with limestone ones.

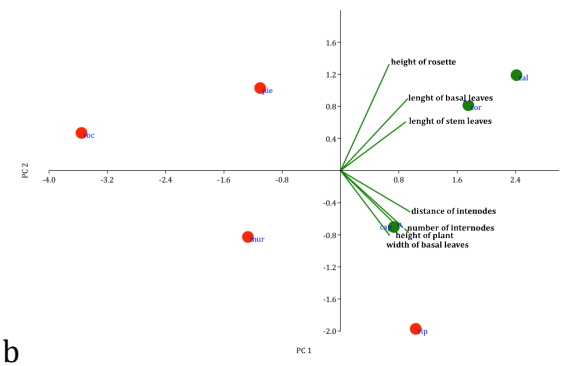
Principal component analysis (PCA) on correlation matrix did not show a completely sub-division of the phenotypes into at least two main groups even if the variations among populations were significantly explained by the first two axes, representing 36% and 25% of the variance, respectively (fig 16a).

However, the same analysis performed using mean value for each population, considering only traits highly significant between serpentine and limestone populations, was able to separate them in two groups according to their edaphic origin by second axis. In this case the PCA1 explain the 61,8% of total variance, and is positively related to all the traits considered, and PC2 explain the 20,1% of total variance, and it is positively correlated to length of stem, length of rosette's leaves and rosette's height, but negatively related to intermodal distance, plant height, rosette leaves diameter and number of internodes (fig 16 c).

Figure 16: PCA analysis on morphological traits; figure a shows the distribution of individuals on two first axis. Figure c shows PCA on mean values of morphological traits.



a



b

Correlation analysis between morphological traits and the content of metals in the soil showed a negative linear correlation between the content of nickel and chromium and the

length of stem and basal leaves and the length of calix, while Cr content is negatively correlated to the height of the plant and the distance between internodes.

Table 11: Spearman correlation among mean values of morphological trait in serpentine and limestone plants and total concentration of heavy metals in soils.

	NF	ID	SLL	PH	CL	BLL
Pb_{tot}	-0.20	0.07	0.21	-0.13	-0.10	0.31
Cu_{tot}	-0.34	0.06	-0.07	0.03	0.28	0.02
Ni_{tot}	-0.18	-0.60	-0.71*	-0.56	-0.70*	-0.82**
Cr_{tot}	-0.10	-0.71*	-0.74*	-0.65*	-0.77**	-0.73*
Cd_{tot}	-0.70*	0.04	-0.30	-0.18	0.15	0.21

NF: number of flowers; ID: distance of internodes; SLL: length of stem leaves; CL: length of calix; BLL: length of basal leaves. (*p<0,05; **p<0,01)

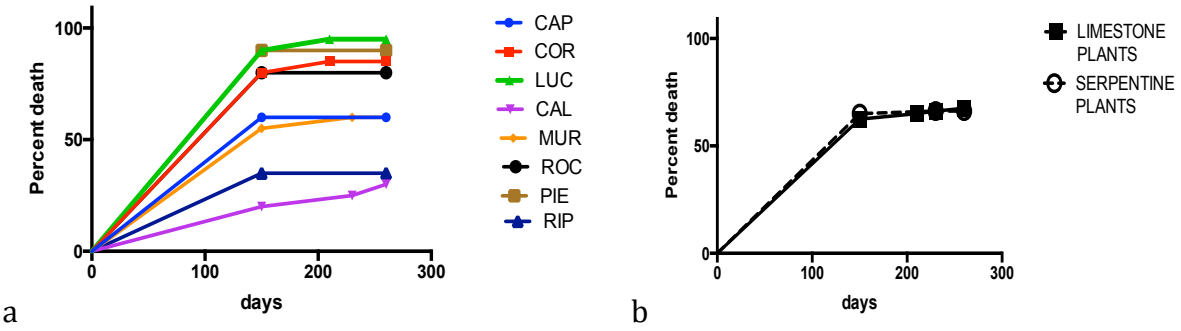
Transplanting experiment

As described above, in Pievescola all plots were lost and this site was not considered in further analysis. Also in Murlo site a lot of individuals died, especially in the plot with ROC-COR populations and the one with PIE-LUC. Thus all individual were grouped in analysis of survivals and in tests that takes in survival, flowering and morphological variation, setting to zero the value for death individuals, in order to avoid unbalance in statistic tests.

In transplanting experiment in Murlo site an overall "χ^2 test" for analysis of survivals showed no significant difference among individuals from serpentine and limestone populations. Also, a pairwise comparison between the two populations in the same plot didn't show difference in survivals. The local population Mur hadn't the highest survival rate.

The pattern of survival and difference among populations in Murlo showed a likely "plot effect", that is, individual planting together had the same mortality not only due to general environment condition but also in relation to locally specific characters, i.e. slope and exposure. To test the possibility of plot effect were performed a linear model with binomial distribution with pop and plot as factor and survival as variable, that however did not

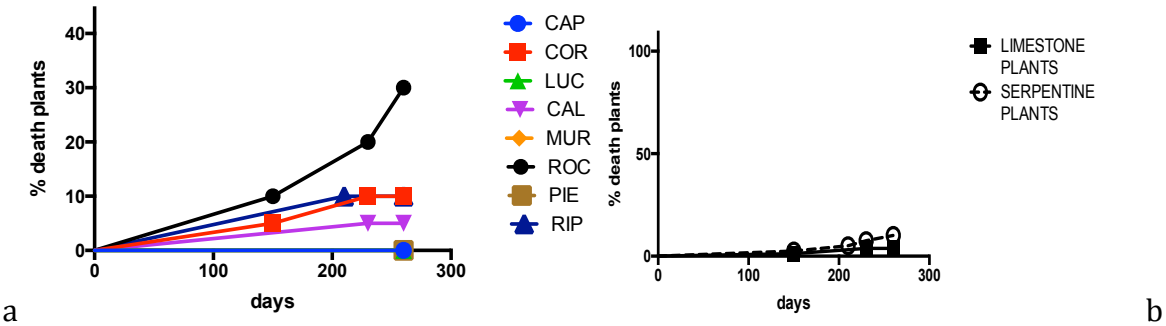
underlined a significant result.



a b

Figure 17: percentage of death plants at Murlo transplanting site. Figure a display percentage for all populations transplanting; figure b display total percentage for serpentine and limestone populations transplanted in Murlo site.

In transplanting sites Capraia and Gerfalco weren't find significant differences in survivals among populations of both serpentine and limestone soil types. Also a pairwise comparison between populations didn't find strong difference.



a b

Figure 18: percentage of death plants at Capraia transplanting site. Figure a display percentage for all populations transplanting; figure b display total percentage for serpentine and limestone populations transplanted in Capraia site.

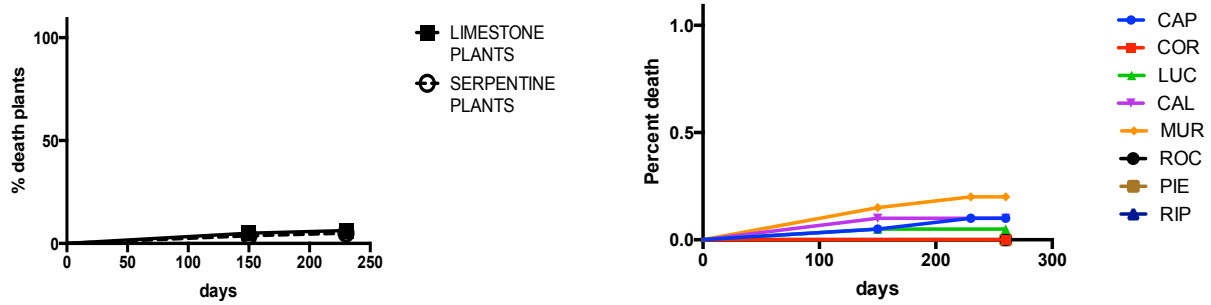


Figure 19: percentage of death plants at Gerfalco transplanting site. Figure a display percentage for all populations transplanting; figure b display total percentage for serpentine and limestone populations transplanted in Gerfalco site.

Comparing survival rate of each local population across the three transplanting sites showed significant differences in their % of survival in the limestone versus serpentine fields plots. The exceptions were local serpentine population (Mur) that had similar survival rates in both habitats. These result means that limestone populations suffer if grow on serpentine site, but however serpentine populations, after transplant, grow better in a limestone site than in a their own environment.

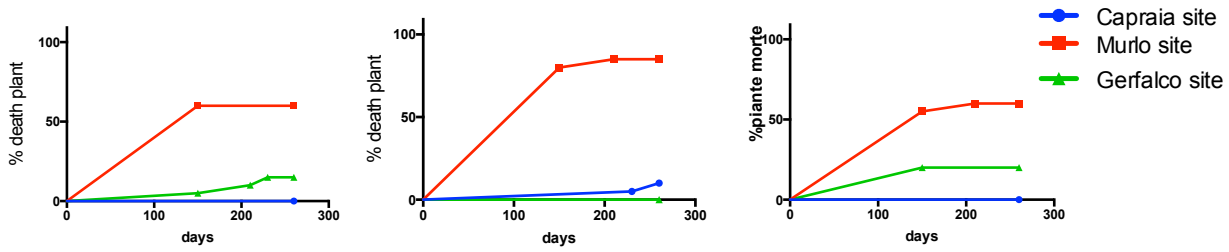


Figure 20: Survival rate of local populations (respectively Capraia, Gerfalco and Murlo) across all three transplanting sites.

While there were no strong survival differences between serpentine and non-serpentine plants when growing at non-serpentine sites, there were significant differences in plant size in both Capraia and Gerfalco sites, where plants from non-serpentine populations were larger than plants from serpentine one. Fitness variations quantified as relative fitness (ratio of rosette area and length at the end of the experiment of non-local population and local one) showed strong evidence in differentiation among populations in limestone sites. The MANOVA analysis underlined significant differences between serpentine and non-serpentine plants for rosette area and plant height at Capraia and Gerfalco non-serpentine sites. However, even if separate one-way ANOVAs conducted on these traits showed that non-serpentine plants were larger than serpentine plants at both

non-serpentine sites, post hoc test underlined that not all limestone populations were different from serpentine ones. In Gerfalco site, local population differs significantly only by PIE serpentine population for both morphological traits. In Capraia site, local population did not differ significantly from serpentine populations.

Table 12: p values of ANOVA (Bonferroni post hoc test) on area and height of rosette of serpentine and limestone populations in Capraia transplanting site.

	CAL	CAP	COR	LUC	MUR	PIE	RIP	ROC
CAL		0.090	0.09	0.651	0.001	0.000	0.000	0.001
CAP	0.00		0.99	0.219	0.082	0.024	0.031	0.079
COR	0.00	0.234		0.222	0.081	0.024	0.030	0.078
LUC	0.00	0.466	0.65		0.004	0.001	0.001	0.003
MUR	0.00	0.020	0.24	0.113		0.622	0.687	0.996
PIE	0.00	0.007	0.12	0.049	0.715		0.927	0.614
RIP	0.00	0.088	0.60	0.335	0.519	0.307		0.680
ROC	0.00	0.038	0.36	0.182	0.784	0.517	0.707	

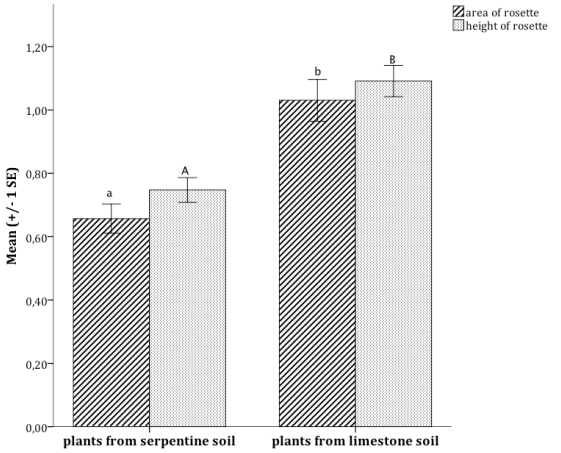


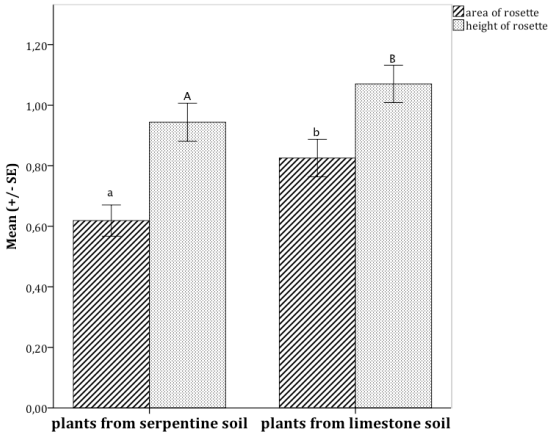
Figure 20: Bars show mean relative values of area and height of rosette of transplanted plants in Capraia site. Relative means ratio among values in plants transplanting and mean value of local populations. (letters mean significant difference on trait between group).

Figure 13: p values of ANOVA (Bonferroni post hoc test) on area and height of rosette of serpentine and limestone populations in Gerfalco transplanting site.

	CAL	CAP	COR	LUC	MUR	PIE	RIP	ROC
CAL		0.002	0.012	0.006	0	0	0.875	0.004
CAP	0.299		0.598	0.781	0.285	0.103	0.002	0.892
COR	0.156	0.701		0.803	0.113	0.032	0.008	0.695
LUC	0.247	0.029	0.011		0.18	0.057	0.004	0.887
MUR	0.112	0.01	0.003	0.653		0.585	0	0.229
PIE	0.122	0.01	0.003	0.695	0.95		0	0.077
RIP	0.527	0.096	0.041	0.598	0.333	0.359		0.002

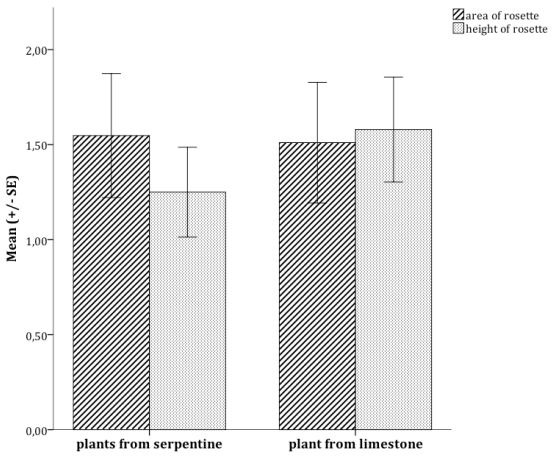
ROC	0.885	0.237	0.118	0.311	0.148	0.161	0.625

Figure 21: Bars show mean relative values of area and height of rosette of transplanted plants in Gerfalco site. Relative means ratio among values in plants transplanting and mean value of local populations. (letters mean significant difference on trait between group).



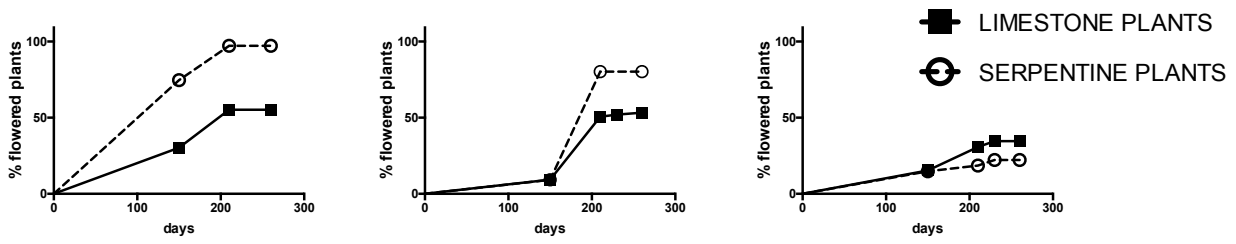
In the serpentine site Murlo, MANOVA analysis did not showed difference in both traits among populations, and local population differ significantly only from RIP and CAL (for the last one only in rosette height), even if this difference favouring the two non local populations who better perform instead of local one.

Figure 22: Bars show mean relative values of area and height of rosette of transplanted plants in Murlo site. Relative means ratio among values in plants transplanting and mean value of local populations.



Comparing the performance of each local population in the three transplanting sites resulted that both CAP and COR populations grow better on limestone soil with no difference between two sites. On the other hand, Mur grew less in its own site than in both Capraia and Gerfalco sites. A GLM, to test if there was a significant effect in transplanting sites based on origin soil, revealed that for these fitness components there was a strong effect of origin soil on plant adaptation.

A "days to flower" analysis between serpentine and non-serpentine plants at any sites shows differences between the planting habitats. Serpentine populations produced much flower than limestone populations in each transplanting sites and a GLM with binomial distribution to analyse the interaction of original soil nested with transplanting showed a significant interaction. Indeed, serpentine population start to flower before limestone population in both non-serpentine sites even if these differences were not found in Murlo site.



a b c

Figure 13: Flowering analysis on transplanting sites. Figure a show variation in flowering plant percentage between serpentine and limestone plants transplanted in Capraia. Figure a show variation in flowering plant percentage between serpentine and limestone plants transplanted in Gerfalco. Figure a show variation in flowering plant percentage between serpentine and limestone plants transplanted in Murlo.

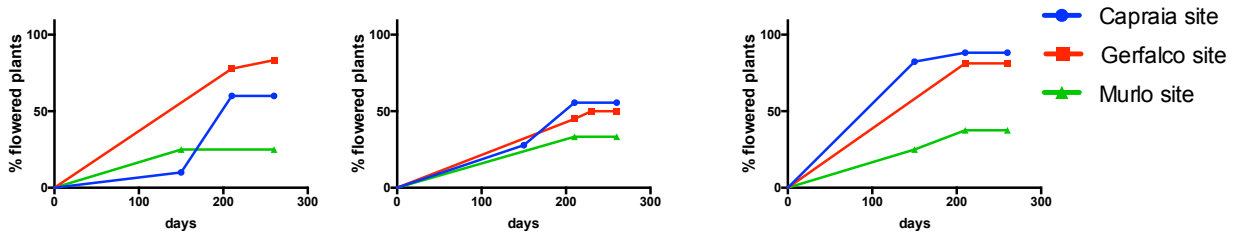


Figure 24: Flowering plant rate of local populations (respectively Capraia, Gerfalco and Murlo) across all three transplanting sites.

Because of serpentine populations flowered more than limestone, it could be possible that strong differences in rosette biomass between serpentine and non serpentine populations in both Capraia and Gerfalco sites was related to different allocation of resource.

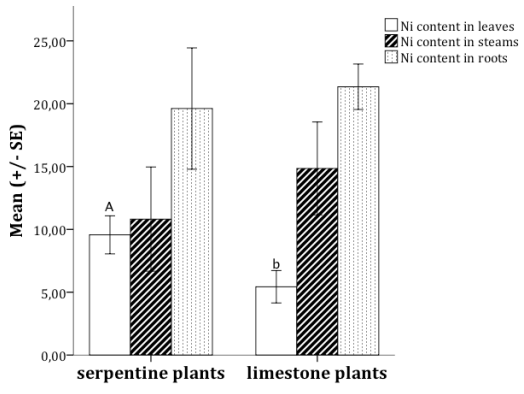
It's already known in literature that a plant that invests more resource in flowering has reduced biomass (reference). Pairwise comparison on investigated traits in each transplanting site were performed between flowered and non flowered plant of serpentine and non serpentine populations and results showed that flowering plant, of both limestone population were larger of non flowering plants, and that flowering limestone plants had higher biomass respect of serpentine plants in both Capraia and Gerfalco site.

In Murlo this tendency was opposed, serpentine flowering plants were larger than limestone one, but this difference wasn't significant. This result allowed concluding that no unbalanced design was used to test difference in plants.

Plant metal content analysis

To verify differences in metal contents in plant tissue, first were analysed plants from common garden experiment, dissected in leaves, stem and roots. Results showed that there were large differences between roots and basal leaves concentrations of metals, meaning an important restriction of the internal transport of metals from root towards basal leaves. Such metal immobilization in root cells as emphasized by the root/leaves > 1, related to an exclusion strategy (Baker, 1981). This mean that *D. sylvestris* plant could store a higher concentration of heavy metals in root than leaves of rosette and this feature is stronger in serpentine plant than in transplanted limestone plant. However plants of limestone origins grown on serpentine soil differ from serpentine plant only in Ni and Cr content of leaves, and were not found difference in accumulation in root and stem.

This was an interesting result because even if *D. sylvestris* seems to accumulate in roots as reported in literature, the translocation of metal in aerial parte makes the difference between serpentine and limestone plants.



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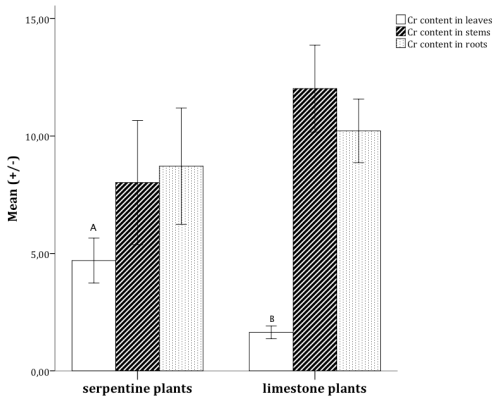

b

Figure 25: metal content in serpentine and limestone plants in common garden experiment. Figure a show variation of Ni in leaves, stems and roots in plants growing on serpentine of both serpentine and limestone origins. Figure b show the amount of Cr in leaves, stems and roots of same plants.

Starting from this point of view analysis of natural plant was done only on leave tissues to verify if the same differences find in transplanting were present also in nature. In plants collected in the field significant differences in elemental concentrations between the investigated populations were found. In shoots, plants from serpentine soils contained

average amounts of Ni and Cr far above those of the accessions from calcareous soils. However, these differences reflect metal concentrations in soil, previously discussed.

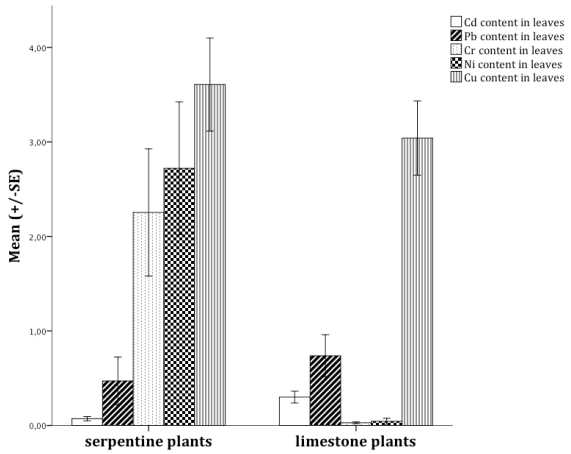

Figure 26: Heavy metal content in natural plants from serpentine and limestone soils.

PCA analysis on leaves content of heavy metal analysed in natural sites underlined that populations clustering in two groups on the basis of their edaphic characteristics. The first two axes explained 78% of variance, especially PCA1 54% related to Ni e Cr and PCA2 24% related to Pb, Cu and Cd.

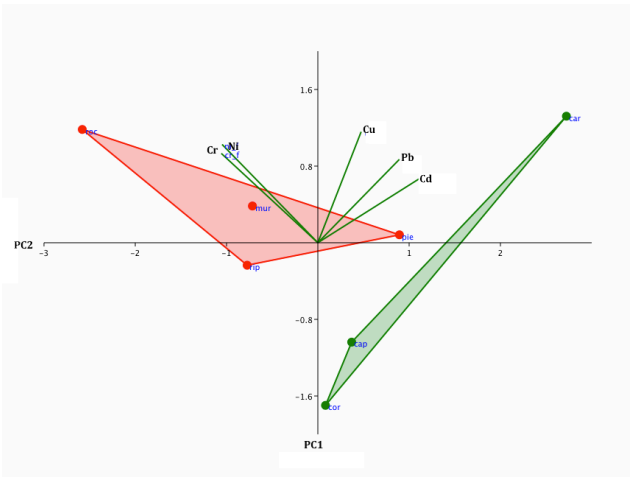

Figure 26: Multivariate analysis on mean values of heavy metal content in natural plants from serpentine and limestone soils.

Discussion

In populations occupying stressful and patchy habitat, as serpentine soil, gene flow has a central role in both enhancing phenotypic plasticity and contrasting local adaptation. Nevertheless, gene flow is the main force increasing gene pool variability in populations and this is a prerequisite for both actions of natural selection and plasticity. However, in such contest, genetic diversity of tolerant populations can result in a significant reduction, expected because of the strong bottleneck as a result of strong selection (Bradshaw 1984; Lefèbvre and Vernet 1990), while genetic differentiation among tolerant populations is expected to be higher compared with non tolerant ones for the founder effect associated with strong selection on toxic soils (Vekemans & Lefèbvre, 1997).

In this study we used SSR markers to investigate pattern of gene flow and genetic differentiation among populations of *D. sylvestris* growing on both limestone and serpentine soils. Our results showed that, as confirmed by polymorphism of investigated loci (PIC range from 0,26 to 0,91), populations growing on both kinds of soils were highly polymorphic with mean alleles number of 68 for serpentine and 65 for limestone populations. Allele richness, calculated on the smallest population size (8 samples), decreased for both population groups (3,22 mean value in serpentine populations, 3,18 mean value in limestone populations). However, the pattern of differences among populations didn't change, that is, populations with higher number of allele also had higher value of allele richness, suggesting that decrease was only due to reduced population size. Despite the great number of alleles (157 shared alleles), only 14 alleles were private for serpentine populations (TRA, IMP, RIP, PIE, SAN, FAL, STE, Roc) and 19 were private for limestone one (IES, TAN, BAG, LUC, CAL, CAR). According to high polymorphism rate of loci investigated, populations presented moderately high level of genetic variability and similar

between the two edaphic groups, as confirmed by observed heterozygosity values, ranging from 0,333 to 0,417 for serpentine populations and from 0,19 to 0,424 for limestone ones; also the mean value of heterozygosity for both edaphic groups are similar underlining a comparable pattern of allelic frequencies. Even the expected heterozygosity was high in all populations. According to this, populations showed a positive level of F (0,302 for both serpentine and limestone populations) and f (0,240, 0,247 for serpentine and limestone groups) indices likely due to some inbreeding rate related to the small size of sampled populations.

Our results proved that there was not a reduction of genetic diversity in serpentine populations, and that the level of diversity is similar among serpentine and limestone populations. This was in accordance with previous study on populations of *Dianthus carthosianorum* growing on metal and non-metal soil in Poland, for which significant differences in intra population genetic diversity was not evidenced, and with study of Słomka (2011) who found even slightly higher genetic diversity within metal populations of *Viola tricolor* in comparison with non-metal ones. In both cases, they explained the results suggesting that metal tolerance wan not always related to decrease in genetic diversity. However, these results were in contrast with other studies on Caryophillacee species growing on metal soil, as for example *Arabidopsis halleri* and *Silene paradoxa*, in which reduced genetic diversity among tolerant and non-tolerant populations were found (Pauwels et al., 2006; Deng et al., 2007; Meyer et al., 2010).

In spite of no proof for reduction of genetic variability in serpentine group respect of limestone one, we detected some evidences of differentiation among populations. Notably, it was observed a mean Fst value of 0,19 among populations examined that could underline a moderate differentiation rate. Indeed, a value of differentiation indices ranging between 0,0 and 0.05 predicts for little genetic differentiation among populations, alternatively a

value ranging between 0.05 and 0.15 predicts for moderate differentiation (Wright 1978; Hartl & Clark 1997). Surprisingly, limestone populations showed higher differentiations (θ=0.14) than serpentine one (θ=0.08). This is supported by mean number of migrants (Nm) detected within serpentine populations (3,22) that was greater than value within limestone (1,67), underlining a greater amount of allelic exchange among serpentine populations. In literature, is proved that a rate of gene flow (estimated by Nm value) smaller than 1 (less than one migrant per generation into a population) is generally considered as a threshold value, beyond which significant population differentiation occurs, conversely a value greater than 1 imply that gene flow among populations is sufficient to encounter the effect of random drifts. This suggested that, even if limestone populations seemed stronger differentiated, gene flow is strong enough to avoid genetic drift. However, neither PCoA nor Bayesian cluster analysis (STRUCTURE) was able to classify populations according to their edaphic origin. As reported by the results, the most likely K detected by Structure analysis, divides populations in 2 groups: each groups includes both serpentine and limestone populations. In particular, PIE, TRA, SAN, ROC, RIP, MUR, COR, IES; BAG, CAP, CAL, which are both serpentine and limestone populations, belonged to first cluster; however, they were distributed in a small spatial scale (distance range) in Suthern West of Tuscany; on the other hand all others populations belonged to second cluster were distributed along Appenine. This underlined a more likely classification of populations in relation to their geographic distribution. Even PCoA analysis divided populations in two main groups on the first axis (PC1 29%); however, the two groups were composed by the same populations resulted in the Bayesian clustering, that is, according to their geographic distributions.

Nevertheless, Mantel test displayed a significant relationship between genetic and geographic distance (r2=0,206). Thus, it seemed that genetic structure of populations *of D.*

sylvestris was shaped mainly by the geographic distribution (distant populations were more differentiated than closely one) rather than by edaphic factors. This explanation may also justify the high Fst values among limestone populations: the mean distance among them was 123 Km, in contrast with the mean distance among serpentine populations of 86 Km. In addition, Fst, calculated only considering limestone populations distributed in Tuscany, drastically decreased (0,07) and became comparable to Fst among serpentine populations, confirming that stronger differentiation of limestone populations was only due to longer distance. Similar evidence also came from the isoenzyme studies of Nyberg Berglund & Westerbergh (2001), according to which serpentine populations of Cerastium alpinum in Scandinavia are genetically more similar to non-serpentine populations within the same geographic region than with distant serpentine populations. Moreover, the possible lack of genetic differentiation on edaphic basis was also confirmed by the results of an AFLP-based study by Mengoni et al (2006), who found close genetic relationships between serpentine and non-serpentine populations of *Onosma echioides* in some of the same sites (Campiglia, Murlo, Riparbella, Capraia).

Despite molecular analysis did not detect strong genetic differentiation on edaphic basis among D. sylvestris populations, chemical analyses underlined significant evidence in metal content in soil and plants growing on ophiolithic outcrops, following the peculiar elemental composition of soil.

Soil analysis reflected, in terms of metal content, serpentine characteristics with levels of Ni and Cr, on average to 263.4 ug g-1 and 191.2 ug g-1, respectively. A minimum Cr concentration about 0.5 mg kg-1 in water and 5 mg kg-1 in soil gives rise to a detrimental effect in plants (Turner & Rust, 1971). Metal content of the examined serpentine soils were lower than the value resulted from other studies in the same area (Mengoni et al., 2000, 2006); however, metal concentration observed exceeded the limit of plant tolerance. Even

if elemental composition of soil is mainly due to geochemical nature of the substrate, trace of heavy metals in soil could be also due to human activities (anthropogenic activity) (Lazaro et al., 2006). Metallurgical, extraction and smelting industries are a very important source for contamination of soils (Alloway, 1995; Adriano, 2001; Commission of the European Communities 2002). This helps to explain the high levels of lead, copper and cadmium found in limestone site Campiglia Marittima, a mining site still active today.

Analysis of metal content of serpentine and limestone plants grown in common garden revealed that limestone and serpentine plants present quite similar concentration of heavy metal in roots and stem, while significant difference was only detectable in basal leaves. Starting form this point, natural plants were investigated in accumulation of heavy metals in rosette's leaves, and results showed that serpentine plants had significantly higher concentrations of Ni and Cr than those from limestone soils. However, in serpentine plants it was observed heavy metal concentration below the mean limit for plant toxicity (Kabata-Pendias & Pendias, 1991), instead, heavy metals in toxic concentrations in limestone plants were not detected. Since plants need chromium and nickel as micronutrients, they have the translocation system from roots to xylem transport. However, if some transport systems were identified for nickel, it is still unknown whether Cr is translocate into leaves. This observation could explains the presence of a greater amount of Ni in the aerial portion of the accumulating plants rather than Cr, that is, conversely, limited to the roots.

High concentrations of Ni reduce growth of roots and buds, determining abnormalities in the development of flowers and leaves and produce a Fe deficiency that leads to chlorosis and leaf necrosis. Furthermore, adaptation to water stress and nutrient deficiency includes slow growth rates and higher root to shoot biomass ratios in order to reduce leaf surface for transpiration and enhance root capacity to absorb water and minerals (Chaves et al., 2003). According to this details, serpentine plants were characterized by a small size of

aerial parts, resulting from significant reduction in length and width of leaf blade as well as in the number of leaves in comparison with plants from limestone soil and no evidences of chlorosis and necrosis were found. This lets assume that serpentine plants have therefore developed defence systems necessary to counteract some of edaphic factors distinctive of serpentine soil, as toxicity of heavy metal and water stress.

Even if PCoA based on molecular marker was not able to classify populations according to their edaphic conditions, multivariate analysis on morphological data and metal content in plants showed some evidence for an edaphic sub division of populations. The amount of variance explained by first axis of phenotypic PCA was mainly due to Ni and Cr content and to some morphological characters negatively correlated to heavy metal content (height of rosette, length of basal and stem leaves).

To figure out if the phenotypic variation is the result of adaptation or an expression of phenotypic plasticity, reciprocal transplant experiment was installed in both serpentine and limestone sites. Surprisingly, our result did not underline survival differences between serpentine and non-serpentine plants at the serpentine and limestone experiment sites. Comparatively, local limestone populations showed the highest survival rate when planted in their origin site; in contrast, serpentine populations better performed in limestone sites than in their own origin site. Nonetheless, the high rate of plants lost in serpentine transplanting site support that strong survival differences in these sites were not detected due to limited sample sizes and significant environmental variance. As reported by results of transplanting experiments in limestone sites, plants of limestone origins were larger than serpentine plants. Thus, even if serpentine plants growing on both limestone and serpentine soils had the same survival rate than limestone ones, the small growth rate could be considered an evidence for cost of tolerance due to intrinsic serpentine "adaptation". In literature, is already known, that other serpentine-tolerant species display

a slower intrinsic growth rate than non-serpentine plants (O'Dell and Claassen 2006, Sambatti and Rice 2006). It has also been shown that serpentine-tolerant plants do not grow as well as non-serpentine plants when planted together on non-serpentine soils (Kruckeberg 1954, Proctor et al. 1975, Jurjavcic et al. 2002). This suggests that there is maybe a trade-off between competitive ability and tolerance to serpentine.

On the other hand, transplanting also confirmed that the serpentine plants flowering more and earlier than the limestone ones on both serpentine and limestone soil. Such tendency towards shortening the life cycle and high reproductive effort is typical for plants exposed to adverse environmental conditions (Chaves et al., 2003; Dechamps et al., 2008). These plants allocate a greater amount of energy for sexual reproduction in order to increase the chance to survive in the extreme environment, paying the cost of reduced size. This is in line with those reported by Kay (2011), that the adaptation, and therefore the isolation between populations of the same species, can also be linked to ecological factors that determine a different affinity for the habitat as well as different flowering times, making unlikely crosses between populations "adapted" and not.

Although, pedological studies (Angelone et al. 1991, 1993) and vegetation analysis (Chiarucci et al 1998, 1998, 2001) suggest that that the main edaphic factor affecting plant growth in Italian serpentine site is drought, even limestone populations originate from xerothermic habitats, therefore, species phenotypes already reflect the acclimation/ adaptation to water deficit. Thus, the differences between the serpentine and non-serpentine populations concerning plant size, general appearance, and phenology, are determined by other concurrent stresses occurring on these soils.

Concluding Remarks

The key role of edaphic factor in addressing phenotypic variation in plants is well documented. However, it could depend on genotype plasticity (to alter the phenotype in response to environmental cues, without changing in allele frequencies), on divergent selection that promotes the evolution of traits adapted to a specific habitat.

Based on our results on genetic and phenotypic differentiation among populations of *D. sylvestris* living on serpentine and limestone soils, we can address the following questions.

- *What are the gene flow dynamics governing the exchange of migrants between populations from serpentine and limestone soils? What are the levels of genetic variability within each population and among populations? Are there barriers that obstacle gene flow?*

We detected high amount of gene flow among serpentine and limestone populations that produced absenc of genetic structure. The geographic distance is likely the main barrier to gene flow. *D. sylvestris* has a wide distribution. Thus, even if serpentine outcrops are patchily distributed, populations are not really isolated. The detected genetic cohesion suggests that ecological connections that facilitate allelic exchange occur among serpentine and limestone populations.

In addition, pollinators may favour pollen flow at large distance, assuring the gene exchange that could explain results of molecular variance analyses. This species is mainly impollinated by two lepidoptera, Microglossun and Hadena, whose distribution range of about 150 km was estimated.

Genetic diversity between the two edaphic groups was comparable even if it strongly contrasts with expectation of reduced genetic variability in tolerant populations. Serpentine soils are considered stressful habitats with more than one limiting edaphic

factors at small spatial scale. In such condition, genetic variability plays an important role in favouring the best allelic pattern for plant survival and adaptation. A population with low levels of genetic variation for ecologically relevant traits would have a reduced ability to adapt to adverse environmental conditions because genetic variation is a prerequisite for adaptive evolution by natural selection (Slatkin, 1987; Hoffmann and Blows, 1994; Gomulkiewicz et al., 1999; Barton, 2001; Lenormand, 2002; Blows and Hoffmann, 2005; Kellermann et al., 2009). In those cases where gene flow can have a facilitating rescue effect on adaptation, it is possible that the negative effects of gene flow (transfer of maladaptative genes) are masked by genetic variation and beneficial mutations provided by the same dispersers, thus helping in maintaining the adaptive potential (Lande, 1995; Holt and Gomulkiewicz, 1997; Gomulkiewicz et al., 1999; Holt, 2003; Garant et al., 2006; Holt et al., 2011).

According to theory, the ability of a population to adapt to local conditions in the face of gene flow depends on the genetic basis of the involved traits (Haldane, 1930; Bulmer, 1972; Yeaman & Otto, 2011). Yeaman showed that local adaptation occurs much more readily with alleles of large effect, which show greater differentiation of allele frequencies under divergent selection (Hedrick et al. 1976). Furthermore, alleles with strong effects are less likely to be lost by drift (Crow & Kimura 1970) and loci with large effects on fitness should disproportionally contribute to local adaptation (Macnair 1991). This is the case in the classic study of local adaptation of plants to sites contaminated with heavy metals (Macnair 1987, 1991). Similarly, an oligogenic basis of adaptation may justify the results of *D. sylvestris* populations investigated here. Values of genetic diversity largely depended on the marker method applied, namely, on portions of the analyzed genome (Mengoni et al., 2001; Gajera et al., 2011), and local adaptation driven by oligogenic traits does not influence gene flow at neutral loci, as for neutral marker employed here.

- ***What are the differences in the accumulation of heavy metals in serpentine and limestone populations compared with concentrations of metals in the soil?***

The content of heavy metals in soils of origins of examined populations confirmed that concentrations of heavy metals were higher in serpentine than in calcareous soils.

The metal content in the aerial part serpentine examined plants was significantly higher than in limestone ones. According to these results, common garden experiment highlighted a significant difference in heavy metal content in leaves of serpentine plants compared to limestone ones. However, fractioned analysis of the metal content in roots, stems and leaves, of both serpentine and limestone plants, displayed that accumulation was mainly in the hypogeal portion of both serpentine and limestone plants: significant differences between leaves and roots content were found in both edaphic groups, but no difference between groups was found at hypogeal level. This suggests that plant accumulation of metal in serpentine and limestone soil is not a genetically controlled trait (i.e. plants accumulate only depending on soil heavy metal concentration), although it is possible to suppose a genetic differentiation in the translocation system (roots / leaves). In addition, the results suggest that *D. sylvestris* mainly shows an exclusionary strategy limiting translocation of metals to leaves.

- ***What is the relationship with habitat of local populations compared to non-local populations? The differences in traits between populations are due to genetic differences or plasticity?***

The results showed in this study highlight an influence of edaphic factors in the phenotypic variation of populations of *D. sylvetrsis* from serpentine and limestone.

In these plants, several morphological traits were found statistically decreased when compared to plants from limestone so highlighting that serpentine is a less permissive

habitat than limestone. Moreover, the absence of phenomena of chlorosis and necrosis in serpentine plants, as well as the reduced biomass of these plants even when grown on limestone, suggest that they have evolved some mechanisms of tolerance to metals. However, the tolerance seems due primarily to a difference in translocation system rather than on accumulation capacity. Nevertheless, in transplanted plants from serpentine to limestone soil, a two-way ANOVA resulted in a significant difference in plant biomass with an effect of the original soil on the transplanting soil. Significant differences were also found in flowering time, as plants from serpentine, when transplanted on limestone, flowered before than resident limestone plants. These differences, persisting independently from the original soil type, should have genetic bases. It can be assumed, therefore, that plant on serpentine are strongly affected by edaphic cues of serpentine soil and the observed phenotypic variation can be ascribed to local adaptation, even if genetic differentiation of populations (likely occurring at few selected loci determining different affinity for the two habitats) is not evident with neutral markers. This divergence persisting among populations from different soil types even in the face of extensive gene flow, confirms the strength of natural selection.

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