**Short title:** Multi-site *PeachRefPop* collection

# The multi-site *PeachRefPop* collection: a true cultural heritage and international scientific tool for fruit trees

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**One sentence summary:** Realization of the *PeachRefPop*, the international multi-site reference collection in peach, provides an invaluable tool for scientific studies in perennial species.

**Author contributions**

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**Abstract**

Plants have evolved a range of adaptive mechanisms that adjust their development and physiology to variable external conditions, particularly in perennial species subjected to long-term interplay with the environment. Exploiting the allelic diversity within available germplasm and leveraging the knowledge of the mechanisms regulating genotype interaction with the environment are crucial to address climatic challenges and assist the breeding of novel cultivars with improved resilience. The development of multisite collections is of utmost importance for the conservation and utilization of genetic materials and will greatly facilitate the dissection of genotype-by-environment interaction. Such resources are still lacking for perennial trees, especially with the intrinsic difficulties of successful propagation, material exchange and living collection maintenance. This work describes the concept, design and realization of the first multi-site peach [*Prunus persica* (L.) Batsch] reference collection (*PeachRefPop: PRP*) located across different European countries and sharing the same experimental design. Other than an invaluable tool for scientific studies in perennial species, the *PRP* provides a milestone in an international collaborative project for the conservation and exploitation of European peach germplasm resources and, ultimately, as a true heritage for future generations.

## Introduction

Since the Roman garden '*hortus'*, fruit tree orchards have represented distinctive features of the Mediterranean rural landscape, a synthesis of the interaction among genotype, environment and human customs (**Biasi et al., 2009**). The diversity of pedo-climatic conditions and production systems, along with plasticity of the genotype and human traditions has shaped the selection of a multitude of local cultivars. These materials represent a cultural and genetic heritage of generations of farmers and a 'common good' to preserve for present and future generations.

Plants have evolved a range of adaptive mechanisms that adjust their development and physiology to variable external conditions, particularly in perennial species subjected to a long-term environmental exposure and interaction. Climate changes are impacting cultivation environments, raising the need for more resilient cultivars able to maintain performances across variable (and often unpredictable) weather conditions (**Varshney et al., 2011; Luedeling, 2012; Ramírez and Kallarackal, 2015**). Also, increasing the sustainability of fruit production (particularly in terms of resource demands and disease management) requires leveraging knowledge of the interactions between plants, soil, and environmental factors and how they affect productivity and end-product quality (**Coakley et al., 1999**; **Singh et al., 2013; Parajuli et al., 2018**).

Peach [(*Prunus persica* L. (Batsch)] originated in China (**Li et al., 2019**), later reaching Persia, the Mediterranean Basin, Europe and the Americas, is now the third most cultivated fruit tree species in temperate regions. Beside its importance as a crop, peach is a recognized model for genetic and genomic studies in fruit trees, representing the ideal system for addressing two main challenges in fruit tree breeding:

1) understanding and harnessing the allelic diversity within available genepools; noteworthy for peach, the intercompatibility with related species of the *Amygdalus* subgenus [almond *(P. dulcis)*, *P. davidiana*, *P. kansuensis,* etc.] has long been considered a source of natural variability, particularly for the introgression of disease resistances (**Gradziel, 2002; Foulongne et al., 2003**). However, interspecific hybrids have had poor applicability in current breeding programs (**Cirilli et al., 2017**), although new genomics-based strategies could change this trend (**Serra et al., 2016**). Conversely, landraces and local ecotypes could be a source of resilience traits more straightforward to introgress, making their preservation and exploitation a suitable strategy for dealing with the changing climatic conditions.
2) systematic dissection of genotype-by-environment (G × E) and/or by-management (G × E × M) interactions as primary sources of variability for several important quantitative traits (**Bassi et al., 2006; Myles, 2013; Chagné et al., 2014**). This is a critical point for genetic analyses of complex traits, such as genome-wide association studies (GWAS) where germplasm collections are characterized to identify quantitative trait loci (QTLs) across different environments, or genome-wide selection (GS), used to predict genomic estimated breeding values.

The comprehension of genetic, epigenetic and physiological mechanisms as well as the estimation of G × E and/or G × E × M effects requires the development of multisite replicated collections and *ad hoc* experimental designs. The availability of such type of resources is rapidly growing in annual species, while it has not yet been implemented in perennial fruit trees.

During the past century, peach orchard systems have changed dramatically following innovations in orchard design, training systems and agronomic management (**Corelli-Grappadelli**

**and Marini, 2008**), other than cultivar evolution. Noteworthy, the first reported 'modern' orchard was a peach plantation established in Massa Lombarda (Ravenna, Italy) at the end of the 19th century using the white fleshed local cultivar-population 'Buco Incavato' (**Bellucci, 1908**). In the last decades, considerable breeding efforts have assisted the intensification of cultivation techniques and the development of horticultural quality concepts with the introduction of novel, fit-for-purpose cultivars (**Byrne et al., 2009**). In Europe, peach has a long cultivation history, tracing back to the Ancient and Middle ages and characterized by the isolation and propagation of chance seedlings operated by farmers and amateurs, through which each country has set its own pool of locally adapted cultivars (**Bassi and Layne, 2009**). The paradigm shift to the modern controlled-crosses approach in early US breeding programs has been the foundation of the dramatic varietal improvement of the last century, beginning with the introduction of seedling materials from China in the mid-19th century (e.g. 'Chinese Cling', progenitor of most modern cultivars) (**Faust and Timon, 1995**; **Byrne et al., 2009**). The worldwide spread of improved US materials, favored also by the limited activities in other countries, has resulted in a rapid replacement of landraces and local accessions, particularly in Europe. From the second half of the 20th century, however, novel programs started in several European countries, although they were mostly based on US breeding stocks with a marginal role for local cultivated germplasm. This led to a consequent loss of many local cultivars, in parallel with a progressive narrowing of the genetic bases in modern cultivars (**Aranzana et al., 2010; Verde et al., 2013**).

As awareness of genetic erosion in modern plant breeding increased (**Fu and Dong, 2015**) the conservation and exploitation of genetic resources has become a fundamental aspect in crop breeding (**Ford-Lloyd and Jackson, 1986**). Considerable efforts have been made in the collection and characterization of many plant germplasms (including fruit tree species), along with the development of approaches for their effective management and utilization (**Gepts, 2006**). The concept of 'core collection', a subset of a germplasm collection of a species that captures most of the genetic diversity while reducing redundancy, has represented an ideal solution for reducing costs and increasing the efficiency of conservation programs (**Frankel and Brown, 1984**). Several allocation methods have been developed for selecting core collections, attempting to maximize allelic richness or allele coverage (MSTRAT, PowerCore, GenoCore), minimize or maximize genetic distance (GDOpt, SimEli) or simultaneously accommodating for multiple criteria (Core Hunter) (**Gouesnard et al., 2001, Kim et al., 2003; Thachuk et al., 2009; Odong et al., 2011; Krishnan et al., 2014**). However, the effectiveness of the sampling strategies varied depending on the objective of the core collection, the statistical approach for its definition and the measures for evaluating its quality (**Odong et al., 2013**). Furthermore, beyond statistical considerations, other aspects are often considered by the institutions hosting the collection, such as historical and socio-economic importance, relevance for breeding activities, popularity among growers and consumers, or distinctive phenotypic characteristics.

In peach, the absence of wild or feral populations makes *ex situ* collections the main valuable reservoirs of allelic variability for many traits not yet exploited in current breeding programs. Remarkable progress has been achieved in the phenotypic and genotypic characterization of peach genetic resources (**Badenes et al., 2015; Cirilli et al., 2018; Yu et al., 2018**), taking advantage of genome sequencing and the development of cutting-edge genotyping tools (**Verde et al., 2012; Verde et al., 2017, Aranzana et al., 2019**). In the framework of the European collaborative project FruitBreedomics (**Laurens et al., 2018**), a coordinated characterization of peach collections has been accomplished across relevant European repositories (**Micheletti et al., 2015; Hernandez-Mora et al., 2017)**, promoting increased utilization of resources and encouraging the sharing of conservation responsibilities. For example, the *Prunus* Working Group within the Fruit Network in the European Cooperative Programme on Plant Genetic Resources (ECPGR) is dealing with *Prunus*, including peach (**Benediková and Giovannini, 2013)**. Nevertheless, long-term maintenance of collections remains particularly challenging due to intrinsic vulnerabilities (e.g. direct exposure to environmental variables and pathogens) and costs for *in vivo* maintenance through vegetative propagation to preserve the original genotypes. Moreover, compliance to phytosanitary requirements hampers the sharing of resources among institutions, each having its own stock of materials, resulting in redundancies or risk of loss for unique accessions.

This article describes the concept, design and realization of the first multi-site peach reference collection (named as *PeachRefPop: PRP*) across five locations in three European countries (Italy, Spain and Greece). Other than an invaluable tool for scientific studies, the *PRP* provides a milestone of an international collaborative project for the conservation and exploitation of European peach germplasms resources and, ultimately, as a true heritage for future generations.

## Results

### 1. Criteria for construction of a reference panel of peach accessions and seedlings

The *PRP* collection was built with the aim of selecting a reduced germplasm pool, reflecting the original genetic and phenotypic diversity (**Figure 1**) and the cultural and socio-economic value of peach cultivation, for its exploitation in future breeding programs. A four-step procedure was followed (exemplified in **Figure 2**):

1. Definition of the *PeachRefPop* domain
2. Establishment of *PeachRefPop* size
3. Identification of the selection criteria
4. Choice and allocation of the entries

#### 1.1 Definition of the *PeachRefPop* domain

To build a resource representing peach diversity and breeding history, the starting point was the genetic material characterized in the framework of the FP7 European project FruitBreedomics (http://fruitbreedomics.com/) in a coordinated effort involving different universities and research institutions across Europe and China. A total of 1,580 *Prunus* accessions (comprising *P. persica* and its hybrids with *P. davidiana* and almond) were phenotyped and genotyped with the ~~IPSC~~9k single nucleotide polymorphism (SNP) array, as previously described (**Micheletti et al., 2015)**. The inclusion of only peach (including *P. ferganensis*, **Verde et al. 2012**) among all the available *Prunus* accessions was the leading concept behind the definition of the *PRP* reference collection. Indeed, as a consequence of many factors (genetic diversity, evolution history, mating system, geographical distribution etc.), sampling strategies for the inclusion of wild relatives (e.g. species of *Amygdalus* subgenus) may substantially differ from those for a cultivated species (e.g. peach) (**Brown and Marshall, 1995**). Moreover, to avoid limitations on the exchange of plant material, the domain was restricted to European repositories. Based on these criteria, the starting panel for building the *PRP* amounted to a total of 1,262 *P. persica* accessions (FB_1262). Besides accessions, seedlings from controlled crosses also represent a valuable source of informative materials for both genetic analysis and breeding (or pre-breeding) activities. For this reason, 1,467 individuals from 18 progenies and their parents (including an interspecific cross with a *P. davidiana* accession), also analyzed during the FruitBreedomics project (**Hernandez Mora et al., 2017**), were considered in the construction process.

#### 1.2 Establishment of the *PeachRefPop* size

The definition of the size is one of the most critical decisions for the establishment of a reference population. For fruit tree crops, the costs of *in vivo* maintenance are particularly onerous, and together with long-term space availability in the field, the main limiting factor of running a germplasm collection. In the perspective of analyzing the interactions between genotype and

**Figure 1**. **Overview of the range of phenotypic diversity in the *PeachRefPop***. Columnar and standard tree growth habit (top left and right panel, respectively). Heart shaped, round and flat fruit (top and third row). Range of fruit flesh, skin color and overcolor (second and bottom rows). Variation in flower morphology and color (third and fourth rows). Fruit size variation (fourth row, first and last panels).

environment and/or management practices, or performing genetic studies such as GWAS and GS, an adequate panel size and experimental design are key factors for the power and reliability of statistical analyses. On the other hand, for agrobiodiversity conservation purposes, the least number of accessions to include in a core set depends on the level of genetic repetitiveness present in the original germplasm pool. The first step towards the establishment of the *PRP* size was the assessment of the allelic richness and redundancy observed at marker loci. Two series of core collections of incremental size were generated, one based on the genetic diversity

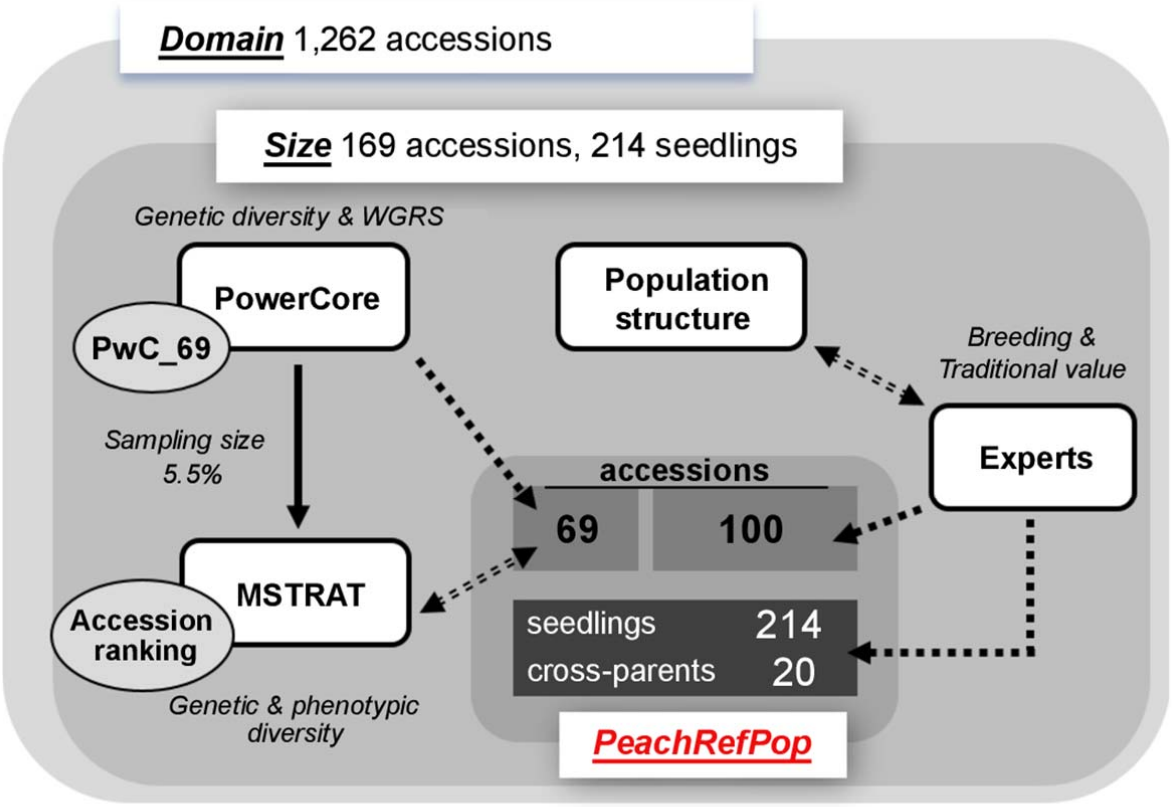


**Figure 2. Graphical summary of the overall scheme followed for selecting the *PeachRefPop* collection**. From the starting panel of 1,262 accessions, 169 accessions were selected combining two sets: 69 accessions extracted from genetic and phenotypic diversity analyses and taking into account availability of whole genome resequencing (WGRS) data; 100 accessions selected by an empirical strategy from an experts panel considering breeding and traditional value along with genetic structure. These were supplemented with 214 seedlings from crossing populations of scientific importance and their respective 20 parents. The total number of entries in the *PeachRefPop* amounts to 403.

(Maximization method, OPT), the other through random sampling (RAN). The maximization procedure (M strategy by **Schoen and Brown, 1993**), is based on the sampling of the total allelic diversity observed at marker loci in the least number of entries. By plotting the genetic diversity measured over the core size, a convex curve was obtained, indicating the presence of redundancy across the European peach germplasm collection. The inflection point, corresponding to a plateau in the increase of diversity, was observed at the level of core 26. At this core size, 99.9% of the total genetic diversity was captured in the core obtained with the M method in comparison to 93.5% with random sampling (**Figure 3**). The outperformance of the optimized versus the random selection was observed across all the core sizes, indicating that the OPT maximization strategy was more efficient and was preferred for conservation purposes in our germplasm.

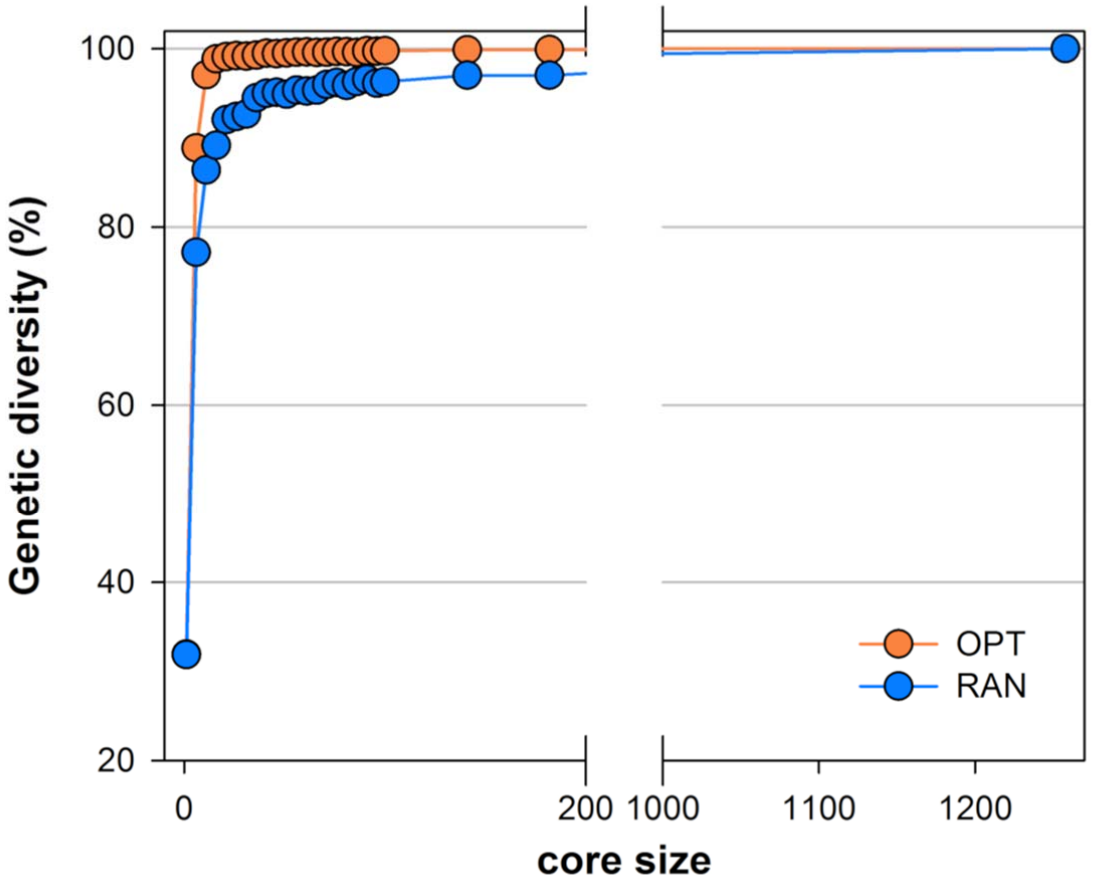


**Figure 3**. **Assessment of allelic redundancy observed at marker loci in the starting panel (FB_1262)**. Core collections of incremental size were generated, based on the Maximization (OPT) and random sampling (RAN) methods in MSTRAT software, using a set of 445 SNPs. Datapoints represent averaged values over 5 independent repetitions for each size.

According to some recent works in peach (reviewed in **Aranzana et al., 2019**), a number of about 100 – 150 unrelated accessions usually provides an adequate resolution for identifying major loci or developing prediction models. In light of all the above premises, an ideal target number of 400 entries was deemed adequate for allocating a minimum of 150 accessions and a maximum of 250 seedlings from progenies (including the parents) based on the outputs of selection criteria.

### 1.3 Identification of the selection criteria

In spite of the genetic redundancy observed and excluding the rare cases of synonymy, the vast majority of the accessions are not overlapped across the various collections, being conserved for a multitude of reasons and purposes, including scientific research, agrobiodiversity preservation or support to breeding activities. To reconcile these reasons with the aim of creating a feasible, usable and multi-purpose reference collection to be shared among European institutions, a mixed

approach was considered for selecting the accessions. A subset of entries was sampled using an analytical strategy, based on the criteria of maximizing genetic (and phenotypic) diversity, also taking into account the availability of whole genome re-sequencing data (WGRS); the remaining entries were selected using an empirical strategy, leveraging the knowledge of an experts panel (e.g. breeders, experienced scientists and curators of each repository) and considering the traditional and historical value at national and/or regional levels, the relevance for breeders, growers and consumers, taking into account agronomical or pomological characteristics. Moreover, to maintain a balanced representation of the genetic structure of the whole collection, the empirical selection of accessions was partially supported by information on population structure [Structure and principal component analysis (PCA) analysis available from **Micheletti et al., 2015**]. Complementing the choice of accessions, seedlings were selected based on the availability of detailed genotypic and/or phenotypic information, genetic background, scientific relevance and, above all, priority traits for breeding.

### 1.4 Choice, evaluation and description of the *PeachRefPop* accessions

Capturing the maximum amount of genetic diversity present in the entire collection while reducing redundancy was the primary driver for sampling the first *PRP* subset (the core set). For this purpose, the advanced M method, implemented in the software PowerCore (**Kim et al., 2007**) through a modified heuristic algorithm, was used to select a core from the initial panel of accessions, based on a set of 3,894 filtered SNPs previously described in **Micheletti et al.,** (**2015**). After superimposing 17 accessions with available whole-genome re-sequencing data, an ideal core of 69 accessions (PwC_69) was extracted, representing a sampling size of 5.5% (**Supplemental Table 1**). Considering the many variables that could affect the actual availability of materials for grafting, a flexible approach was further developed to rank each accession of the whole panel based on genotypic and phenotypic diversity. Four different sets made up of 100 cores of 70 entries each were constructed with MSTRAT by setting different combinations of genotypic (9 subsets of SNPs extracted approximately every 1.8 Mb to avoid linkage between them) and phenotypic data (7 qualitative and 10 quantitative traits, following transformation of the latter into categories) (**Supplemental Table 2**). Accessions were ranked in groups according to the average frequency of inclusion across the four sets (**Supplemental Table 3**). Combining the core population extracted by PowerCore with the MSTRAT ranking list resulted in a shortlist of 69 accessions (41 and 28, respectively, indicated as Core_69), ensuring the inclusion of the maximum possible level of genetic diversity. For the completion of the final *PRP_X* panel, the remaining 100 accessions (Priority_100) were empirically selected by experts, following the above specified criteria.

Estimates of genetic diversity were used to compare the starting panel FB_1262, the core collection obtained by PowerCore (PwC_69) and the final set of *PRP* accessions (*PRP_X*),

composed by joining Priority_100 and Core_69 subsets. In addition, Core Hunter software was used to create additional core sets, either of 69 and 169 entries, based on the optimization of various criteria, including allelic coverage (CV_169) and three distance-based algorithms A-NE (AN_69 and AN_169), E-NE (EN_69 and EN_169) and E-E (EE_69 and EE_169). Concerning parameters accounting for allelic diversity, all sets showed high and similar values for the allelic coverage (CV), while the number of effective alleles ($N_e$) and expected heterozygosity ($H_e$) were slightly lower for the Priority_100 subset (**Table 1**). The Shannon-Weaver diversity index (SH) was comparable among the different subsets, ranging between 0.595 in EE_169 and 0.534 in Priority_100. SH generally displays higher values in the presence of a reduced redundancy (**Peet, 1975**). In contrast, values of observed heterozygosity ($H_o$) tended to be more variable, ranging from a minimum of 0.202 in PwC_69 to a maximum of 0.318 in AN_69. According to **Odong et al. (2013)**, distance-based criteria were used for further evaluations, such as the minimization of A-NE distance, particularly indicated for generalist collections (as the *PRP*), and maximization of either E-E or E-NE, both suitable for core collection representing the extremes of the entire collection. A-NE distance generally tends to decrease along with the increase of core size, being minimized in the AN_169 and AN_69 core sets (0.137 and 0.172, respectively), *a priori* optimized using this selection criterion. Despite the relative low performance of both Priority_100 and Core_69 (0.188 and 0.195, respectively), the *PRP_X* set showed low values for this index (0.165), most probably as a consequence of the increased size. Regarding E-E and E-NE, *PRP_X* (as well as Priority_100) showed lower values, particularly for E-NE distance, indicating the presence of a certain redundancy within the panel.

The population structure of peach germplasm was well represented in the *PRP_X*, in agreement with the presence of clusters of breeding-derived accessions (further separated in peach- and nectarine-type groups), Occidental traditional and admixed entries with prevalent Oriental origins (**Figure 4A**). Structure was also preserved in the other core sets, except for that selected through the E-E distance algorithm, tending to oversample the admixed group (**Supplemental Figure 1)**. PCA was also run to check the distribution of the *PRP_X* with respect to the other sets, and the first two components explained 15.9 and 8.4%, respectively, of the total variance detected. In the scatter plot, 95% confidence ellipses show almost overlapping areas (except for EE_169), confirming that the *PRP_X* panel was well distributed to represent the structure of the starting germplasm (**Figure 4B**). Finally, a Neighbor-joining (NJ) tree, based on the dissimilarity matrix between the whole FB_1262 panel, was also built to assess the distribution of *PRP* accessions (**Figure 4C**).

A number of accessions of historical and regional importance, mostly belonging to the Occidental traditional cluster, were included. For example, French cultivars dating from late Middle Age (‘Grosse Mignonne’, ‘Millecoton de Septembre’, ‘Reine des Verges’, ‘Brugnon Violet’) (**Okie et al., 2008**), traditional non-melting Spanish cultivars (‘Amarillo de Agosto 1’, ‘Calante’, ‘Campiel’,

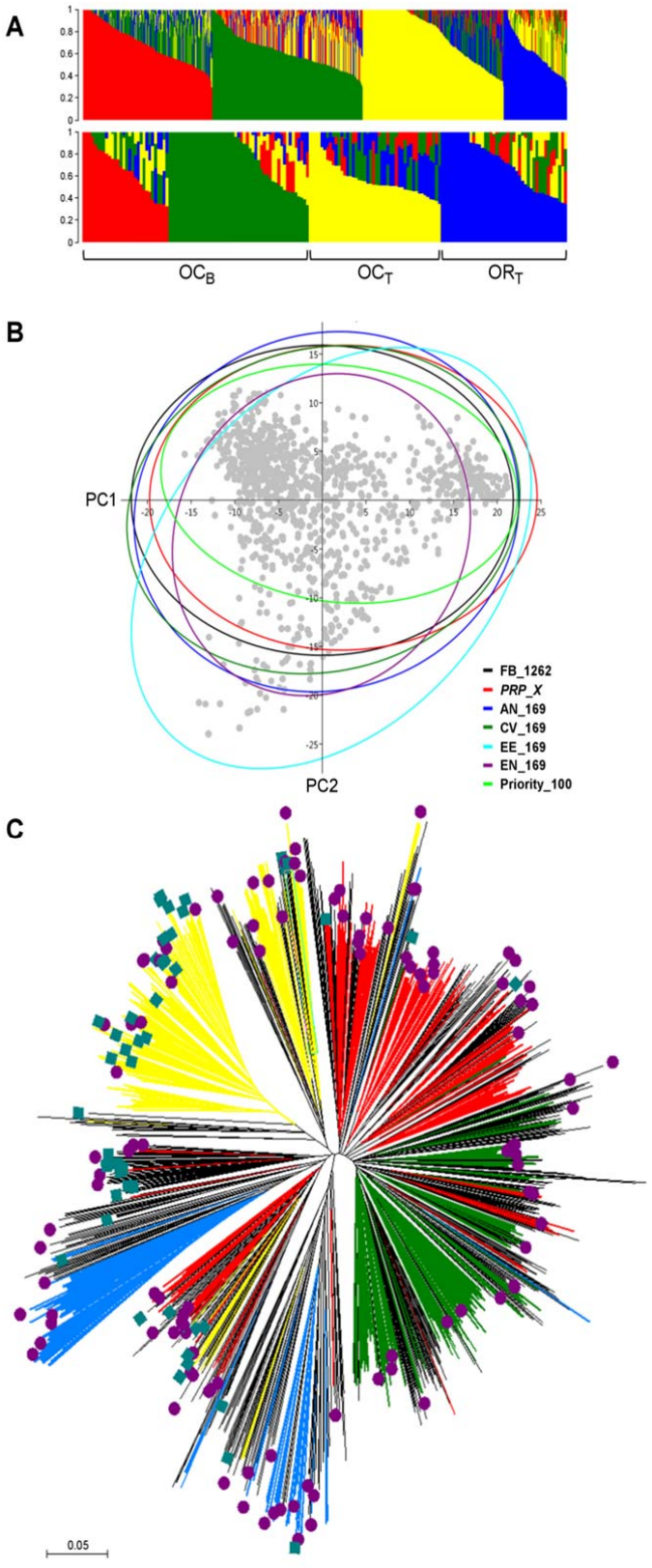


**Figure 4. Genetic structure and phylogenetic analysis of *PeachRefPop* accessions.** A) Population structure estimated in the whole panel (FB_1262) and *PeachRefPop* accessions (*PRP_X*), as estimated for K (number of a priori cluster) equal to 4; B) PCA analysis of the subsets with a core size of 169 entries. Scores for each accession were obtained from the work of Micheletti *et al.*, 2015. The 95% confidence ellipses in the scatter plot were estimated using PAST software. C) NJ phylogenetic tree. Blue squares indicate accessions with traditional and historical value, violet circles indicate the other *PeachRefPop* accessions, and colors reflect the population structure.

248 'Jesca', 'Groc Abel', 'Groc Alto') (**Badenes et al., 1998**; **Wünsch et al., 2006**) and the Italian

249 'Crasiommolo Rosso' (a white fleshed nectarine belonging to the 'Sbergie' type) and 'Poppa di
250 Venere', first reported at the end of eighteenth century (**Majoli, 1790 - 1810**). The richness of the
251 Italian peach germplasm was also widely represented by materials from several regions, including
252 Sicily ('Imera', 'Tardiva di Ficarazzi', 'Settembrina di Bivona', 'Gialla di Moavero') (**Marchese et al**
253 **2005**), Campania ('Zingara Nera'), Apulia ('Percoco di Turi'), Liguria ('Michelini'), Emilia-Romagna
254 ('Buco Incavato', 'San Varano 2' and 'San Varano 3', 'Rosa del West', this last used for the
255 preparation of the famous cocktail 'Bellini') and Tuscany ('Regina di Londa') (**Gallesio, 2003;**
256 **Monte et al., 2006; Liverani and Giovannini, 2016**). Early breeding materials, mainly from US
257 programs and founders of most of the currently cultivated materials are also included, along with
258 worldwide commercial cultivars (**Supplemental Table 4**).
259 Finally, *PRP* accessions encompassed a wide range of phenotypic variability for traits
260 related to fruit quality, resistance or tolerance against major diseases (brown rot, powdery mildew,
261 leaf curl, aphids and Sharka disease), tree growth habit and phenology (**Figure 5 and**
262 **Supplemental Table 4**).
263
264 **1.5 Choice and description of the *PeachRefPop* progenies**
265 Seedlings from 15 cross populations from the research and breeding activities of some European
266 universities and institutions were also added. Most of these materials were already described in
267 depth (**Hernandez-Mora et al., 2017**). The leading criterion for the choice of seedlings was the
268 effective segregation of priority traits in peach, mainly related to phenology (fruit developmental
269 period, maturity date), fruit quality (fresh weight, soluble solid content, titratable acidity, texture and
270 aroma) and disease resistance (brown rot, powdery mildew, green peach aphids and Plum Pox
271 Virus (PPV)) (**Table 2**). A range of breeding materials was considered, such as F1, F2, BC1
272 populations as well as hybrids with *P. davidiana*, particularly interesting as a source of PPV
273 resistance (**Decroocq et al., 2005**).
274
275 **2. Experimental design and orchard sites description**
276 The *PRP* was established in 5 institutions from 3 countries (Greece, Italy and Spain) (**Figure 6A**):
277 I. Institute of Agrifood Research and Technology (IRTA) in Gimenells, Catalonia region, Spain
278 (ES)
279 II. Murcia Institute of Agri-Food Research and Development (IMIDA) in Mula, Murcia region,
280 Spain (ES)
281 III. Centro di Ricerca per le Produzioni Vegetali (CRPV) in Imola, Emilia-Romagna region, Italy
282 (IT)
283 IV. Institute of Plant Breeding and Genetic Resources (IPB&GR) in Naoussa, Imathia region,
284 Greece (GR)
285 V. Research Centre for Olive, Fruit, and Citrus Crops (CREA) in Rome, Italy (IT).

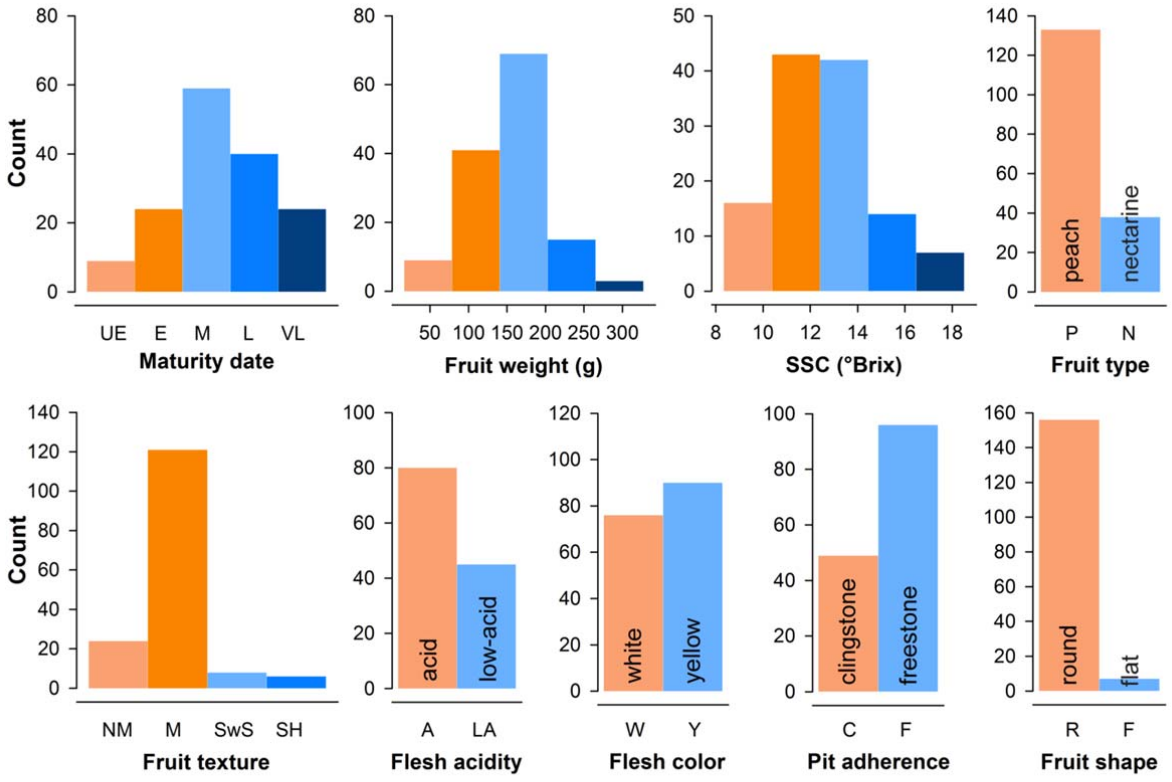


**Figure 5**. **Distribution of main phenotypic traits in the *PeachRefPop* accessions**. In the maturity date plot, UE, E, M, L and VL indicates ultra-early, early, medium, late and very late ripening accessions, respectively; SSC, soluble solid content; in the fruit texture plot, four major texture groups are shown: non-melting (NM), melting (M), slow-softening (SwS) and stony hard (SH).

For each accession and seedling, a single mother plant was propagated through grafting on a common 'GF677' rootstock by the same nursery. Plants were grafted in the same year (2015) to obtain trees of the same age. To ensure an adequate compromise between the number of replicate trees and sustainable costs of maintenance, an augmented design with replicated control checks was adopted in all sites except Rome, hosting a partial copy of the *PRP* (accessions only, without randomization).

Accessions and seedlings were arranged in two blocks (M1 and M2) according to the following design (**Figure 6B**):

- the **M1 block**, composed of two sub-blocks (M1.1 and M1.2) each including the entire *PRP* collection of 169 accessions plus 20 cross parents (A group) and 214 seedlings (S group) for a total of 403 genotypes in each sub-block. Taking into account the physical layout of the design for each location/field combination (i.e., the number of rows and the number of positions per row) each accession and seedling was randomly assigned to a position within a sub-block (as illustrated for Gimenells location in **Supplemental Table 5**). To assess and correct for spatial variation within and between experimental sites, the three accessions 'Big Top', 'Springcrest', and 'Nectaross' from the A group were included with a higher replication and randomly distributed over M1.1 and M1.2 sub-blocks (at least 5 additional trees of each genotype for each sub-block).

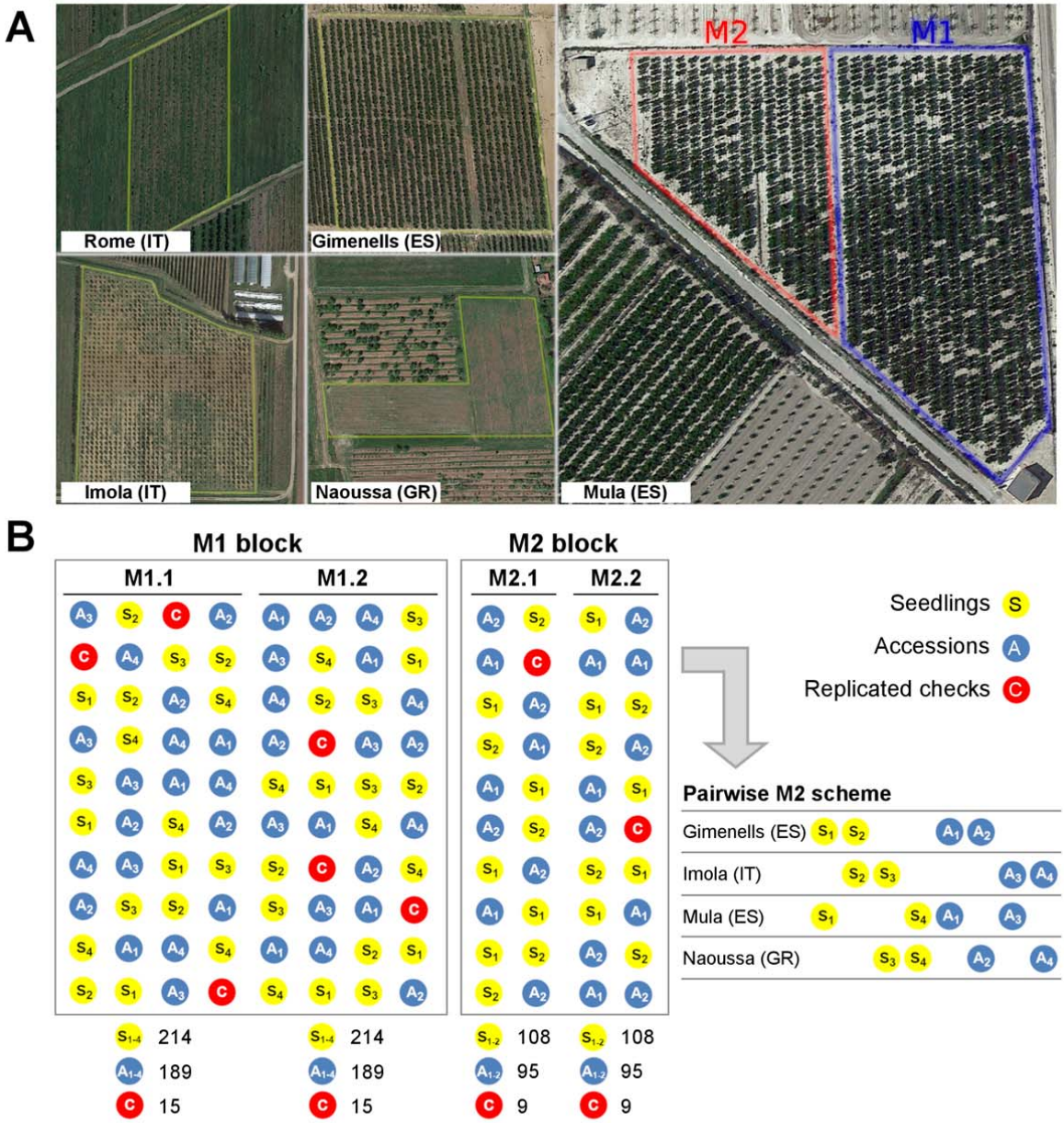


**Figure 6**. **Experimental design and *PeachRefPop* orchards layout**. A) Google maps satellite images of the established *PeachRefPop* orchards across the different European sites; B) Experimental design of multisite *PeachRefPop*. A schematic example is provided for Gimenells location. Accessions (A) and seedlings (S) in each block and sub-block were completely randomized. The M1.1 and M1.2 sub-blocks each include a full copy of the collection (189 accessions, 214 seedlings) plus replicate checks (C) of the accessions 'Big Top', 'Nectaross' and 'Springcrest' (5 additional trees for each sub-block). The M2.1 and M2.2 sub-blocks include half of the *PeachRefPop* collection and each site has a different half, chosen according to a pairwise design scheme. To this end, accessions (excluding control checks), and seedlings were randomly assigned to eight disjoint subgroups (A1 to A4 and S1 to S4) of approximately equal size and 4 of them assigned so that each site shares at least one A or one S group with the other sites. In the example, each M2 sub-block at Gimenells is composed of A1 and A2 (46 and 46 accessions, respectively, plus the 3 checks for a total of 95), S1 and S2 (54 and 54 seedlings, respectively, for a total of 108) other than 3 additional replicates for each of the 3 checks (9 trees). Gimenells shares the A1 and S1 groups with Mula, S2 with Imola and A2 with Naoussa.

- the **M2 block**, composed of two sub-blocks (M2.1 and M2.2) each including half of the *PRP* collection (85 accessions plus 10 cross-parents and 112 seedlings). In each site, the M2.1 and M2.2 sub-blocks include the same set of entries (i.e. the same half of the collection), randomly assigned in each sub-block, plus the replicated control checks previous described (at least 3 additional trees of each entry for each sub-block). The composition of the M2 block is not the same

across sites (i.e. each site has a different half of the collection), chosen according to a pairwise design scheme (**Figure 6B**): firstly, excluding control checks, accessions and seedlings of the *PRP* collection were randomly divided into eight disjoint subgroups - A1 to A4 (of 48, 47, 47 and 48 accessions, respectively) and S1 to S4 (of 54, 53, 54 and 53 seedlings, respectively) - then 4 subgroups were assigned so that each site shares at least one A or one S with the other sites. For example, Imola location shares A4 and S3 with Naoussa, A3 with Mula and S2 with Gimenells. This partial replication design is such that, within the full design, all subgroups were well connected.

The geographic location of each site as well as the basic climate and soil parameters are shown in **Table 3**. The sites covered a range of latitude from about 38˚ N in Mula (south-eastern Spain) to 44˚ N in Imola (northern Italy), while altitude spanned from near sea level in Imola and Rome (53 and 73 m, respectively) to 278 m at the Mula site. Although all sites are included in the Mediterranean zone, climates widely range from semi-arid in Mula (warm winter and hot summer) to sub-continental in Imola and Naoussa (with moderately cold winter). Average monthly temperatures (1999 - 2018 time series) varied from the colder regimes of Naoussa (1.7 ± 3.9˚C and 23.5 ± 2.9˚C in the coldest and hottest month, January and August, respectively) to the warmer conditions of Mula (7.9 ± 3.7˚C and 27.1 ± 2.6˚C, respectively) (**Figure 7A**). The fulfillment of chilling requirement (i.e. the period of cold temperatures needed for overcoming endo-dormancy) is a parameter of utmost relevance for peach reproductive phenology. According to the Chilling Hours (CH) model (**Weinberger, 1950**), assigning one hour for each hourly temperature between 0 and 7.2° C threshold, accumulation patterns widely ranged from 1,762 ± 124 CH at Naoussa to 693 ± 159 at Mula. Also, precipitation differently affected the selected sites, with Imola having the wettest conditions (964 ± 218 mm per year) and both Spanish locations having the driest (Gimenells 361 ± 95 and Mula 336 ± 75) (**Figure 7B**).

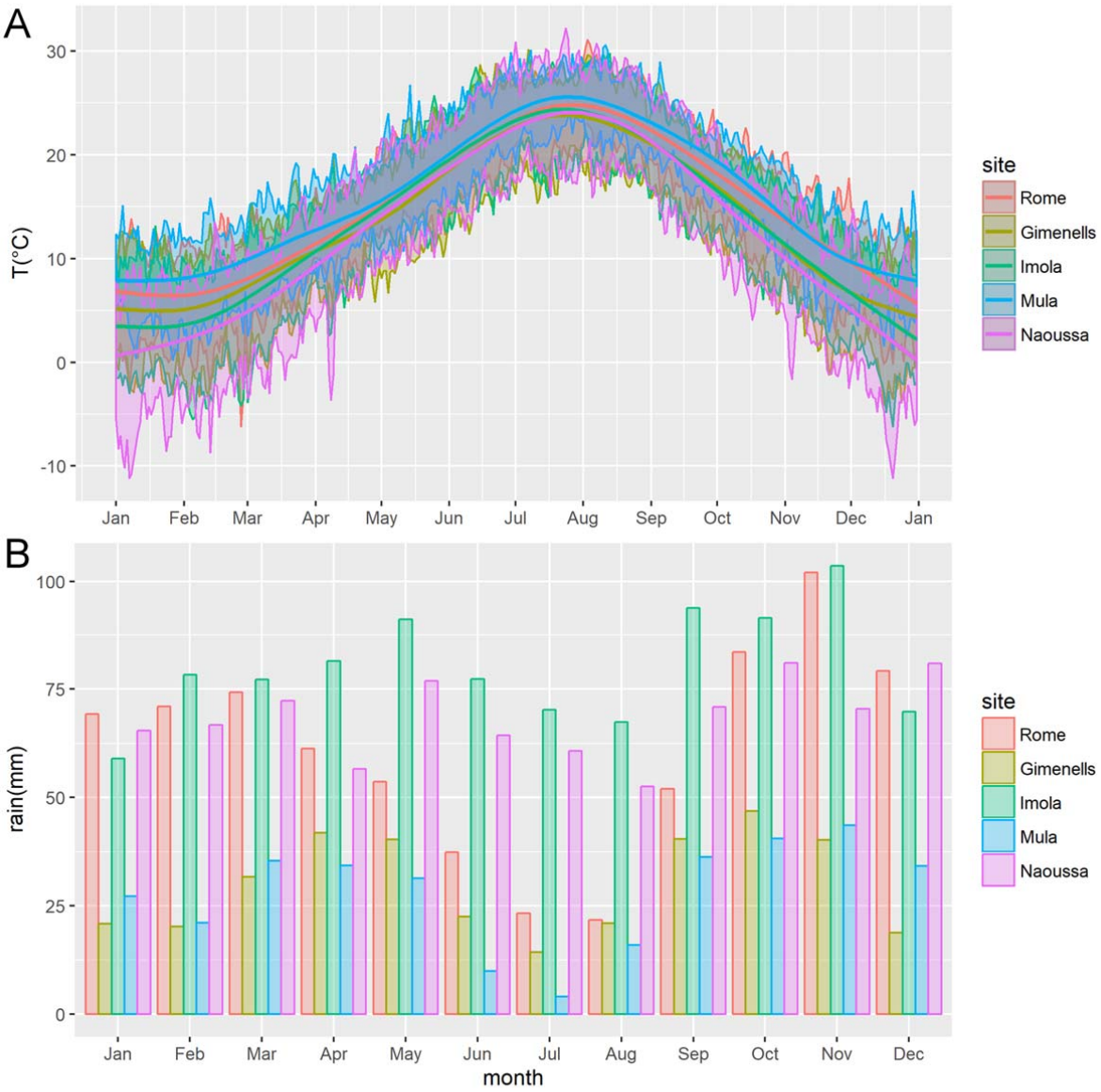


**Figure 7**. **Climatic profiles of *PeachRefPop* sites.** A) Trend of minimum and maximum daily air temperatures at the five locations (averaged from 1999 – 2018 time series). Thick lines show smoothed mean temperatures. B) Average monthly precipitations (in mm).

## Discussion

The concept of the *PRP* arises from the growing awareness about current and common issues on *ex situ* peach conservation across European institutions. Fluctuations in funding availability and intrinsic constraints of living orchard collections threaten the long-term preservation of diversity resources, causing a progressive loss of valuable materials. Reference or core collections have been designed for several fruit tree species, for example olive (**Khadari et al., 2003; El Bakkali et al., 2012; Belaj et al., 2012**), grape (**Laucou, et al., 2011**), cherry (**Campoy et al., 2016**), apple (**Gross et al., 2013; Lassois et al., 2016**) and apricot (**Krichen et al., 2012**). Nevertheless, they have mainly been created for improving resource allocation in the context of a single institution or repository. The development of a trans-national and shared strategy provides

the most promising opportunity in the conservation approach. Actual establishment of the *PRP* has required huge coordination efforts and faced the effective availability of materials, the difficulties of their exchange and the success of clonal propagations (particularly for old, often unique, accessions). The sampling strategy for the *PRP* has been defined to accommodate multiple purposes while maintaining the maximum possible diversity compared to the starting panel. The final panel of accessions was assembled by the combination of two different subsets: the first (Core_69), ensuring the preservation of the total allele number with the minimum number of accessions, was extracted by widely adopted maximization strategies, either using a class coverage criterion (in PowerCore) or ~~Shannon-Weaver index (~~SH index (in MSTRAT), with the latter penalizing redundancy. The second subset, accommodating for other scopes (Priority_100), was chosen by experts with a robust knowledge on the genetic structure in peach, providing a reliable criterion for assisting selection. As a whole, genetic analysis supports that *PRP* composition is highly representative of the diversity of peach germplasms present in European collections, as it retains all the allelic variability present within the starting panel, specifically targets defined genetic clusters according to the genetic structure and includes most relevant phenotypic traits. Indeed, differences among the various sampling strategies were negligible for allelic coverage (CV), expected heterozygosity and SH index, revealing a buffer effect towards optimization criteria. Such effect could be expected, since peach has experienced a severe domestication bottleneck with a reduction of genetic diversity, followed by a strong artificial selection during domestication and modern improvement (**Verde et al., 2013; Yu et al., 2018; Li et al., 2019**). This is also reflected in the narrow genetic bases of peach germplasm available across main European repositories. Thus, the high level of allelic redundancy allows selecting many different subpopulations able to retain the same amount of genetic variation. In spite of this, a preliminary validation using distance-based criterion not used in the selection stage showed a minimized A-NE index, the most indicative for evaluating the quality of multipurpose collections **(Odong et al., 2013**). Conversely, E-E and, particularly, E-NE indices resulted less optimized, due to a certain redundancy on the Priority_100 subset (i.e. a higher number of genotypes providing unique alleles). This was mainly due to the inclusion of accessions of traditional and breeding values, respectively belonging to the Occidental Traditional and Occidental breeding clusters, characterized by a very narrow genetic background. Clearly, the inclusion of these materials is crucial in the overall perspective of balancing diversity and usefulness, as they integrated various fundamental qualities, such as popularity, prestige, tradition and breeding. A similar mixed strategy was also recently optimized for creating a core collection for Swiss pear germplasm (**Urrestarazu et al., 2019**).

Climate challenges in peach growing areas increase the need for resilient cultivars able to maintain productivity while showing an enhanced capacity for adaptation to sub-optimal conditions. Nevertheless, resilience and adaptive traits often have a complex inheritance and a strong

interaction with the environment or cultivation practice (Kissoudis et al., 2016). The partitioning of phenotypic variation into genotypic, environmental and their interaction components involves *ad hoc* experimental designs and integration of field data on a common set of genetic materials under a range of different environmental/management conditions. Multi-environment trials (METs) have been extensively used to study GxExM interactions, carry out GWAS and develop GS models for complex traits in annual crops (**Malosetti et al., 2013; Gutierrez et al., 2015; Zhu et al., 2018; Bustos-Korts et al., 2019**) or study GxE interactions in forest trees (Li et al., 2017). In contrast, such experimental designs are lagging in fruit trees, largely because of the need for large and diverse germplasm sets for quantitative genetics analyses and the above-mentioned difficulties in material propagation and exchange. The *PRP* aims to fill this gap, as the replicated design and the different pedo-climatic conditions across sites are particularly indicated for the dissection of interactions between genotype and environment and/or management practice. The *PRP* locations cover major climatic zones of the Mediterranean area, from semi-arid conditions of southern Spain to sub-continental of northern Italy and Greece determining a broad range of temperatures and precipitation patterns. In particular, sites are characterized by a different rate and amount of chilling and heat accumulation, which will be particularly useful for the dissection of traits associated to reproductive phenology, such as blooming and fruit ripening time. The proximity of experimental sites to major production areas provide an added value for the translation of scientific outcomes. The inclusion of both accessions and seedlings from various crosses allows development and testing of statistical approaches for genomics-assisted breeding, such as joint linkage-association analysis (**Yu et al., 2008; Lu et al., 2010**) and genome-wide selection (**Resende et al., 2012; van Nocker and Gardiner, 2014**), or systematic QTL validation (**Peace et al., 2014**). Also, the integration of omics (including epi-genomics) data may improve our understanding of physiological changes in response to environmental stimuli and constraints.

The *PRP* multi-site experimental design was established with a complete randomization of genotypes (accessions and seedlings) within each sub-block and replicate checks to account for spatial variability. The rationale behind the choice of such design mainly derived from the possibility of a direct comparison with standard reference varieties. A drawback of this approach is the relatively few degrees of freedom for experimental errors, lowering the power to detect differences among genotypes. The use of alpha designs (**Patterson and Williams, 1976**) and derived row-column designs (**John and Eccleston, 1986**) might be statistically more powerful, especially to estimate contrasts between genotypes and improve estimation of spatial variation, e.g. due to different soil composition within the orchard. The identification of optimal designs for a large number of genotypes is still challenging (**Cullis et al., 1998**). The *PRP* will allow validation of the performance of this experimental design on fruit trees and a foundation for future planning of multisite collections.

The *PRP* has been grafted on a single 'GF677' rootstock, a *P. amygdalus* × *P. persica* hybrid. 'GF677' is the most widespread, mainly for its growth vigor, excellent affinity, adaptation to limestone soils and tolerance to drought and replanting (**Reighard and Loreti, 2008**). While the choice of a single rootstock is justified by the need of simplifying the experimental design, this precludes assessment of scion-by-rootstock interaction. A number of *Prunus* rootstocks are currently available for peach, some of them harboring interesting traits for resistance to soil pathogens or abiotic stress conditions. Their integration into feasible experimental designs will be the next challenge.

In perspective, the *PRP* should fulfill several purposes, from research to education and traineeship of young breeders. A better understanding of diversity is expected to encourage the use of broad-ranging germplasm (maybe also in other existing *ex situ* collections) in breeding programs. In the last decades, the mission of many agriculture-oriented institutions has shifted from the traditional focus of establishing horticultural collections to a wider target of preserving germplasm resources and agricultural heritage (**Hammer et al., 2003; Havens et al., 2006**). This objective is of utmost importance for fruit tree species of ancient cultivation history, such as peach. For these reasons, a number of traditional and local cultivars (either old or relatively modern) has been included in the *PRP*, as a safeguard of an integral part of the rural landscape and collective memory. Since information and descriptions about local germplasms are scarce and often restricted to cultivation areas, their choice has been directly handled by curators of each repository, with the aid of experienced breeders.

## Materials and Methods

### Datasets

A set of 1,262 accessions was selected as representative of the peach [*Prunus persica* (L.) Batsch] germplasm maintained in collections of four different European countries (Supplemental Table S3). The complete list of institutions providing plant materials, SNP genotyping and phenotypic data for seven monogenic traits have been previously described (**Micheletti et al., 2015**). SNP genotyping data were obtained from the Genome Database for Rosaceae (https://www.rosaceae.org/publication_datasets accession number tfGDR1013). Phenotypic data reported in Supplemental Table 4 were obtained from Micheletti et al. (2015) and the FruitBreedomics database (http://bioinformatics.tecnoparco.org/fruitbreedomics/).

### Construction of core subsets

The advanced M (maximization) strategy implemented in PowerCore v. 1.0 (**Kim et al., 2007**) using 3,894 SNP markers was carried out to extract a core subset able to capture all the alleles observed in the entire collection. The size of the final core collection depends on the level of variability and redundancy present in the whole panel and cannot be set *a priori*. Seventeen kernel accessions with available whole-genome re-sequencing data were superimposed through the

454 'preferential selection' tool, which retains the accessions defined by the user without validation.
455 The standard M strategy implemented in MSTRAT (**Gouesnard et al., 2001**) was also applied.
456 MSTRAT algorithm selects a subset of *n* accessions from the *N* accessions of the entire collection
457 by maximizing the number of alleles (and/or trait classes) at each locus. The sampling size
458 estimated with PowerCore was set as default parameter and four sets of 100 core collections were
459 constructed by using different combinations of genotypic and phenotypic data. Due to the restraints
460 in the number of variables MSTRAT is able to manage, different subsets of approximately 100
461 SNPs each were obtained through an *ad hoc* developed Perl script program, by extracting 1 SNP
462 every 1,800 Kbp, corresponding to the max boundary for linkage disequilibrium (LD) found in some
463 subpopulations of the original plant material (**Micheletti et al 2015**). Seven qualitative and 10
464 quantitative traits (these last transformed into qualitative categories) were used as phenotypic data.
465 For each run, the core size was set to 70 and 100 independent replicates with 100 iterations were
466 generated. The Shannon-Weaver diversity index was used as a second criterion to classify core
467 subsets. Redundancy was assayed through the 'Redundancy' tool implemented in MSTRAT, which
468 samples two different sets of core collections of increasing size, as defined by the user, through
469 the application of the maximization strategy or random sampling. For this analysis a subset of 445
470 SNP markers was pruned from the whole set of 4271 using Plink v1.07 with a window size of 50, a
471 shift of 7 and a variance inflation factor (VIF) of 2. Redundancy was assayed in the whole panel of
472 accessions with a step of 5 in the first 100, 5 repetitions and 50 iterations. The Mixed Replica
473 search algorithm implemented in the Core Hunter II software (**De Beukelaer et al., 2012**) was
474 used to generate a core collection of fixed size (either of 69 and 169 entries) based on the
475 optimization of the Modified Rogers' (MR) distance measure (**Wright, 1978**), with a weight of 1.0.
476 For the evaluation of the quality of the different core subsets, genetic distance-based criteria were
477 considered: the average genetic distance between all the entries of each core collection (E-E); the
478 average distance between each entry and the nearest neighboring entry for each core collection
479 (N-E); the average distance between each genotype of the entire collection and the nearest entry
480 in each core collection (A-NE). The quality of each collection increased for lower value of A-NE
481 (the maximum representation is obtained for AN = 0, when each accession is represented by itself
482 or by an identical duplicate), and higher value both for E-NE (maximizes the average distance
483 between each selected individual and the closest other selected item in the core) and E-E
484 (maximizes the average distance between each pair of selected individuals in the core.).

485 **Genetic diversity and population analyses**

486 Genetic diversity measures were performed using GenAlex 6.41 (**Peakall et al., 2006**) and include:
487 number of effective alleles ($N_e$, the number of equally frequent alleles required to give the observed
488 level of heterozygosity), levels of observed ($H_O$) and expected ($H_E$) heterozygosity, and the
489 Shannon-Weaver index (SH). Allelic coverage was calculated by the function CV implemented in
490 Core Hunter II software. Population structure was inferred using a model-based clustering

algorithm ADMIXTURE v1.22 (**Alexander et al., 2009**). From SNP data, the software identifies K *a priori* genetic clusters provided by the user, and for each individual it estimates the probability of membership to each cluster. A preliminary analysis was performed by inputting successive values of K from 2 to 6. The value of K that maximized the predictive accuracy was chosen based on a 10-fold cross-validation procedure with 10 different fixed initial seeds (**Supplemental Figure 2**). Data of Principal Component Analysis (PCA) were retrieved from a previous work (**Micheletti et al., 2015**). The 95% confidence ellipses in the scatter plot were estimated using PAST software (**Hammer et al., 2001**). Phylogenetic tree was built from a pairwise genetic distance matrix between individuals clustered with NJ method in TASSEL (**Bradbury et al., 2007**). Bootstrap replicate and tree reconstruction were performed in MEGA7 software (**Kumar et al., 2016**).

**Experimental design and pedo-climatic analyses**

In the experimental design, randomization was performed with the Genstat software (https://genstat.kb.vsni.co.uk/knowledge-base/hcitegen/). Meteorological time-series from 1999 to 2018 were obtained from ECMWF (European Centre for Medium-Range Weather Forecasts), except for Mula (Murcia, Spain), for which data were available from a nearby weather station. Hourly temperature series were obtained by linear interpolation of available tri-hourly data and expressed in degrees Celsius. Cumulative precipitations were averaged and expressed in mm per month or year. Chill accumulation was calculated according to the Chilling Hours Model (**Weinberger, 1950**) as the sum of hourly temperatures between 0 and 7.2 °C during the dormant season (15 November - 31 March). Soil texture was expressed according to USDA (United States Department of Agriculture) classification. Mineral composition, pH and organic matter content were determined according to standard procedures for soil analysis.

**Supplemental Data**

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**Supplemental Figure S1**. Population structure estimated in the core sets AN_169, EE_169, EN_169 and CV_169.

**Supplemental Figure S2**. Predictive accuracy (cross-validation error) of population stratification in both *PRP_X* and FB_1262 as determined by Admixture software.

**Supplemental Table S1**. PowerCore output.

**Supplemental Table S2**. MSTRAT outputs for the four settings.

**Supplemental Table S3**. Accession ranking by MSTRAT frequencies.

**Supplemental Table S4**. *PeachRefPop* accessions description.

**Supplemental Table S5**. Layout of sub-block M1.1 as illustrated for Gimenells location

**Acknowledgments**

We wish to thank Claudio Buscaroli and Martina Lama for field assistance, Remo Chiozzotto for lab assistance and Michela Troggio (Fondazione Edmund Mach) for genotypic analyses. We are grateful to Fosco Vesely for extracting climate series. This work has been partially supported by the PRIMA-FREECLIMB international project (DB, IE, PD, BQT), the European Union-funded project "FruitBreedomics: Integrated approach for increasing breeding efficiency in fruit tree crops" (Grant #FP7-265582) (DB, LR, PA, IV, TB, BQT, WG), and in the framework of MAS.PES, an Italian project aimed at apricot and peach breeding (DB, SF). We thank the INRAE's 'Prunus Biological Resources Center' for preserving and managing the peach collections and the Fruit Tree Experimental Unit (UEA) of INRAE-Nouvelle Aquitaine-Bordeaux for growing the trees and grants from the Spanish Ministry of Economy and Competitiveness (MINECO/FEDER projects AGL2012-40228-C02-01 and RTA2015-00050-00-00) and the CERCA Programme-Generalitat of Catalonia (PA). We also thank the Italian National Centre of Fruit Germplasm (Centro Nazionale per il Germoplasma Frutticolo, CNGF) at CREA – Rome for preserving and maintaining peach collections, and the Italian Ministry of Agriculture (MIPAAF) for its financial support through the RGV-FAO program (IV).

## Dedication

This work is dedicated to the memory of our colleague Chiara Ferrandi, who recently passed away.

## Tables

**Table 1**. Genetic analysis and parameters for the different core subsets. $N_e$: number of effective alleles); SH: Shannon-Weaver diversity index; $H_o$: observed heterozygosity; $H_e$: expected heterozygosity; CV: percentage allelic coverage; MR distance: average Modified Rogers genetic distance; E-E: average entry-to-entry distance; A-NE: average distance between each genotype of the collection and the nearest entry, E-NE: average distance between each entry and the nearest entry.

| Set name | $N_e$ | SH | $H_o$ | $H_e$ | CV | MR distance | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | EE | A-NE | E-NE |
| FB_1262 | 1.621 | 0.547 | 0.292 | 0.367 | 0.995 | 0.285 | - | 0.131 |
| PwC_69 | 1.675 | 0.574 | 0.202 | 0.39 | 0.987 | 0.318 | 0.203 | 0.237 |
| EE_69 | 1.705 | 0.587 | 0.234 | 0.401 | 0.992 | 0.347 | 0.209 | 0.229 |
| AN_69 | 1.645 | 0.560 | 0.318 | 0.378 | 0.977 | 0.286 | 0.172 | 0.210 |
| EN_69 | 1.704 | 0.587 | 0.269 | 0.402 | 0.991 | 0.334 | 0.207 | 0.275 |
| CV_169 | 1.638 | 0.556 | 0.285 | 0.375 | 0.995 | 0.302 | 0.163 | 0.212 |
| EE_169 | 1.721 | 0.595 | 0.224 | 0.408 | 0.994 | 0.330 | 0.183 | 0.191 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| AN_169 | 1.643 | 0.559 | 0.300 | 0.377 | 0.987 | 0.290 | 0.137 | 0.203 |
| EN_169 | 1.683 | 0.578 | 0.277 | 0.394 | 0.993 | 0.315 | 0.175 | 0.256 |
| Core_69 | 1.713 | 0.593 | 0.247 | 0.406 | 0.988 | 0.303 | 0.195 | 0.212 |
| Priority_100 | 1.597 | 0.534 | 0.283 | 0.356 | 0.979 | 0.277 | 0.188 | 0.179 |
| ***PRP_X*** | **1.647** | **0.563** | **0.270** | **0.379** | **0.988** | **0.290** | **0.165** | **0.180** |

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**Table 2**. Description of the progenies used for establishing the *PeachRefPop* collection. Trait abbreviations: FD, flowering date; MD, maturity date; SSC, soluble solid content; FW, fruit weight; BR, brown rot; TA, titratable acidity; SwS, slow-softening texture; PM, powdery mildew; PPV, Plum Pox Virus; GPA, green peach aphid; SH, stony hard texture.

| Cross (parents) | Acronym | Institution | Type of Progeny | Seedlings # | Trait(s) |
|---|---|---|---|---|---|
| **'Bolero' × 'Oro A'** | B × O | UMIL – Milan | F1 | 9 | MD, SSC, FW, skin overcolor, aroma |
| **'Contender' × 'Elegant Lady'** | C × EL | UMIL – Milan | F1 | 14 | BR, MD |
| **'Max 10' × 'Rebus 028'** | M × R | UMIL – Milan | F1 | 9 | MD, TA, SSC, FW, SwS |
| **'Sweetfire' x 'Garcica'** | Sf × G | UMIL – Milan | F1 | 15 | MD, TA, SSC, FW, SwS |
| **'Belbinette' × 'Nectalady'** | Bb × Nl | IRTA – Lleida | F1 | 20 | FD, MD, TA, SSC, FW |
| **'Big Top' × 'Nectaross'** | Bt × Nr | IRTA – Lleida | F1 | 19 | FD, MD, TA, SSC, FW |
| **'Big Top' × 'Armking'** | Bt × Ak | IRTA – Lleida | F1 | 18 | FD, MD, TA, SSC, FW |
| **'Subirana' x 'Feraude'** | PN643 | IRTA – Lleida | F1 | 7 | Fruit shape |
| **'Summergrand' x '*P. davidiana* P1908'** | SD | INRA – Avignon | F1 | 6 | PM, PPV |
| **'Zephyr' × [(('Summergrand' (S) × '*P. davidiana* P1908')) x S]** | BC2 | INRA – Avignon | BC2 | 13 | FD, PM, PPV, TA, SSC, FW |
| **'Pamirskij 5' × 'Rubira'** | P × R | INRA – Avignon | F2 | 13 | PM, GPA, foliage colour |
| **FRF 1495 x FRF 1148 (Ma 16-03-059)** | POP1376 | CREA – Forli | F1 | 17 | PM; fruit pubescence |
| **IFF 983 x Ma 25-01-042** | POP1115 | CREA – Forli | F1 | 17 | TA, SwS and aroma |
| **FRF 1695 x FRF 1681** | POP1095 | CREA – Forli | F1 | 19 | SH |
| **FRF 813 × FRF 691** | POP1039 | CREA – Forli | F1 | 18 | skin overcolor |

562 **Table 3**. Basic pedo-climatic features of the five *PeachRefPop* locations. Features include geographic coordinates, altitude, average annual
563 minimum and maximum temperatures and cumulative annual precipitations (data series 1999 – 2018). Chilling accumulation was calculated
564 according to the Chilling Hours (CH) model as the sum of hourly temperatures between 0 and 7.2 °C during the dormant season; S.O.M, soil
565 organic matter content.

| Site | Geographical coordinates | Altitude (m) | Climate | | | Soil | | |
|---|---|---|---|---|---|---|---|---|
| | | | Avg. Annual Temperature (min - max, °C) | Cumulative Precipitation (mm) | Chilling accumulation (CH) | Texture | pH | S.O.M. (%) |
| CREA - Rome (IT) | 41°47’ N - 12°33’ E | 79 | 11.2 - 18.9 | 731 ± 155 | 1171 ± 224 | sandy - loam | 7.7 | 1.9 |
| CRPV - Imola (IT) | 44°20’ N - 11°45’ E | 53 | 9.5 - 17.9 | 964 ± 218 | 1753 ± 195 | silty - loam | 7.2 | 1.5 |
| IMIDA - Mula (ES) | 38°3’ N - 1°25’ O | 278 | 12.0 - 24.5 | 336 ± 75 | 693 ± 159 | clay | 7.8 | 2.6 |
| IPB&GR - Naoussa (GR) | 40°37’ N - 22°06’ E | 119 | 8.1 - 17.2 | 818 ± 160 | 1762 ± 124 | sandy - loam | 6.8 | 2.5 |
| IRTA - Gimenells (ES) | 41°39’ N - 0°23’ E | 259 | 9.1 - 18.9 | 361 ± 95 | 1637 ± 133 | sandy - loam | 7.7 | 2.6 |

## Figure Legends

**Figure 1**. Overview of the range of phenotypic diversity in the *PeachRefPop*. Columnar and standard tree growth habit (top left and right panel, respectively). Heart shaped, round and flat fruit (top and third row). Range of fruit flesh, skin color and overcolor (second and bottom rows). Variation in flower morphology and color (third and fourth rows). Fruit size variation (fourth row, first and last panels).

**Figure 2**. Graphical summary of the overall scheme followed for selecting the *PeachRefPop* collection. From the starting panel of 1,262 accessions, 169 accessions were selected combining two sets: 69 accessions extracted from genetic and phenotypic diversity analyses and taking into account availability of whole genome resequencing (WGRS) data; 100 accessions selected by an empirical strategy from an experts panel considering breeding and traditional value along with genetic structure. These were supplemented with 214 seedlings from crossing populations of scientific importance and their respective 20 parents. The total number of entries in the *PeachRefPop* amounts to 403.

**Figure 3**. Assessment of allelic redundancy observed at marker loci in the starting panel (FB_1262). Core collections of incremental size were generated, based on the Maximization (OPT) and random sampling (RAN) methods in MSTRAT software, using a set of 445 SNPs. Datapoints represent averaged values over 5 independent repetitions for each size.

**Figure 4.** Genetic structure and phylogenetic analysis of *PeachRefPop* accessions. A) Population structure estimated in the whole panel (FB_1262) and *PeachRefPop* accessions (*PRP_X*), as estimated for K (number of a priori cluster) equal to 4; B) PCA analysis of the subsets with a core size of 169 entries. Scores for each accession were obtained from the work of **Micheletti et al., 2015**. The 95% confidence ellipses in the scatter plot were estimated using PAST software. C) NJ phylogenetic tree. Blue squares indicate accessions with traditional and historical value, violet circles indicate the other *PeachRefPop* accessions, and colors reflect the population structure.

**Figure 5.** Distribution of main phenotypic traits in the *PeachRefPop* accessions. In the maturity date plot, UE, E, M, L and VL indicates ultra-early, early, medium, late and very late ripening accessions, respectively. SSC, Soluble solids content. In the fruit texture plot, four major texture groups are shown: non-melting (NM), melting (M), slow-softening (SwS) and stony hard (SH).

**Figure 6.** Experimental design and *PeachRefPop* orchards layout. A) Google maps satellite images of the established *PeachRefPop* orchards across the different European sites; B) Experimental design of multisite *PeachRefPop*. A schematic example is provided for Gimenells location. Accessions (A) and seedlings (S) in each block and sub-block were completely randomized. The M1.1 and M1.2 sub-blocks each include a full copy of the collection (189 accessions, 214 seedlings) plus replicate checks (C) of the accessions ‘Big Top’, ‘Nectaross’ and

603 ‘Springcrest’ (5 additional trees for each sub-block). The M2.1 and M2.2 sub-blocks include half of
604 the *PeachRefPop* collection and each site has a different half, chosen according to a pairwise
605 design scheme. To this end, accessions (excluding control checks), and seedlings were randomly
606 assigned to eight disjoint subgroups (A1 to A4 and S1 to S4) of approximately equal size and 4 of
607 them assigned so that each site shares at least one A or one S group with the other sites. In the
608 example, each M2 sub-block at Gimenells is composed of A1 and A2 (46 and 46 accessions,
609 respectively, plus the 3 checks for a total of 95), S1 and S2 (54 and 54 seedlings, respectively, for
610 a total of 108) other than 3 additional replicates for each of the 3 checks (9 trees). Gimenells
611 shares the A1 and S1 groups with Mula, S2 with Imola and A2 with Naoussa.
612 **Figure 7.** Climatic profiles of *PeachRefPop* sites. A) Trend of minimum and maximum daily air
613 temperatures at the five *PeachRefPop* locations (averaged from 1999 – 2018 time series). Thick
614 lines show smoothed mean temperatures. B) Average monthly precipitations (in mm).
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