**UNIVERSITÀ DEGLI STUDI DI SASSARI**
**CORSO DI DOTTORATO DI RICERCA**
**Scienze Agrarie**

Curriculum Produttività delle Piante coltivate

Ciclo XXIX

# Morphological, chemical and genetic characterization of Citrus monstruosa, an endemism of Sardinia

Dott.ssa Grazia Viglietti

| | |
|---|---|
| *Coordinatore del Corso* | Prof. Antonello Cannas |
| *Referente di Curriculum* | Prof.ssa Giovanna Attene |
| *Docente Guida* | Dott.ssa Grazia Maria Scarpa |
| *Tutor* | Dott. François Luro |

Anno accademico 2015- 2016

## Acknowledgments

We would like to thank the president of the **INRA Centre** and also Yann Froelicher, **AGAP Unit** Director, in Corsica, France, for the period spent in their laboratories.

For experiments and analysis thanks to UMR 1390 INRA-AGAP, San Giuliano and, in particular, my tutor François Luro. Franck Curk for his assistance and support and all INRA laboratory technicians: Gilles Constantino, Elodie Marchi and Isabelle Tur. We also want to thank **University of Corse**, CNRS UMR 6134 SPE, "chemistry and biomass" team, Ajaccio, in particular prof. Félix Tomi and Dr. Sylvain Sutour for the essential oil analysis.

We also would like to thank the Department of Agronomy, food, natural resources, animals and environment (**DAFNAE**) in Padova for the period spent in their laboratories and, specially, prof. Gianni Barcaccia and dr. Giulio Galla for their support and their help about work made with them.

Finally, we wish to acknowledge the collaboration of dr. Giovanna Becca and dr. Pietro Paolo Urgeghe for analysis performed by them.

# Index

# AIM AND ORGANIZATION OF THE WORK

Characterize an unknown species means to protect and enhance its existence, especially if it is closely linked to the territory and its traditions. Sardinia has a rich plant heritage, probably due to its geographical isolation. This biodiversity is threatened by agriculture standardized resulting in loss of local landraces.

The main purpose of this work is the characterization of a species of unknown origin in Sardinia, *Pompia*, through the study of its morphological, chemical and genetic characteristics.

The **Chapter 1** is introduced the data of a first genetic analysis approach, performed with DNA barcoding technique to identify which of the species of the genus *Citrus* are more similar to Pompia. It is also investigated the ploidy level through chromosomal counts, karyotype and flow cytometry.

In **Chapter 2** it was decided to make more detailed genetic analysis using SSR and InDel molecular markers with the aim of identifying which of the *Citrus* species and varieties are Pompia's parents and if there is genetic variability inside Sardinian specimens.

Finally, **Chapter 3** contains the Pompia phenotype and its essential oil composition in leaves and fruits, comparatively with its ancestors and lemon, a same interspecific hybrid origin, to estimate the inheritance of parental characters. In second step, the variability of Pompia phenotype was investigated by the sampling of different orchards and locations in Sardinia.

The present work has been corrected by a english, mother tongue, professor.

# GENERAL INTRODUCTION

## Origin and diffusion of citrus

The term "agrume", citrus, derives from Medieval Latin *acrumen* (der. *acer*: *agro*) and indicates the acidic fruits. It is used commonly in the plural form to indicate either the trees or the fruits. This term enters quickly, part of the spoken language, thanks to the growing economic value of citrus. Citrus originates from South-East Asia, precisely in the area that stretches from the Himalayas to Indonesia, passing through China, Vietnam, Thailand and Malaysia (Calabrese, 2009a).

The oldest historical evidences on citrus can be found in China and India. In the fifth century BC, in "Shih Ching" documents, during the Chou dynasty, terms as *chu* (kumquats and mandarins), *yu* (pummelo, *Citrus maxima*), *yuzu* (*Citrus junos*) and *chih* (*Poncirus trifoliata*) are mentioned. In India, in an anthology of sacred texts Brahmanic previous to the 800 BC the citron is mentioned for the first time.The citron was sacred in India because it is associated with the elephant-headed deity, Ganesh, god of wisdom and intelligence. In Chinese references, the citron will only appear in 304 AD in a book of botany, indicated with the name of *yuan-kou-tzu*. The citron "Hand of Buddha" at that time was known as *kou han*. Chinese civilization reserved for citrus a key role: they were a symbol of prosperity and good wishes, and were offered as a gift. Some emperors created even institutional figures, charge of tax collection due to the emperor in *kan*. The term *kan* literally means, "tree from the sweet fruit" and was used to refer to mandarin (Calabrese, 2009a).

The citron was the first citrus to reach Mesopotamia, probably brought by the caravans who were trading with India and China. It was found for the first time by the Greek botanists in the wake of Alexander the Great in 327 BC. Citron was described by Theophrastus from Ereso (372-287 BC) in his *Historia Plantarum*. In Greek times the fruit was not eaten, the plant was used for purely decorative purposes. The leaves and flowers perfumed the sheets and kept away moths, while the juice was used as an antidote to many poisons and was used to counter bad breath (Calabrese, 2009a).The Romans identified originally citron, lemon and lime all under the terminology of "yellow skin citrus". References to citrus is various in the Roman age, but only at the third or fourth century AD cultivation of citrus has been described by Macrobius and Palladium. This latter, in his *Opus agriculturae*, dedicates an entire chapter to citrus treating grafting, planting, growing, pruning and protection.

Due to expansion of the Arabs in Spain, Italy and North Africa sour orange reached the West. It is believed erroneously that the Arabs broughtcitrus intoEurope; they actually introduced and valorized only sour orange that was used for adorning mosques and patios. They were, however, the first to lay the bases for the rational cultivation of this fruit. There were many literary references, where the citrons, lemons, pummelos and limes in addition to sour orange are also mentioned. In the Middle Ages, the information on citrusis enriched by the Crusaders; gradually crops have spread all over the Mediterranean and, in particular, on the Ligurian Riviera, in eastern Spain, in Sicily and Crete (Calabrese, 2009a).

The sweet orange was introduced only later. In fact, it is very likely that the Greeks and Romans did not know the common orange. The studies lead to two hypotheses: the Portuguese introduced orange in Europe either in the fourteenth or by the Genoese in the fifteenth century of the modern era. In the sixteenth century, for sure, the orange was known all over the Mediterranean under the name of "Orange China", "Orange of Malta" and "Orange Portugal". In the Renaissance, a period of cultural splendor and exaltation of beauty, citrus had a purely aesthetic role, as well as scientific, in the gardens and plant collections from all over Europe. In this time, citrus were grown in pots that in the hottest period were exposed to the sunshine, while in colder periods were protected in a sheltered environment. This kind of cultivation became a cult especially among the aristocrats, with preference for the most strange and bizarre citrus. Even the References, poetic, narratives and scientific, accompanied the unique attention to these plants throughout the modern period, with a number of fairly well-known authors who devote to citrus depictions in major paintings, poetry monographs and book chapters.

Citrus followed Europeans in their migrations to the Americas. In Barbados islands the new hybrid of pummelo and sweet orange, grapefruit (*C. paradisi* Macf.) was born around the end of the eighteenth century. During or years of grapefruit born citrus begin to be cultivated for commercial purposes in the Ligurian Riviera. In addition, their use in the confectionery industry for the production of candied fruit began to emerge.

Later, the cultivation spread most southern, reaching eastern Spain, Sicily and Calabria. In 1810 in Malta, and almost simultaneously in Palermo, was introduced the cultivation of mandarin, which soon became one of the most popular fruit.

Citrus was now the most popular fruit products and was sold worldwide. The range of varieties has been enriched and the range of uses, colors, and flavors has been greatly

expanded. The limit to the presence of citrus in a territory is now linked to the climatic characteristics of the region that the best fit to a species or variety. The orange is among all the more widespread, even for the large number of cultivars. For pummelo, grapefruit and lime the best quality is observed in hot environments, while citron, lemon, and clementine prefer temperate environments. Citrus was rich in principles (vitamins, flavonoids) useful to combat the occurrence of diseases, which is why these fruits are defined nutraceuticals (Calabrese, 2009a). Citrus was used not only entirely for human consumption, but also in cosmetics.

**Citrus in Italy: history for a cultural identity**

The full expansion of citrus in Italy took place simultaneously with the birth of the Italian garden in Renaissance age, in particular by means of the Medici family. Painters and poets who wish to honor the Medici family always chose citrus such as ornamental element in their pictures or their stories, as symbol of eternal youth (Fatta del Bosco, 2009).

The cultivation of citrus began in Italy by means of purely decorative purposes: above all the beauty and bizarreness on the loaves have been considered, with particular regard to those monstrous defined "teratological" who had, over time, enriched "genetic matrix" of citrus (Pozzana, 1990).

The diffusion of these plants led to the need for sheltered accommodation for the colder periods, and so it was that gave birth to the first greenhouses: the *orangeries*, true and proper buildings where exotic species were housed. Inside them, the citrons had to be placed in front of windows because of the greater need of the sun; lemons were placed against the wall while orange trees were in the center. Soon the orangeries became also the favorite place to host art exhibitions, concerts, banquets and dance. At the end of the '700 fashion of orangeries declined and glass-iron structures were built most apt to a commercial food use. The old structures remained as a noble and unique architectural evidence of the past (Fatta del Bosco, 2009).

The Italian agricultural landscape is strongly characterized by the presence of citrus since the introduction of the first among them, the citron. Several environmental, economic and social conditions resulted in different citrus landscapes throughout the peninsula, subjects in the years to changes and dropouts due to evolution of cultural practices and market changes. It is still possible to find traces of the historic Italian citrus landscapes like "Jardino di Pantelleria",

the “Conca d'Oro” in Sicily, the orange groves of the Gargano, the lemon groves of Amalfi and Garda, which barely survived despite the economy of the market, and established the identity of aesthetic places.The protection of such important landscapes should be strongly supported and encouraged to revitalize the traditional landscape of citrus in Italy, also preserving biodiversity (Barbera, 2009).

**Citrus in Sardinia: in search of the past**

The first historical information about the presence of citrus in Sardinia is dated from the fifth century AD. Palladio described in *Opus agriculturae* a species that identifies as citron. From the Middle Ages up to and over the thirteenth century, the citrus cultivation it’s supposed to be practiced in Giudicali gardens of San Vero Milis, in Villacidro, San Sperate and in Milis. In Ogliastra citrus was present in Tortolì, as described in a document, into the Codice Diplomatico Sardo, with which "the ortu de su kidru de Turrele" (Chessa et al., 1994) was given as a gift. The first mentionin the Sardinian language is in “Condaghe di Santa Maria di Bonarcado”, dating back to the mid-twelfth century, whose writings record contracts, transactions, and various topics of the period Arborense and which quotes literally: “*Comporei fundamentu in sanctu Iorgi de Calcaria et posi ad ortu de cedru et de omnia pomu*” (Virdis, 2002).

The existence of various acts of purchase as the previous one suggests that the citrus production of these lands was far higher than the local demand, which suggests that, already at that time, the product was exported.

Following the conquest of the Aragonese in the fourteenth century, appeared in Sardinia also lemon and sour orange which were, first, sold for local consumption and, secondly, used for decorative purposes, as evidenced by a document which quotes the large garden of oranges in Villa Lotzorai owned by the municipality of Pisa (Chessa et al., 1994).

During the domination of Spain, despite the state of neglect of countrysides, the cultivation of citrus continued to expand from the most typical areas in the neighborhoods of Sassari, Alghero, Bosa and Ogliastra. In 1700, citrus cultivation occupies a prominent role compared to other fruit. Although the most cultivated species was the citron, lemon and orange also played an important role. In addition to fresh consumption, the products were often used in industry for confectionery, especially citron and lemon; it was even prepared a distillate of orange flowers for export to Switzerland, which unfortunately had little success. In this period there were very accurate studies undertaken by the botanist from Sassari Manca dell’Arca (1780) and, later, by Moris (1837). The introduction of mandarin, with the name “agrume

arancio dè mandarini", is due, in the first 800, to the marquis of Villahermosa.The expansion of this crop continued to consolidate itself over time, by increasing its spread in the island and increasing production and yield standards, especially in areas less suitable: Sassari and Nuoro (Chessa et al., 1994).

The continuous introduction of cultivars from different citrus areas has, on one hand, improved the varietal standards, and on the other, caused a replacement of the old local varieties, less suited to market standards. Some of the plants grown in Sardinia are likely clones of oldest varieties other derived from cultivars introduced in less remote times (Chessa et al., 1994) also for this, the regional segment is structured in a complex and rich germplasm which need to be protected. Many varieties of citrus are disappeared or represented by a very small number of specimens. In fact, there are few old varieties that still inhabit the Sardinian territory and represent a genetic heritage well adapted to local ecological conditions, strongly linked to the territory and its traditions.

According to a survey carried out in 1980 in Sardinia, a list of varieties of native citrus has been redated, most of themare grown on marginal lands, and they are an important source of biodiversity. From this survey, performed by Chessa et al., (1994), resulted that the "old" variety of local citrus are:

- "Arancio comune": cultivar present only in older plants, its origin is not known; it was described by Milella (1960) as "Aranzu sardu". The virtues of this fruit are the high content of juice and its late ripening;
- "Arancio dolce a buccia sottile": only few individuals remainingin the area of Posada old about 60-70 years. It produces a fruit with good juice yield, thin skin, few seeds;
- "Miele": uncommon orange cultivar, it could be useful at crossroads for its early ripening and the absence of seeds;
- "Ovale corda": probable local orange clone, originated from ancient varieties, it could be useful in breeding programs for the absence of seeds and good size;
- "Pisu": consists of a set of biotypes forming a clonal population arising from a variety of orange " common blond";
- "Tardivo di Cabras": cultivar of orange that has no attractive merchandise nor organoleptic qualities;

- “Tardivo di San Vito”: orange cultivar on average diffused, has interesting features such as the small number of seeds, the delay in ripening, high juice yield and high productivity;
- “Vaniglia comune”: orange cultivar highly appreciated in the market, is one of the oldest Italian varieties originated in Sicily;
- “Vaniglia rosato”: orange cultivar uncommon, is the unique variety of sweet oranges of the pigmented group;
- “Dolce di Muravera”: lemon variety with low acidity, slightly juicy, medium size;
- “Limone di Santu Ghironi”: ancient variety of Sardinian lemon, particular for the absence of seeds, high juice yield and good fruit size;
- “Pompia”: probable natural hybrid of unknown origin sporadically growing in the Baronia area (East Sardinia).

**Figure 1:** Iconography of Pompia according to Camarda et al., (2013)

## Pompia

### *Origin and diffusion*

*Pompìa, pumpìa* is not an italian word. The idiom is present in dictionaries as Sardinian etymological Dictionary (Wagner) and Giovanni Spano Sardinian Dictionary, but not in the Great Dictionary of the Italian language (Battaglia). It is not present even in Spanish dictionaries. It is useless to track the origins of the name in the word "pompelmo" (grapefruit), because this, according to the Etymological Dictionary of the Italian Language, is derived from the Dutch "pompelmo(es)", composed of "pompelm"(big) + "limoes" transcript of the Malaysian "limous"(lemon).It is easy, however, find its etymology in Sumerian "pu" "fruit garden" + akkadian "pium" fodder. The compound "pu(m)pium" in high antiquity meant "fodder gardens", perhaps because it was edible, although not among the most desired fruit (Dedola, 2016).

The Pompia is a typical citrus of Sardinia, some authors place its cultivation in different countries: Villacidro, San Sperate, Oliena, Milis and Siniscola. The earliest records of its existence date back to the eighteenth century in an essay on plant and animal biodiversity of Sardinia, written by the botanist Andrea Manca Dell'Arca from Sassari, published in 1780. Listing the citrus in Sardinia Dell'Arca cites lemons, oranges, and citrons among which places Pompia without adding anything else. Moris in 1837 calls it *Citrus medica monstruosa* and indicates, doing a careful morphological description, *C. medica tuberosa* Risso and Poiteau (1818) and *C. medica* s.l. Gallesio (1811) as its probable synonyms. Moris reports, also, the Italian name *China citron* and the vernacular *Spompia*, a view shared by many authors (Chessa et al., 1994).

Among the historical citations, a description of Pompia is ascribed to Theophrastus, which describes it as a thorny *Citrus* (*Citrus medica cetra*). Palladio signals also the citron cultivation in Sardinia and Dioscorides describes the cooking of a fruit, oblong and wrinkled, in wine or honey, saying that was edible only if transformed.

According to Fancello (2012) Pompia could be a hybrid of citron and grapefruit, Camarda et al. (2013) suggested *C. limon* var. *Pompia* var. nova as a new name of Pompia and concluded that Pompia is an hybrid between lemon and citron. In 2015, Mignani et al. supported this hypothesis. However, it is different from these species for aspects of both axle and the fruit and may be a natural hybrid originated within the local citrus population (Chessa et al., 1994). Some descriptions of some species present in the paintings of Bartolomeo Bimbi (late seventeenth century) could be associated to Pompia, in these are paited an assortment of

citron types, or similar fruits to the citron; today we know that the described and illustrated citrus were citron hybrids. Many of these described citrus, have been lost.

In addition to these hints other historical documents are missing, it is not possible to say with certainty, how long this plant exists, or if it originated in Sardinia and, as a consequence, it is an endemic sardinian citrus or arrived in the island from somewhere else. Inspired from the historical sources, it is likely that the Pompia originated from Milis, since it testifies to its presence in that territory in 1760, in a statistic compiled by order of the Viceroy, and then from Milis has moved towards the center and the oriental coast. Its ancient origin and little knowledge either on taxonomic position and bio-agronomic characteristics, make it a particularly attractive citrus. The Pompia is still known and appreciated only by an expanding nichemarket.

It is mainly diffused in the Baronia, in the eastern coast of the island, where it is most valued and commercialized in nurseries, from Torpè to Orosei. Is also found in Oliena and sporadically in other areas of Sardinia more or less adjacent to places of presence as Bitti, Milis, Budoni and more. Today plants grown from seed are cultivated into the nursery of the forest site of Sa Unnichedda and, by vegetative propagation, at the Experimental Field Technical Institute for Agriculture of Siniscola. But isolated plants in urban and peri-urban private gardens are grown.

It uses are purely family and related to local traditions, especially in the confectionery sector. In fact, it is the main ingredient of candied sweet "Sa Pompia" obtained by cooking the albedo in honey for several hours.

**Figure 2:** Cultivation of Pompia trees

From 2004 the sweet called "Sa Pompia" is under supervision by Slow Food, with the aim of spreading the culture of Biodiversity.

***Agronomic aspects and morphology of plant and fruit***

The Pompia tree presents medium size (2-3 m high), has an expanded erect posture, it is also grafted on sour orange.The leaves have a non-winged petiole 6-10 mm and a green-dark foil, leathery, elliptical with apex rounded to ovate-lanceolate with entire margin and with a very marked midrib.The flowers are white solitary or grouped in inflorescences of 2-8 flowers mostly abortive. The have numerous stamens and dorsifixed anthers, stigma rounded greenish is irregularly lobed. Fruiting starts generally in October and lasts until February-March (D'Aquino et al., 2005). The fruits have sub-spherical to oval shape in the juvenile stages and in fruits of small size, crushed-compressed in the mature forms, by more or less strongly to smooth lumpy and irregular with a apex delimiting, at times, a circular depression, and depression in the insertion of the stalk, which it is hardly removable. The have a tall standard: the fruit can reach a weight of 600-700 g (Chessa et al., 1994). The colour changes from green to yellow-citrine according to the degree of fruit maturity, it is rough with longitudinal ribs prominent and thick, although there are cases in which it is smooth and thin. It is rich in essential oils that are reminiscent of lemon, but with more delicate fragrance. Albedo is very thick (4-15 mm), white with consistent parenchyma. The endocarp slices are light yellow, 13-14 in number, with large vesicles and stubby, seedless or with 1-3 polyembryonic seeds (D'Aquino et al., 2005) long 8-12 mm, having irregular trigona shape, with slight ribs and pointed apex (Camarda et al., 2013).The quantity of juice is low as well as its sugar content, while the acidity is high (Mignani et al., 2004).

**Figure 3:** Pompia flowering

**Figure 4:** Ripening of fruits

## Taxonomy of citrus and genetic relationship among taxa

The taxonomy of citrus still stir up serious doubts in the scientific community, because of the intergeneric sexual compatibility, the high frequency of buds mutations and the long history of cultivation and spread (Nicolosi et al., 2000). The taxonomists, after classifying the citrus in the order of *Geraniales* (Swingle and Reece, 1967), now seem to agree that citrus belong to the order of *Sapindales* Berchtold & J.Presl, of the *Rutaceae* family Jussieu (Stevens, 2013 ; NCBI, 2014). *Rutaceae* family, whose name comes from the genus *Ruta* (rue - smelling plants), includes herbaceous plants, shrubs and trees witch are characterized by the presence of essential oils that make them just "smelling".

According to Swingle and Reece (1967) citrus belong to the family *Rutaceae*, subfamily *Aurantioideae* divided into two tribes: the *Clauseneae* (with 5 genera) and *Citreae* (28 genera). The first includes the most primitive species than the second. The *Citreae* are divided into 3 subtribes: *Balsamocitrinae*, *Triphasiinae* and *Citrinae*. The *Citrinae* are divided into three groups, one of which contains the so-called real citrus consisting of 6 botanical genera: *Microcitrus*, *Eremocitrus*, *Clymenia*, *Poncirus*, *Fortunella* and *Citrus* (see Figure 5). The subtribes of *Citrinae* is characterized by the structure of its fruit, divided into segments with inside bags of succulent flesh. This typical fruit, not present in any other plant, is called “hesperidium” (Praloran, 1971).

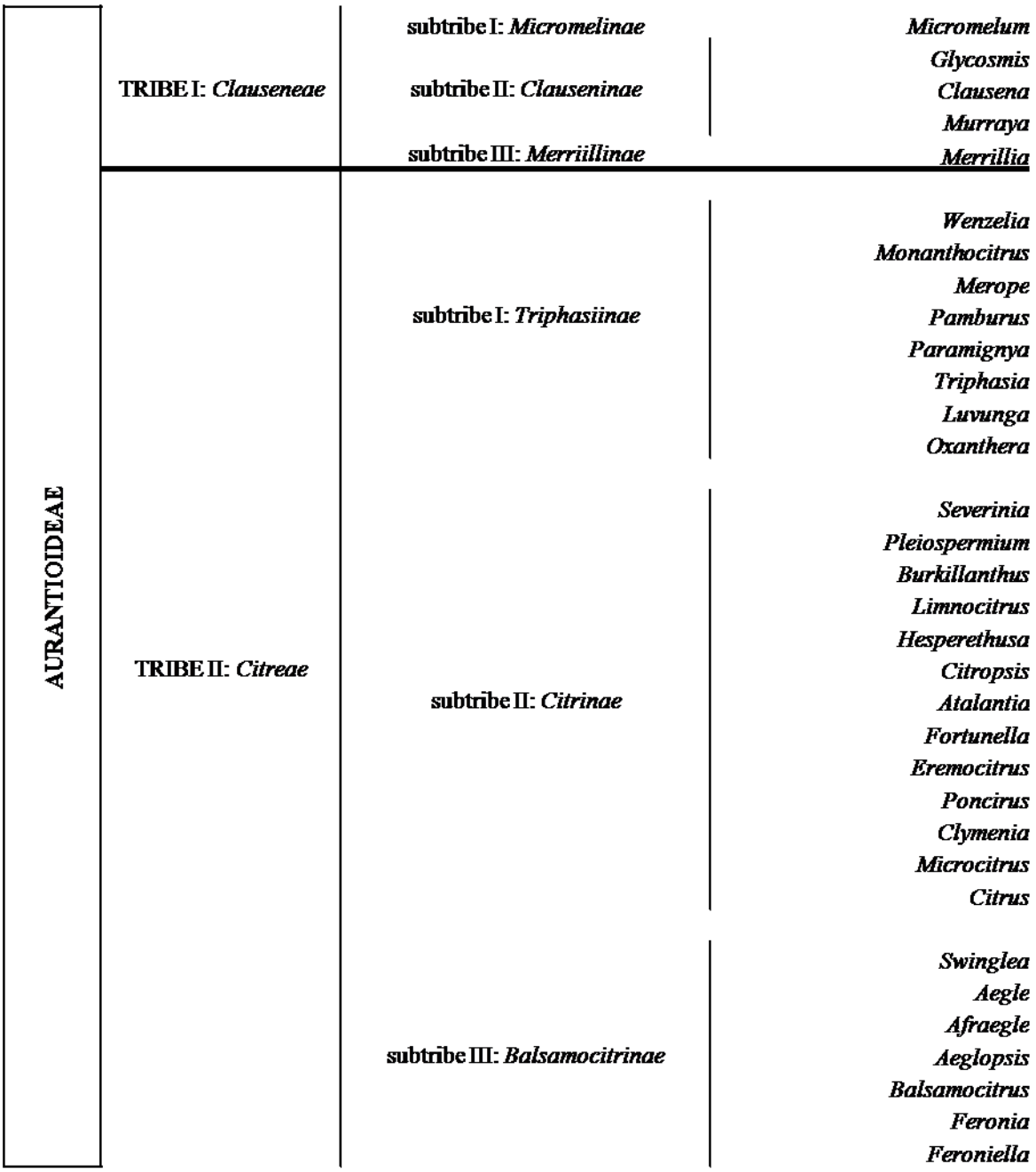



**Figure 5:** *Aurantioideae* classification according to Swingle and Reece (1967)

**The citrus: 6 botanical genera**

***Microcitrus Swingle***

*Microcitrus* exhibit leaf dimorphism with smaller, pointed leaves in younger plants. These leaves are perennial and more or less coriaceous. The flowers are tiny with free stamens, ovaries having 4 to 8 lodges with 4-8 eggs per loggia. The juice vesicles are rounded and uncompressed into segments, from this derives the name that is often associated with "lemon caviar".The fruits are round, oval or cylindrical depending on the species, endowed of essential oil glands on the skin. The tree is often shrubby and bushy.

***Eremocitrus Swingle***

*Eremocitrus* is a monospecies genus: *Eremocitrus glauca (Lindl.)*Swingle, better known as "Desert lime" from Eremon = desert. The plant is bushy and thorny with thin branches and stalk. The leaves are perennials, small, very elongated, erect or pendulous. The green parts of the plant are covered with a glaucous pruina, blue-green, waxy from which the species name derives. The flowers are very small, often isolated; more rarely can be grouped in inflorescences. The fruits are small, yellow-green when ripe and have a diameter of 18-20 mm. The glands present in the rind are rich in essential oils and taste of the flesh recalling that of lemon, so very acidic.

***Clymenia Swingle***

*Clymenia* are shrubs without thorns, with perennial leaves, coriaceous and sharp at both ends. The flowers are white and fragrant, formed at the base of the leaves and have numerous stamens. The fruits have a shape similar to the lemon with a small umbo at the apex, orange red when ripe; the pulp is sweet and contains numerous seeds. The genus contains only one species *Clymenia polyandra* (Tan.) Swing. sin. *Citrus polyandra* Tan. first classified in *Citrus*, later Berhow (2000) recognized as a hybrid of *Fortunella* and *Citrus*. The hypothesis has been confirmed by recent molecular studies that highlight how *Clymenia polyandra* is part of a genus in itself (Garcìa-Lor et al., 2013a).

***Poncirus Rafin***

This genus presents bushy shrubs, thorny plants, with deciduous, trifoliate leaves and white flowers, which bloom before all other citrus species; the fruits are small and inedible.The skin tends to turn yellow when ripe, is covered with a light fluff. The plants are mainly used as an ornamental species or, in the case of *Poncirus trifoliata*, as a graft to produce hybrids resistant to cold and "Citrus Tristeza" (Davies and Albrigo, 1994).

***Fortunella Swingle***

Genus native of China, his name is dedicated to the British botanist Robert Fortune (Swingle and Reece, 1967) who introduced it to Europe in 1846 at the London Horticultural Society. It includes four species with evergreen leaves, coriaceous and rich in essential oils. The trees, of variable size depending on the species, are thorny and resistant to cold, although less of *Poncirus*. It has white flowers and rather small round fruits. It is a genus little known in Italy, mainly used for ornamental purposes (Davies and Albrigo, 1994).

***Citrus L.***

Belong to this genus all species of cultivated citrus and is generally more diversified in species, varieties and cultivars. Of this genus belong trees or shrubs with evergreen leaves, coriaceous, oval-elliptic, with possible stipules, important for systematic. The morphology of the trees varies from one species to another in shape, size and demeanor.The size, the shapes and colors of the leaves are also variables such as the size of the fruits. The shape of the fruit can vary from spherical, such as into orange, the piriformis, to finish the form of fingers in citron "Buddha's Hand". The species that compose this genus are sexually compatible with each other and among other related genera.

There are various classifications of the *Citrus* genus, but the most widely accepted are those of Swingle (1943) and Tanaka (1954). The first includes 16 species in the *Citrus* genus divided into two subgenus: *Eucitrus* (10 species) and *Papeda* (6 species) divided according to the morphological characteristics and chemical composition of flowers and fruits; the second includes many more in a meticulous division into 2 subgenera (*Archicitrus* and *Metacitrus*), 8 sections, 13 subsections, 8 groups, 2 microgroups for a total of 145 species. Of these, 55 refer to citrus propagated by humans, 20 are extinct and 19 are of uncertain classification (Calabrese, 2009b). In 1961 Tanaka adds two new subsections, a new group and 12 new species to arrive at a total of 157 species. It is considered that Tanaka has given a more detailed description of the one made by Swingle in 1943 by classifying, for example, the mandarins in 36 species, while they were grouped under one species *Citrus reticulata* Blanco, in the classification of Swingle.

The natural or artificial hybridization has probably played an important role in the development of many, or most species of *Citrus*. Scora in 1975 and Barrett and Rhodes in 1976 claimed that among the cultivated *Citrus* only exist 3 main species. Through hybridization amond them and the species of the subgenus *Papeda* or closely related genera,

all other species, called satellites, would arise.The species identified as the main ones are represented by citron (*C. medica* L.), mandarin (*C. reticulata* Blanco) and pummelo (*C. maxima* (Burm.) Merr.). This hypothesis was confirmed by a several studies on the phenotypic and genetic diversity (Herrero et al., 1996; Nicolosi et al., 2000; Ollitrault et al., 2003; Fanciullino et al., 2006). In addition to the 3 species defined as ancestral, today many authors consider native even *Citrus micrantha* Wester (Papeda) (Federici et al., 1998; Nicolosi et al., 2000; Ollitrault et al., 2012a). It is thought that it is the origin of the green limes such as Mexican lime (*Citrus aurantifolia* (Christm.) Swing.).

***The Citrons [Citrus medica L.]***

Originating in Southeast Asia, the citrons present plant thorny shrubs, gown content, and irregular posture. They are normally used for jams or for purely ornamental purposes. The leaves are quite large, coriaceous. The young shoots and flowers have a purple tint, as most of the lemons, except in Corsican citron (Calabrese and Barone, 2009). It is not rare to find sterile female flowers, without pistils. In addition, the male and female reproductive organs mature at the same time and always before the opening of the flower. The fruits are usually large, with an albedo very thick highly adherent to the endocarp. The seeds are mono embryonic (Swingle and Reece, 1967).

They are mainly divided into 2 groups: acid pulp citrons, how the Diamante variety and Etrog, and sweet pulp, as the Corsican variety. It is further recalled the citron "Buddha's Hand" that has a particular fruit shape as it is divided into segments that resemble the fingers of one hand, this explains its name. This variety, with almost no endocarp, is cultivated in China for purely ornamental and traditional medicine use.

***The Mandarins [Citrus reticulata Blanco]***

Originating in Asia, the mandarins present a more complicated classification than the other analyzed groups, due to numerous biotypes, crosses and interspecific hybrids difficult to place. The first who divided them into 36 species was Tanaka in 1954, but even today their classification is always evolving. They consist of variable size trees, shrubs, with small thorny branches. The leaves are lance-shaped, small and fragrant. The flowers occur singly or grouped in inflorescences. The fruits are generally spherical in shape and can be compressed to 2-pole; they have thin skin easy to peel off from the endocarp. The seeds can be mono or polyembryonic. The monoembryonic belongs to hybrids of mandarins having regressions from pummelo conferring them the character (García-Lor et al., 2013b).

***The Pummelos [Citrus maxima (Burm.) Merr.]***

Cultivated for centuries in China and neighboring countries, the only pummelo of some significance obtained in the West is the Chandler, having pink pigmentation. The group is clearly different from the others: only monoembryonic species. The trees are vigorous and continuously productive. The young shoots and leaves are often pubescent. The leaves are very large, with winged oval stipules. The flowers are large, elongated; the fruits are of size, color and shape vary widely, but generally large, spherical, pear-shaped or flattened at the poles.They generally have a very thick albedo and elongated juice vesicles that very easily detach (Swingle and Reece, 1967). The fruits are used for both fresh consumption, and as religious offerings in Vietnam and China.

***The Papeda***

This group comprises a large number of wild species.The plants are small, spiny, the leaves vary in size, but generally bilobed. The flowers are small, with the stamens not welded. The fruits vary in size and present drops of essential oil into the flesh. The fact that it gives the fruit a bitter flavor making the fruit unappetizing. Many varieties are grown mainly for essential oil extraction that has a strong scent of citronella, greatly appreciated by the perfumery and cosmetics.

**Genetic origin of the most important cultivated species**

The study of citrus began from the acceptance of the hypothesis that there are 4 ancestral taxa. Many have been, over the centuries, the cases of cross-pollination that have created fertile hybrids with new and unique features (orange, grapefruit, lemon, sour orange, clementine). It can be said that all secondary species represent a botanical group of hybrids, derived from the 3 or 4 species representing the real and oldest specimens of the genus *Citrus* (D'Aquino et al., 2005). For limes are listed in multiple origins, one of which involves hybridization with citrons, the other with *Papeda* (Nicolosi et al., 2000, Curk et al. 2016).

All the secondary species are producing polyembryonic seeds. The apomixis of all secondary species allowed to establish and multiply the highly heterozygous structures and led taxonomists to consider these groups as species (Scora, 1975; Barrett and Rhodes, 1976).The genus *Citrus* has, in fact, experienced a period of allopatric differentiation in the 3 ancestral taxa, followed by interspecific recombination events which direct products have been set for optional apomixis, for this, the groups are defined species by taxonomists. The result of these processes has led to highly heterozygous genotype structures, formed by a mosaic of large

fragments of different phylogenetic origin. For example, *Citrus sinensis* (sweet orange) is a mosaic of DNA fragments inherited from *C. reticulata* and *C. maxima*. Studies based on the chloroplast (Green et al., 1986; Nicolosi et al., 2000) and mitochondrial genome (Froelicher et al., 2011) show that the cytoplasm of the orange, sour orange and grapefruit, derives from pummelos. The use of molecular markers has made a significant contribution to decipher the citrus phylogeny, that which could clarify the intricate taxonomic relationships among species of *Citrus.* Remarkable studies have yet to be made in this direction, to have a clear and unambiguous outlook on the citrus taxonomy.

The origins of the cultivated species of citrus are supported by works based on different molecular markers: isoenzymes (Herrero et al., 1996), RFLP (Yamamoto et al., 1993; Federici et al., 1998), RAPD and SCAR (Nicolosi et al., 2000), AFLP (Liang et al., 2007), SSR (Luro et al., 2001, 2008; Barkley et al., 2006; Ollitrault et al., 2010) SNP (Ollitrault et al., 2012a) and genome sequences (Wu et al. 2014).

Many were the assumptions made by various researchers using a huge variety of methods genetic origin of cultivated species. For the sweet orange and grapefruit has been reached fully agree, among the researchers, that they are hybrids originated from crosses between pummelo and mandarin.

The hypotheses on the origin of the lemon are contradictory: based on morphological characteristics Swingle (1943) and Malik et al. (1974) felt that it was a hybrid of citron and lime. Barrett and Rhodes (1976) have suggested that lemon was a hybrid of citron, pummelo and a species belonging to the genus *Microcitrus*; some argue that the lemon can be a hybrid of citron and sour orange, hypothesis corroborated by Curk et al. (2016).

As regards to the origin of the Mexican lime (*C. aurantifolia*) it was considered by Barrett and Rhodes (1976) a trihybrid. Torres and co-workers (1978) hypothesized that the lime could be a hybrid of papeda and citron. Hypothesis later confirmed by Curk et al. (2016).

The Palestine sweet lime (*C. limettoides*) could be a hybrid between the Mexican lime and a sweet lemon or a sweet citron. Barrett and Rhodes (1976) consider this lime born from a crossover between Mexican lime and sweet orange (Nicolosi et al., 2000). Molecular studies today has accurately to identified the *C. limettoides* as a hybrid of citron, male parental, and an unknown hybrid pomelo x mandarin as his female parent (Curk et al., 2016).

It has been supposed that clementine is a hybrid of mandarin and sweet orange (Deng et al., 1996) and the consistency of this hypothesis has been demonstrated.

Citron, ancestral species, takes partat the crosses that generate of many citrus species, always representing the pollinator species. Examples are the “Rough” lemon (*C. jambhiri*) and "Volkamer" lemon that are mandarin and citron hybrids (Nicolosi et al., 2000, Curk et al. 2016).

***Citrus sinensis (L.) Osb.***

Genotypically sweet orange is very close to *C. reticulata*, however, it has introgressions of *C. maxima*. The great proximity to the mandarins, suggests that it is not a direct hybrid, but a result of the first or second generation backcrossing with the genome of the mandarines (Barrett and Rhodes, 1976; Nicolosi et al., 2000). More recently, Wu et al. (2014), via the complete genome sequencing, demonstrated that the two parents of the sweet orange are of interspecific origin (*C. maxima / C. reticulata*).

***Citrus aurantium L.***

This citrus is known internationally as Orange Seville since around this city is the most important cultivation center in the world. It seems to be a direct cross between a pummelo (*Citrus maxima*) and a mandarin (*Citrus reticulata*) (Swingle and Reece, 1967; Scora, 1975; Barrett and Rhodes, 1976; Green et al., 1986; Yamamoto et al., 1993; Nicolosi et al., 2000; Wu et al., 2014). In Italy it is used as a rootstock for its outstanding agronomic characteristics, but is sensitive to the “Citrus Tristeza Virus”. If prior to the advent of the virus it was normally used, today can it only be used in countries where the virus is not present or in association with acidic varieties such as lemos limes or citrons that are not sensitives to this virus.

***Citrus paradisi Macf.***

Discovered at the beginning of nineteenth century in the Caribbean (Webber, 1943), the grapefruit is a close species to pummelos, probably a result of spontaneous hybridization between a pummelo and a sweet orange. (Barrett and Rhodes, 1976; Scora et al., 1982; Li et al., 2010; Ollitrault et al., 2012a).

***Citrus clementina Hort. Ex Tan.***

*C. clementina* was described for the first time in 1902. It is considered to be a natural hybrid between a mandarin (*C. reticulata*) and an orange (*C. sinensis*) (Ollitrault et al., 2012a; Ollitrault et al., 2012b).

***Citrus limon (L.) Burm.***

The geographic origin of yellow lemons is still unclear. Some place its origin in southern China and in India (Tanaka, 1929; Biraghi, 1935). Webber in 1967 indicates possible origin as a center of southern China and northern Nyanmar. The doubts arise mainly from the fact that very often the description of the fruit does not distinguish them from a citron or other hybrids (Malik et al., 1974).

Scora (1975), Barrett and Rhodes (1976), Federici et al. (1998) assert that lemons are a direct hybrid between citron and lime, but more recent studies describe them as a direct hybrid between sour orange and citron (Gulsen and Roose, 2001a; Curk et al., 2016).

***Citrus aurantifolia (Christm.) Swing.***

The classification of Swingle includes *Citrus aurantifolia* Swing. in a single species. Tanaka, however, distinguishes a number of species: *C. aurantifolia* Swing. (Mexican lime, small fruit), *C. latifolia* Tan. (Persian or Tahiti kind, the fruit almost as big as a lemon), *C. limettioides* Tan. (sweet juice), *C. limetta* Risso (Mediterranean lime).

Some authors consider Mexican lime is a direct hybrid between *Citrus micrantha* and *Citrus medica* (Scora, 1975; Nicolosi et al., 2000). In 1969, Tanaka claims that it could derive from the *Papeda* group. This variety produces small green fruit, a thin skin with pale green flesh with one or two seeds per fruit.

Some limes are triploid. Among these, the Tahiti lime (*C. latifolia*) previously described as a hybrid between citron and lemon (Mabberley, 2004; Bayer et al., 2009) is in fact originated from fertilization of lemon ovule by an unreduced pollen gamete (diplogamete) of Mexican lime (Curk et al. 2016). The origin of Tanapeo lime is linked to a across between a diploid egg (diplogamete) of *C. aurantifolia* - Mexican lime - and a regular pollen gamete of *C.medica* (Curk et al., 2016).These two limes difference for the presence of seeds in the first and their absence in the second.

***Citrus bergamia Risso & Poit.***

The origin of bergamot, *C. bergamia* Risso & Poit, is still very controversial.The area would seem to be approximately the cost of the province of Reggio Calabria, where it is known from 1700. According to Di Giacomo (1989), it was introduced in Calabria, from the town of Berga (hence would derive its name) and grafted on a lemon tree in 1400 (Calabrese and Barone, 2009).In the most recent studies, it has seen that the bergamot and sour orange have the same cytoplasmic origin and so the same maternal origin (Gulsen and Roose, 2001b), the paternal origin is attributable to *C.limon* (Curk et al., 2016).

The bergamot is cultivated for the extraction of its essential oil, whose best quality is achieved in the province of Reggio Calabria. Attempts to make cultivation in other parts of the world have never given good results. The commerce of the essence is regulated by specific rules, at the buyer's warranty, verify by the specific consortium of Calabrian bergamot (Calabrese and Barone, 2009).

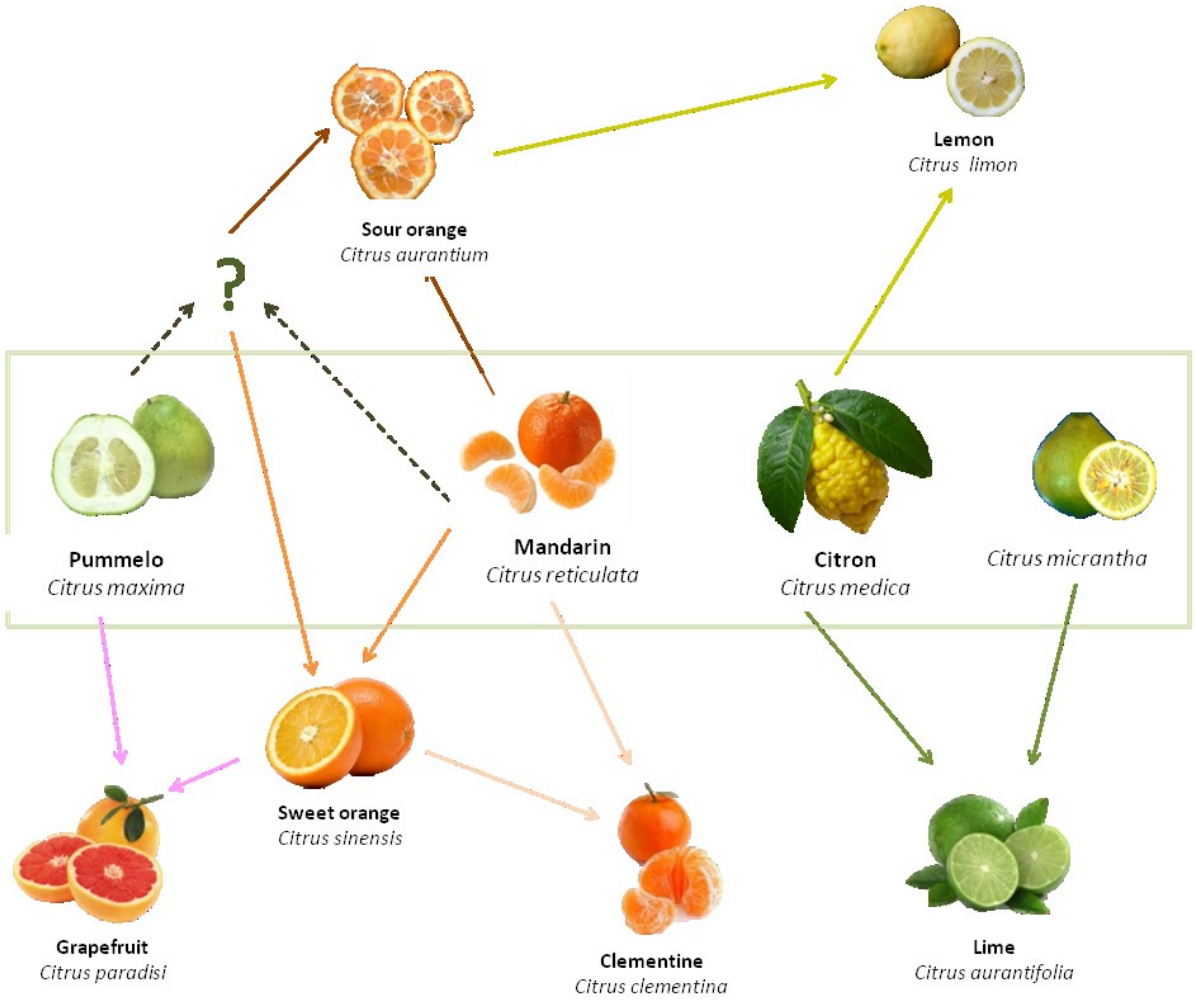



**Figure 6**: Phylogeny of cultivated citrus (secondary species) from ancestral taxa

## Citrus essential oils and their properties

### Essential oil composition

Essential oils are complex mixtures of organic substances of different nature, characterized by the presence of one or a few major aromatic compounds. It always knows their biological activities, including antimicrobial and antioxidant activity (Fancello et al., 2016). Once extracted appear to be oily substances, liquid, volatile and fragrant, recalling, for their scents, the botanical origin of the plant they come from. The chemical classes most represented in a citrus oil are the terpenes, the benzene compounds and other compounds containing sulfur and nitrogen. The d-limonene is the most represented compound in zest essential oil. In the essences of oranges, clementines and grapefruit, d-limonene is present for 90-95%, while down to 60-75% in lemon and mandarin essences. In bergamot it is contained in percentages about 30-50. The characteristic flavor of an essential oil depends on the content in oxygenated terpenes, aldehydes, ketones, esters and alcohols; in fact they give the essence of freshness contributions. In all citrus essential oils it is a non-volatile component consists of waxes, carotenoids, chlorophyll, flavonoids and coumarins. From a physical point of view are insoluble, or almost, in water, but transported by water vapor in spite of their high boiling point (150-300°C) (Poiana, 2009).

In citrus, essential oils are found mainly in fruits, located in the outer part of the fruit (exocarp), in oil glands, called utricles. They can also locate in the albedo and the pulp though to a much lesser degree. Moreover, they are present in varying degrees in leaves, flowers and twigs.

It is therefore also possible to obtain essences or distillates starting with other parts of the plant: neroli, from the distillation of the flowers; the petit-grain, from the distillation of the leaves; aromatic water, by distillation of other parts.

It has already been said, that the chemical component affects the aroma of an essential oil. Among these one of the most known is that of the lemon. In the volatile fraction the most found component is the limonene, followed by α-pinene and γ-terpinene, all molecules of terpene hydrocarbons. Among the oxygen compounds, 2-7% of the total composition, the most represented are aldehydes: neral and geranial. In general, the most precious oils are that obtained from winter lemons, since a greater presence of aldehydes (Poiana, 2009).

In the oil of sweet orange, limonene constitutes 90% of the volatile component. It was also observed that among blonde pulp fruits and pigmented pulp fruits there is a difference in the proportion of aldehydes, greater in the first, and in the percentage of valencene, characteristic component of this essence, higher in seconds.

In the essential oil of sour orange, made up almost entirely by limonene, only the linalyl acetate, among the oxygenated compounds, exceed 1%.

The bergamot essential oil is the most peculiar. In this oil the hydrocarbon fraction decreases to benefit of oxygenated compounds, and in particular terpene alcohol linololo, in the laevorotatory optically active form and its ester linalyl acetate. The essential oil is produced in a defined area of Calabria and in a very narrow time span between December and February. The time of harvest affects the ratio of linalool and linalyl acetate. At the beginning of season, the alcohol is in greater quantities and tends to decrease in the following months, while its ester has opposite trend.

The essential mandarin oils are very variable in their composition, as they are influenced by the variability among the different species sousually denominated, and by the yield time, very wide for mandarins. The volatile fraction of the mandarin consists of limonene and γ-terpinene and is characterized by the presence of N-methyl anthranilate of methyl, an ester content in concentrations of less than 1%, but important marker of this oil.

Grapefruit is not suitable to the extraction of the oil. In addition to the presence of elevated limonene concentration, it is important to highlight the presence of a compound characterizing this oil: the nootkatone.

The essential oil extraction of citron has never been grown since the characteristics of the fruit (% high albedo and irregular shape of the flavedo) lend little oil extraction. It is very similar, for qualitative characteristics, to lemon.

**Uses and virtues**

Among the properties of citrus essential oils, antioxidant and antimicrobial are the most important and interesting for human health. The antioxidant capacity was the subject of several studies (Sahin et al., 2004; Malhotra et al., 2009; Dambolena et al., 2010; Viuda-Martos et al., 2010). Among the terpenic compounds, the highest antioxidant activity is given by carvacrol, thymol and cresol (Dapkevicius et al., 1998). Sanches-Silvan et al. (2014)

suggest the use of some essential oils as a natural source of antioxidants, in food packaging. Essential oils are recognized also as powerful antimicrobial agents (Burt, 2004; Lang and Buchbauer, 2012; Tongnuanchan and Benjakul, 2014). In recent years several studies have demostrated their effectiveness against species of *Aspergillus* and *Penicillium*. It was analyzed each oil constituent in order to identify the most effective components. Some authors correlate the antimicrobial activity of citrus oil to the major constituent of the oil, whereas others claim that the activity is linked to synergistic or antagonistic effects of the diverse components present in it (Fancello et al., 2016).

Citrus essential oils are used in several fields. The essential oil of bergamot, for example, is used mainly in the fragrance industry. It has become an important constituent and fixative for cologne, soaps and perfumes. Only in recent years, it has found, in addition, application as a flavoring of foods.

The essential oil of lemon, for its characteristics of freshness, finds application in the formulation of beverages, as a flavoring in baked goods and candies. Its more important use is found in pharmaceutical products as a flavoring additive in formulations.

The essential oil of sweet orange, typical for the aroma that reminds the sweet tones of the fruit, is used as a flavoring agent in drinks, baked goods, candy, and pastries. Unlike other citrus, its use in the pharmaceutical and cosmetics is limited.

The sour orange oil is widely used in perfumery and cosmetics industry for its bitter tone reminiscent of mandarin. The neroli oil, obtained from the distillation of sour orange flowers, is widely used in the production of perfumes for its floral and sweet characteristics.The oil of "petit-grain", obtained by the distillation of leaves and twigs of sour orange, produces a more strong and fruity oil respect to neroli and it is used in the fragrance industry for the production of soaps and detergents.

Important studies were conducted using the essential oil of the leaves, aimed to the characterization of varieties and species within the genus *Citrus* and the environmental impact assessment in oil chemical variability (Lota et al., 1999, 2002; Tomi et al., 2008). This is because the use of citrus leaf essential oil has enormous advantages compared to that obtained from the fruits. Firstly, because the leaf is an ever-present element in the tree, and secondly because the essential oil of the leaf has a very different composition, not dominated by

limonene or limonene / γ-terpinene, which represent more than 70% oil of the rind of the fruit (Luro et al., 2012).

**Extraction techniques**

In citrus the most commonly methods used for the extraction of the essential oils are mainly two: hydrodistillation and the squeezing. The hydrodistillation or steam distillation is a method mainly used for the production of the essential oil of the leaves, young fruits and leaves (petit-grain) and flower (neroli). This type of extraction exploits the properties of essential oils to be volatile, easily vaporized and dragable by water vapor. Using this method the drug for distillation can be used both fresh and dried. Usually it is used material in the fresh state and the time that elapses between the collection and the distillation must be as short possible so as to not alter the characteristics of the oil and prevent its dispersion. Squeezing or pressing is used for the essential oil extraction from the rind of the fresh fruit. Once it has done manually, now by means of special equipment (hydraulic press, squeezer), by which the endocarp juice is extracted before and then the essence from the rind.

**Essential oil characterization**

***Gas Chromatography and Mass Spectrometry (GC/MS)***

To date, the most widely used analytical technique for the characterization of an oil is the one that matches the gas-chromatography (GC) and mass spectrometry (MS) allowing the identification of different analytes present in complex matrices (Qiao et al., 2008; Reinhard et al., 2008; Song et al., 2000; Smith et al., 2001; Cevallos-Cevallos et al., 2011; Barrek et al., 2003; Malhotra et al., 2016; Azimi and Fatemi, 2016; Prasad et al., 2016; Luro et al., 2012; Dar et al., 2016; Duan et al., 2016; Deng et al., 2017). The GC/MS applications are very broad and cover both the identification of unknown substances and the analysis of trace substances. It decides, generally, to pair to take advantage the advantages of both techniques, carry out a synergic study, fast, simple, sensitive, not over consumption of material use and with a great power of separation and resolution.

The gas chromatography was used for the first time in 1941 by Nobel prizes Martin and Synge, and only after ten years it became customary in the laboratory. The features that made a wide use of gas chromatography technique can be summarized as follows:

- very reduced time for analysis

- possibility to separate any mixture of substances, by operating under appropriate conditions
- possibility to carry out analyzes in series, since the same column can be regenerated continuously through the carrier gas
- high sensitivity (amount of analyzable substance$10^{-5}$-$10^{-12}$ g)

The GC analyzes are applicable to volatile substances or rendered such by vaporization, and are carried by the gas chromatograph. The separation process is based on the distribution of the mixture components between two phases: a fixed phase (stationary) constituted by a granular solid or liquid on a support of inert particles and a moving (mobile). The mobile phase is an inert gas that does not react with the analyte, but it works as a carrier and pass through a column in which is located the fixed phase. The columns can be capillaries, and then the stationary phase is deposited as a film on the thin wall, and the gas (hydrogen, helium, nitrogen, argon) passes into the lumen of the tubular structure; or they can be packed by a porous solid (silica gel, alumina) or liquid (oil, polyethylene glycol) that soaks into inert substances such as diatomaceous substratum. The mixture is introduced into the top of the mobile phase of the separation system such that the components have the opportunity to interact with the stationary phase. After the sample has been injected, the mobile phase carries it through the chromatographic system. The components present in it can interact with the stationary phase to which will have different affinities. The transport of the analytes will occur only in the moment in which they will be present in the mobile phase. At the end of the column is placed a detector capable of highlighting the various substances that emerge at different times, emitting a signal with an intensity proportional to their concentration, the signal is recorded by a tool so which will result in a chromatogram.

With the resolution of the GC capillary columns it is of better quality to other methods, and also, it is easily interfaced with the mass spectrometer (Skoog et al., 1996). In this case, the output of the separation column, the different components of the mixture, are sent to the detector to mass spectrometry.

The MS is used to measure the molecular masses and determining the structural formula of unknown compounds, also having small quantities available. But it is a destructive method of analysis and is not based on the interaction between radiation and materials. The principle on which it is based is the following: a molecule of which you wish to measure the mass properties, must first be volatilized and ionized in a component of the spectrometer called

ionization source. It can not be obtained a mass spectrometry unless you produce ions in the gas phase that will subsequently be accelerated until reaching a specific speed by means of an electric field, and then screened in a mass analyzer which separates appropriate entities of different masses and, finally, it will be so distinguished. The result is a mass spectrum, which represents the relative abundance of the ions as a function of their relationship mass/charge characteristic of each compound, since it is directly related to its chemical structure and to the ionisation conditions which it was subjected, which will be an fingerprint of the identified compound. It presents itself as a set of vertical lines (peaks) of different intensity, corresponding each one to the value of mass of a fragment ion. The higher mass peak value is related to the molecular ion. Generally, the ionic current is normalized to 100, being the highest peak (base peak) of value equal to 100, regardless of its absolute value. From the mass spectrum, the structure of an unknown compound can be traced back, attributing to each ion an elementary composition and rebuilding fragmentation mechanisms that follow typical patterns for the various classes of compounds.

## Evolution of the molecular tools for identifying the species

The correct identification of the species is at the basis of the study of plants and it is also essential for the preservation of resources. Modern techniques, based on DNA analysis, exploiting the high degree of polymorphism, the independence from the environment, cultivation and the degree of development of the plant, as well as the reproducibility of the results, clarify many doubts regarding existing relationships among the various genotypes of citrus (Germanà, 2009). Different tools, in constant evolution, are already available, some widely used. In 20 years, the evolution of molecular markers was greatly enriched the study of diversity and phylogeny of plants. Many studies in recent years have focused on citrus giving a multitude of information that can be used and reproduced.

### *Analysis of the nuclear genome*

#### *SSR (Simple Sequence Repeats)*

The SSR markers, or microsatellites most commonly, allow to highlight possible polymorphisms at the level of DNA repeated sequences. Dispersed through the genome of plants, many sequences exist, also structurally very simple, which are repeated a variable number of times. SSR markers are very effective tools and suitable for the genotypic typing, varietal identification and conduct of evolutionary studies, as they are single-locus markers of

co-dominant type. They allow distinguishing homozygous from heterozygous loci represented by a single size of amplified DNA fragment in the case of homozygote and from two different size of amplified DNA fragment in the case of heterozygotes. They are generally highly polymorphic detecting many alleles at the same locus.The SSR markers present, however, some limitations in the study of the structure and distribution of genetic variation within and among populations (Barcaccia and Falcinelli, 2006). They, in fact, are sensitive to homoplasy, or identity of situation (identity-in-state), but not of derivation identity (identity-by-descent). This means, that species, or populations within a single species, apparently close to the fact that they possess similar alleles at the same locus SSR, may actually differ for the origin of the repeated sequences. Identical alleles can then actually be different for independent genetic events. In addition, these markers do not allow getting to the specificities of the research level. Genotyping studies show, in fact, very frequent occurrence of common alleles at least to two ancestral taxa (García-Lor et al., 2012; Ollitrault et al., 2012b; García-Lor et al., 2013b), but it's easy also to find alleles specific to ancestral species (Curk et al 2016). Despite these restrictions, the SSR markers still find many phylogenetic applications and evolutionary investigations. Today there are many SSR markers available for genetic research of citrus (Novelli et al., 2000; Roose et al., 2000; Corazza-Nunes et al., 2002; Liu et al., 2002; Ahmad et al., 2003; Golein et al., 2005; Barkley et al., 2006 ; Jiang et al., 2006; Novelli et al., 2006; Caruso et al., 2008; Chenet al., 2008; Froelicher et al.,2008 ; Luro et al., 2008; Nematollahi et al., 2009; Ghorabaie et al., 2010; Gulsen et al., 2010; Ollitrault et al., 2010; Amar et al., 2011; Biswas et al., 2011; Cristofani-Yaly et al., 2011; El-Mouei et al., 2011; Kamiri et al., 2011; Ollitrault et al., 2011; Singh et al., 2011; Uzun et al., 2011; Biswas et al., 2012; García-Lor et al., 2012; Golein et al., 2012; Ollitrault et al., 2012b; Polat et al., 2012; Snoussi et al., 2012; Chai et al., 2013; Kacar et al., 2013; Liuet al., 2013; Yildiz et al., 2013; Hou et al., 2014).

***InDel (Insertion/Deletion)***

The term InDel indicates Insertion or Deletion in the DNA sequence locatable through the comparison of the length of the fragments of a same area of the genome, amplified by PCR. The InDel normally appear as a result of insertion of transposable elements, or for a shift of a single sequence during replication or in response to events of genetic recombination. Unlike the SSR, the chances of homoplasy are very low so they are very suitable for phylogenetic studies (Britten et al., 2003). Moreover, they are easily highlighted by electrophoresis targeted products of the PCR (Vasemägi et al., 2010). Recently, they have also found ample space in genetic studies of the citrus, since they are considered one of the major initial divergence

factors and probably represent the most rapid and significant form of variation of the sequence in the evolution of eukaryotes (Britten et al., 2003; Ollitrault et al., 2011; García-Lor et al., 2012; Ollitrault et al., 2012; Ollitrault et al., 2012b; Snoussi et al., 2012; García-Lor et al., 2013b). Compared to the coding regions, non-coding regions show a higher frequency of InDels (Tian et al., 2008). These markers have been significantly been ignored in phylogenetic reconstruction and the gaps created by InDel were punctually removed from the alignment of the sequences as they are considered missing data (Mahadani and Ghosh, 2014). Recent studies have shown that InDel greatly increase the number of informative phylogenetic characters (Blair and Murphy, 2011). Nevertheless, the degree of polymorphism of InDel markers is lower than SSR markers (Garcia-Lor et al. 2012).

***Analysis of cytoplasmic genomes***

The analysis of the chloroplast DNA (cpDNA) is very usefull for phylogenetic and evolutionary studies in plants, because of its non-recombinant nature, its high presence in the tissues, the small size and predominantly uniparental inheritance (Olmstead and Palmer, 1994). The maternal phylogenetic study can be carried out on citrus through the use of chloroplast markers, either RFLP (Gulsen and Roose, 2001b), InDel (De Araújo et al., 2003), CAPS (Nicolosi et al., 2000; Lotfy et al., 2003; Yamamoto et al., 2013), cpSSR (Cheng et al., 2005; Li et al., 2006; Deng et al., 2007) and comparing the sequences of the chloroplast genes (Jung et al., 2005; Bayer et al., 2009; Lu et al., 2011; Wali et al., 2013). In addition to chloroplast markers, mitochondrial markers have been developed (Yamamoto et al., 1993; Froelicher et al., 2011; Snoussi et al., 2012). A greater number of polymorphisms are generally found in the chloroplast and mitochondrial genomes of the *Citrus* genus(Lotfy et al., 2003). The mtDNA genetic role seems to be universally conserved, but this genome shows remarkable variation of shape and dimensions (Gray et al., 1999). The PCR markers show a low level of discrimination when it comes to mtDNA (Engelke and Tatlioglu, 2004), probably due to a low of nucleotide substitution (Muse, 2000).

Since 2006, complete sequence of the chloroplast genome of the sweet orange is available (Bausher et al., 2006).

***DNA barcodes***

DNA barcoding was proposed for the first time as a system for identification of the species by Hebert et al. (2003a). It is a technique, which uses DNA sequences arising from small fragments of the genome to identify the species. It is capable, therefore, to contribute to the

study of biodiversity, which, shortly before, was mainly based on the morphology according to the Linnaean classification system (Nicolè et al., 2011).

Hebert et al. (2003b) analized the divergence of the sequence in more than 13,000 congeneric pairs representing the 11 phyla, and found that the diagnosis at the species level could be obtained by analyzing the CO1 (cytochrome c oxidase 1). It was, therefore, determined that the identification of biological animal diversity could be obtained through the use of short DNA sequences of the CO1 genes (Hebert et al., 2003a; Hebert et al., 2003b; Hebert et al., 2004a ; Hebert et al., 2004b; Hogg and Hebert, 2004).

The study of the barcode in plants is much more complex than that of animals because of the greater hybridization attitude and the more complex evolutionary history (Kress et al., 2005; Newmaster et al., 2006).

Disputes concerning the value of DNA barcoding (Holmes, 2004) arise mostly because of the perception that determinations of the species, based solely on the amount of genetic divergence, could lead to an incorrect detection of the species and that DNA barcoding could help identify, as a means for phylogenetic reconstruction, rather than as a tool to be mainly used for identification purposes (Scotland et al., 2003; Seberg et al., 2003; Will and Robinoff, 2004). Along with doubts, in the same years, many of the technical support to the studies were used as a process of identification at the species level(Besansky et al.,2003; Janzen,2004; 2005; Hebert et al.,2003a;b; 2004a;b; Kress,2004). If the DNA barcoding found immediate success in the field of animals, the same did not happen to plants. The reason for this lies in the fact that the mitochondrial genes of plants are not good candidates for discrimination to the species level, due to their low rate of change of the sequences. In fact, the divergences of the coding regions CO1 among the families of the plants was detected be just few bases (Cho et al., 1998;2004). Moreover, plants change quickly mitochondrial genomic structure (Adams and Palmer, 2003) thereby denying the existence of intergenic spacers that would be the unique universal identifiers of variability at the species level (Luo et al., 1995).

In 2009, the working group of plants of Consortium for the Barcode of Life (CBOL) establishes the association of two cloroplastic gene regions, *rbcL* and *matK*, as a universal guideline for the habitual use of barcode data, for distinguishing samples at the species level. The *rbcL* sequence is remarkably easy to align and amplify. In fact, there are a large number of available sequences in GenBank. The use limit of this region is that it can discriminate well

enough at genus and family level, but not at the species level. The sequence *matK* shows a higher level of variation of the sequence, and thus can discriminate better at the species level, but it is amplified with ineffective primers (Luo et al., 2010). Some authors also suggest to combine the spacer inter-gene *psbA-trnH* to *rbcL* gene as an alternative to *matK* (Kress and Erickson, 2007). Finally, even if the DNA sequences of the organelles are the primary source for a barcoding system, one or more nuclear genes are normally added to the analysis in the case of hybrids (Nicolè et al., 2011).

The nuclear locus most commonly used for systematic studies in plants at the species level, is the region of the internal transcribed spacer (ITS), between the genes coding for ribosomal RNA (rRNA) (Alvarez and Wendel, 2003). Generally, the rDNA intergenic spacers group evolves rapidly and is highly polymorphic. It provides, therefore, an excellent tool for the evaluation of genetic variability, taxonomic and phylogenetic population studies (Singh et al., 2008).

The region ITS, comprising the ITS1 (including sequences between the 18S and 5.8S rDNA) and ITS2 (including sequences between the 5.8S and 28S rDNA), is an area of particular importance for the discrimination at the species level. The ITS sequences show a greater divergence of sequence with respect to the coding regions that accompanying them (Kollipara et al., 1997), in fact they are excised from the RNA in a splice-post-transcriptional process and therefore, not coding for a gene product, are subject to high variability of sequence. They are used to distinguish among related plant species and to infer phylogenetic relationships among populations from families (Kolchinski et al., 1991). Moreover, their location between conserved regions (18S and 28S rDNA) allows the use of universal primers. For all these reasons it has been identified as a possible plant locus barcode.

In general, a region to be considered barcode must satisfy three requirements: significant variability and divergence at the species level; possess of appropriate size to facilitate DNA extraction and amplification; possess of flanking conserved regions to develop universal primers (Kress et al., 2005).

## References


Adams K.L., Palmer J.D., 2003. Evolution of mitochondrial gene content: gene loss and transfer to the nucleus. Molecular phylogenetics and evolution, 29(3): 380-395

Ahmad R., Struss D., Southwick S.M., 2003. Development and characterization of microsatellite markers in Citrus. Journal of the American Society for Horticultural Science, 128(4): 584-590

Álvarez I., Wendel J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Molecular phylogenetics and evolution, 29(3): 417-434

Amar M.H., Biswas M.K., Zhang Z., Guo W.W., 2011. Exploitation of SSR, SRAP and CAPS-SNP markers for genetic diversity of Citrus germplasm collection. Scientia Horticulturae, 128(3): 220-227

Azimi F., Fatemi M.H., 2016. Multivariate curve resolution-assisted GC-MS analysis of the volatile chemical constituents in Iranian Citrus aurantium L. peel. RSC Advances, 6(112): 111197-111209

Barbera G., 2009. Paesaggi degli agrumi. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Barcaccia G., Falcinelli M., 2006. Genetica e genomica. Genomica e Biotecnologie genetiche Vol. III

Barkley N.A., Roose M.L., Krueger R.R., Federici C.T., 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theoretical and applied genetics, 112(8): 1519-1531

Barrek S., Paisse O., Grenier-Loustalot M.F., 2003. Analysis of pesticide residues in essential oils of citrus by GC–MS and HPLC–MS after solid-phase extraction. Analytical and bioanalytical chemistry, 376(2): 157-161

Barrett H.C., Rhodes A.M., 1976. A numerical taxonomic study of affinity relationships in cultivated Citrus and its close relatives. Syst Bot 1: 105-136

Bausher M.G., Singh N.D., Lee S.B., Jansen R.K., Daniell H., 2006. The complete chloroplast genome sequence of Citrus sinensis (L.) Osbeck var'Ridge Pineapple': organization and phylogenetic relationships to other angiosperms. BMC Plant Biology, 6(1): 21

Bayer R.J., Mabberley D.J., Morton C., Miller C.H., Sharma I.K., Pfeil B.E., Rich S., Hitchcock R., Sykes, 2009. A molecular phylogeny of the orange subfamily (Rutaceae: Aurantioideae) using nine cpDNA sequences. American Journal of Botany, 96(3): 668-685

Berhow M.A., Hasegawa S., Kwan K., Bennett R.D., 2000. Limonoids and the chemotaxonomy of Citrus and the Rutaceae family. In: M.A. Berhow, S. Hasegawa, G.D. Manners (eds.). Citrus limonoids: Functional Chemicals in Agricolture and Foods, American Chimical Society, Washington D.C.

Besansky N.J., Severson D.W., Ferdig M.T., 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. Trends in parasitology, 19(12): 545-546

Biraghi A., 1935. Rilievi su alcuni citrus a frutto acido presenti in India in relazione alla ricerca di forme resistenti al mal secco. Bollettino della Reale Stazione di Patologia Vegetale, Roma Bol. (n.s.) 15: 424-441

Biswas M.K., Chai L., Amar M.H., Zhang X., Deng X.X., 2011. Comparative analysis of genetic diversity in Citrus germplasm collection using AFLP, SSAP, SAMPL and SSR markers. Scientia horticulturae, 129(4): 798-803

Biswas M.K., Chai L., Mayer C., Xu Q., Guo W., Deng X., 2012. Exploiting BAC-end sequences for the mining, characterization and utility of new short sequences repeat (SSR) markers in Citrus. Molecular biology reports, 39(5): 5373-5386

Blair C., Murphy R.W., 2011. Recent trends in molecular phylogenetic analysis: where to next?. Journal of Heredity, 102(1): 130-138

Britten R.J., Rowen L., Williams J., Cameron R.A., 2003. Majority of divergence between closely related DNA samples is due to indels. Proceedings of the National Academy of Sciences, 100(8): 4661-4665

Burt S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. International journal of food microbiology, 94(3): 223-253

Calabrese F., 2009a. Gli agrumi nella storia. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Calabrese F., 2009b. Tassonomia. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Calabrese F., Barone F., 2009. Lime e Limette. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Camarda I., Mazzola P., Brunu A., Fenu G., Lombardo G., Palla F., 2013. Un agrume nella storia della Sardegna: Citrus limón var. Pompia Camarda var. nova. Quad. Bot. Amb. Appl. 24: 109-118

Caruso M., Federici C.T., Roose M.L., 2008. EST–SSR markers for asparagus genetic diversity evaluation and cultivar identification. Molecular Breeding, 21(2): 195-204

CBOL Plant Working Group, 2009. A DNA barcode for land plants. Proceedings of the National Academy of Sciences, 106(31): 12794-12797

Cevallos- Cevallos J.M., García- Torres R., Etxeberria E., Reyes- De- Corcuera J.I., 2011. GC- MS Analysis of Headspace and Liquid Extracts for Metabolomic Differentiation of Citrus Huanglongbing and Zinc Deficiency in Leaves of 'Valencia'Sweet Orange from Commercial Groves. Phytochemical Analysis, 22(3): 236-246

Chai L., Biswas M.K., Yi H., Guo W., Deng X., 2013. Transferability, polymorphism and effectiveness for genetic mapping of the Pummelo (Citrus grandis Osbeck) EST-SSR markers. Scientia Horticulturae, 155: 85-91

Chen C., Bowman K.D., Choi Y.A., Dang P.M., Rao M.N., Huang S., Soneji J.R., McCollum T.G., Gmitter Jr F.G., 2008. EST-SSR genetic maps for Citrus sinensis and Poncirus trifoliata. Tree Genetics & Genomes, 4(1): 1-10

Cheng Y., De Vicente M.C., Meng H., Guo W., Tao N., Deng X., 2005. A set of primers for analyzing chloroplast DNA diversity in Citrus and related genera. Tree physiology, 25(6): 661-672

Chessa I., Mulas M., Pala M., 1994. Gli agrumi. In: (edited by M. Agabbio) Patrimonio Genetico di Vecchie Specie Arboree da Frutto: Le vecchie varietà della Sardegna: 339-360. Carlo Delfino Editore, Sassari

Cho Y., Mower J.P., Qiu Y.L., Palmer J.D., 2004. Mitochondrial substitution rates are extraordinarily elevated and variable in a genus of flowering plants. Proceedings of the National Academy of Sciences of the United States of America, 101(51): 17741-17746

Cho Y., Qiu Y.L., Kuhlman P., Palmer J.D., 1998. Explosive invasion of plant mitochondria by a group I intron. Proceedings of the National Academy of Sciences, 95(24): 14244-14249

Corazza-Nunes M., Machado M., Nunes W., Cristofani M., Targon M., 2002. Assessment of genetic variability in grapefruits (Citrus paradisi Macf.) and pummelos (C. maxima (Burm.) Merr.) using RAPD and SSR markers. Euphytica, 126(2): 169-176

Cristofani-Yaly M., Novelli V.M., Bastianel M., Machado M.A., 2011. Transferability and level of heterozygosity of microsatellite markers in Citrus species. Plant Molecular Biology Reporter, 29(2): 418-423

Curk F., Ollitrault F., Garcia-Lor A., Luro F., Navarro L., Ollitrault P., 2016. Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. Annals of Botany 117 (4), 565-583. Doi:10.1093/aob/mcw005

D'Aquino S., Fronteddu F., Usai M., Palma A., 2005. Qualitative and physiological properties of 'Pompia', a citron-like fruit. Plant Genetic Resources Newsletter 143: 40-45

Davies F. S., Albrigo L.G., 1994. Citrus. Crop Production Science in Horticulture. Ed. CAB International

Dambolena J.S., Zunino M.P., Lucini E.I., Olmedo R., Banchio E., Bima P.J., Zygadlo J.A., 2010. Total phenolic content, radical scavenging properties, and essential oil composition of origanum species from different populations. Journal of agricultural and food chemistry, 58(2): 1115-1120

Dapkevicius A., Venskutonis R., van Beek T.A., Linssen J.P., 1998. Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. Journal of the Science of Food and Agriculture, 77(1): 140-146

Dar M.S., Lawrence R., Khan A.N., 2016. Evaluation of the chemical constituens and the antibacterial activity of essential oil of Citrus karna fruit peel. International Journal of Pharmaceutical Sciences and Research, 7(3): 1245

De Araújo E.F., de Queiroz L.P., Machado M.A., 2003. What is Citrus? Taxonomic implications from a study of cp-DNA evolution in the tribe Citreae (Rutaceae subfamily Aurantioideae). Organisms Diversity & Evolution, 3(1): 55-62

Dedola S., linguist. Personal Communication, 2016

Deng G., Craft J.D., Steinberg K.M., Li P.L., Pokharel S.K., Setzer W.N., 2017. Influence of Different Isolation Methods on Chemical Composition and Bioactivities of the Fruit Peel Oil of Citrus medica L. var. sarcodactylis (Noot.) Swingle. Medicines, 4(1): 1

Deng Z., La Malfa S., Xie Y., Xiong X., Gentile A., 2007. Identification and evaluation of chloroplast uni-and trinucleotide sequence repeats in citrus. Scientia horticulturae, 111(2): 186-192

Deng Z.N., Gentile A., Nicolosi E., Continella G., Tribulato E., 1996. Parentage determination of some citrus hybrids by molecular markers. In Proc Int Soc Citricul (2): 849-854

Di Giacomo A., 1989. Il bergamotto. Baruffa (ed), Reggio Calabria

Duan L., Guo L., Dou L.L., Zhou C.L., Xu F.G., Zheng G.D., Li P., Liu E.H., 2016. Discrimination of Citrus reticulata Blanco and Citrus reticulate 'Chachi' by gas chromatograph-mass spectrometry based metabolomics approach. Food Chemistry, 212: 123-127

El-Mouei R., Choumane W., Dway F., 2011. Characterization and Estimation of Genetic Diversity in Citrus Rootstocks. International Journal of Agriculture & Biology, 13(4)

Engelke T., Tatlioglu T., 2004. The fertility restorer genes X and T alter the transcripts of a novel mitochondrial gene implicated in CMS1 in chives (Allium schoenoprasum L.). Molecular Genetics and Genomics, 271(2): 150-160

Fancello F., Petretto G.L., Zara S., Sanna M.L., Addis R., Maldini M., Foddai M., Rourke J.P., Chessa M., Pintore G., 2016. Chemical characterization, antioxidant capacity and antimicrobial activity against food related microorganisms of Citrus limon var. Pompia leaf essential oil. LWT-Food Science and Technology, 69: 579-585

Fancello G., (2012) Taccuinistorici.it – Pompia. Avaliable at : http://www.taccuinistorici.it/ita/news/moderna/prodotti-tradizionali-italiani/Pompia.html

Fanciullino A. L., Dhuique-Mayer C., Luro F., Casanova J., Morillon R., Ollitrault P., 2006. Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. Journal of Agricultural and Food Chemistry, 54(12): 4397-4406

Fatta del Bosco G., 2009. Gli agrumi nei giardini storici. In: Citrus: Trattato di agrumicoltura. (a cura di V. Vacante e F. Calabrese). Edagricole, Milano

Federici C. T., Fang D. Q., Scora R. W., Roose M. L., 1998. Phylogenetic relationships within the genus Citrus (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theoretical and Applied Genetics, 96(6-7): 812-822

Fenu G., Carai A., Foddai M., Azara E., Careddu S., Usai M., 2010. Composition and seasonal variation of Citrus mostruosa essential oil from Sardinia. Int. J. Essent. Oil Therap, 4: 23-25

Fraternale D., Giamperi L., Bucchini A., Cara P., Ricci D., 2010. In vitro plant regeneration from callus of Citrus x monstruosa (Pompia), an endemic citrus of Sardinia. Natural product communications, 5(6): 927-930

Froelicher Y., Dambier D. ,Costantino G. , Lotfy S. , Didout C. , Beaumont V. , Brottier P. , Risterucci A.M. ,Luro F.and Ollitrault P.,2008Characterization of microsatellite markers in *Citrus reticulata* Blanco. Molecular Ecology Notes 8 (1):119-122

Froelicher Y., Mouhaya W., Bassene J.B., Costantino G., Kamiri M., Luro F., Morillon R., Ollitrault P., 2011. New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. Tree Genetics & Genomes, 7(1): 49-61

Gallesio G., 1811. Traitè du Citrus. Louis Fantin, Paris

Garcìa-Lor A., Ancillo G., Navarro L., Ollitrault P., 2013a. Citrus (rutaceae) SNP markers based on competitive allele-specific PCR; transferability across the aurantioideae subfamily. Applications in plant sciences, 1(4): 1200406

Garcìa-Lor A., Curk F., Snoussi-Trifa H., Morillon R., Ancillo G., Luro F., Navarro L., Ollitrault P., 2013b. A nuclear phylogenetic analysis: SNPs, indels and SSRs deliver new insights into the relationships in the 'true citrus trees' group (Citrinae, Rutaceae) and the origin of cultivated species. Annals of botany, 111(1): 1-19

García-Lor A., Luro F., Navarro L., Ollitrault P., 2012. Comparative use of InDel and SSR markers in deciphering the interspecific structure of cultivated citrus genetic diversity: a perspective for genetic association studies. Molecular genetics and genomics, 287(1): 77-94

Germanà M.A., 2009. Il miglioramento genetico degli agrumi: metodi tradizionali ed innovativi. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Ghorabaie H.R., Ghazvini R.F., Golein B., Nabipour A.R., 2010. Identification of some Citrus accessions in a Citrus germplasm utilizing simple sequence repeat markers (SSRs). Horticulture Environment and Biotechnology, 51(4): 343-347

Golein B., Bigonah M., Azadvar M., Golmohammadi M., 2012. Analysis of genetic relationship between 'Bakraee'(Citrus sp.) and some known Citrus genotypes through SSR and PCR-RFLP markers. Scientia horticulturae, 148: 147-153

Golein B., Talaie A., Zamani Z., Ebadi A., Behjatnia A., 2005. Assessment of genetic variability in some Iranian sweet oranges (Citrus sinensis [L.] Osbeck) and mandarins (Citrus reticulata Blanco) using SSR markers. International Journal of Agriculture and Biology, 7(2): 167-170

Gray M.W., Burger G., Lang B.F., 1999. Mitochondrial evolution. Science, 283(5407): 1476-1481

Green R.M., Vardi A., Galune E., 1986. The plastome of Citrus. Physical map, variation among Citrus cultivars and species and comparison with related genera. Theor Appl Genet 72 (2): 170–177

Gulsen O., Roose M. L., 2001b. Determination of genetic diversity and phylogenetic relations to citrus ancestors in lemons by DNA markers. Bahce, 30 (1-2): 53-63

Gulsen O., Roose M.L., 2001a. Lemons: diversity and relationships with selected Citrus genotypes as measured with nuclear genome markers. Journal of the American Society for Horticultural Science, 126(3): 309-317

Gulsen O., Uzun A., Canan I., Seday U., Canihos E., 2010. A new citrus linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers. Euphytica, 173(2): 265-277

Hebert P.D., Cywinska A., Ball S.L., deWaard J.R., 2003a. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B: Biological Sciences, 270(1512): 313-321

Hebert P.D., Penton E.H., Burns J.M., Janzen D.H., Hallwachs W., 2004b. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences of the United States of America, 101(41): 14812-14817

Hebert P.D., Ratnasingham S., de Waard J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London B: Biological Sciences, 270(Suppl 1): S96-S99

Hebert P.D., Stoeckle M.Y., Zemlak T.S., Francis C.M., 2004a. Identification of birds through DNA barcodes. Plos biol, 2(10): e312

Herrero R., Asins M. J., Pina J. A., Carbonell E. A., Navarro L., 1996. Genetic diversity in the orange subfamily Aurantioideae. II. Genetic relationships among genera and species. Theoretical and Applied Genetics, 93(8): 1327-1334

Hogg I.D., Hebert P.D., 2004. Biological identification of springtails (Hexapoda: Collembola) from the Canadian Arctic, using mitochondrial DNA barcodes. canadian Journal of Zoology, 82(5): 749-754

Holmes B., 2004. Barcode me. New Sci 2453: 32–35

Hou X.J., Liu S.R., Khan M.R., Hu C.G., Zhang J.Z., 2014. Genome-wide identification, classification, expression profiling, and SSR marker development of the MADS-box gene family in Citrus. Plant molecular biology reporter, 32(1): 28-41

Janzen D., 2004. Now is the time. Philosophical Transactions of the Royal Society B: Biological Sciences, 359(1444): 731-732

Janzen D., 2005. In Plant Conservation: A natural History Approach, eds. Krupnick G.A., Kress W.J.. Univ. Chicago Press, Chicago

Jiang D., Zhong G.Y., Qi-Bing H., 2006. Analysis of microsatellites in citrus unigenes. Acta Genetica Sinica, 33(4): 345-353

Jung Y.H., Kwon H.M., Kang S.H., Kang J.H., Kim S.C., 2005. Investigation of the phylogenetic relationships within the genus Citrus (Rutaceae) and related species in Korea using plastid trnL-trnF sequences. Scientia horticulturae, 104(2): 179-188

Kacar Y., Uzun A., Polat I., Yesiloglu T., Yilmaz B., Gulsen O., Tuzcu O., Kamiloglu M., Kurt S., Seday U., 2013. Molecular characterization and genetic diversity analysis of mandarin genotypes by SSR and SRAP markers. Journal of Food, Agriculture & Environment, 11(1): 516-521

Kamiri M., Stift M., Srairi I., Costantino G., El Moussadik A., Hmyene A., Bakry F., Ollitrault P., Froelicher Y., 2011. Evidence for non-disomic inheritance in a Citrus interspecific tetraploid somatic hybrid between C. reticulata and C. limon using SSR markers and cytogenetic analysis. Plant cell reports, 30(8): 1415-1425

Kolchinski A., Kolesnikova M., Ananiev E., 1991. "Potraying" of plant genomes using polymerase chain reaction amplification of ribosomal 5S genes. Genome (34): 1028-1031

Kollipara K.P., Singh R.J., Hymowitz T., 1997. Phylogenetic and genomic relationships in the genus Glycine Willd, based on sequences from the ITS region of nuclear rDNA. Genome (49): 57-68

Kress W.J., 2004. Paper floras: how long will they last? A review of Flowering Plants of the Neotropics. Am. J. Bot. 91: 2124-2127

Kress W.J., Erickson D.L., 2007. A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding psbA-trnH spacer region. PLoS one, 2(6): e508

Kress W.J., Wurdack K.J., Zimmer E.A., Weigt L.A., Janzen D.H., 2005. Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences of the United States of America, 102(23): 8369-8374

Lang G., Buchbauer G., 2012. A review on recent research results (2008–2010) on essential oils as antimicrobials and antifungals. A review. Flavour and Fragrance Journal, 27(1): 13-39

Li X., Xie R., Lu Z., Zhou Z., 2010. The origin of cultivated citrus as inferred from internal transcribed spacer and chloroplast DNA sequence and amplified fragment length polymorphism fingerprints. Journal of the American Society for Horticultural Science, 135(4): 341-350

Li Y.Z., Cheng Y.J., Yi H.L., Deng, X.X., 2006. Genetic diversity in mandarin landraces and wild mandarins from China based on nuclear and chloroplast simple sequence repeat markers. The Journal of Horticultural Science and Biotechnology, 81(3): 371-378

Liang G., Xiong G., Guo Q., He Q., Li X., 2007. AFLP analysis and the taxonomy of Citrus. Acta Horticulturae 760: 137-142

Liu J.H., Pang X.M., Cheng Y.J., Meng H.J., Deng X., 2002. Molecular characterization of the nuclear and cytoplasmic genomes of intergeneric diploid plants from cell fusion between Microcitrus papuana and rough lemon. Plant Cell Reports, 21(4): 327-332

Liu S.R., Li W.Y., Long D., Hu C.G., Zhang J.Z., 2013. Development and characterization of genomic and expressed SSRs in citrus by genome-wide analysis. PloS one, 8(10): e75149

Lota M.L., de Rocca Serra D., Tomi F., Bessiere J.M., Casanova J., 1999. Chemical composition of peel and leaf essential oils of Citrus medica L. and C. limonimedica Lush. Flavour and fragrance journal, 14(3): 161-166

Lota M.L., de Rocca Serra D., Tomi F., Jacquemond C., Casanova J., 2002. Volatile components of peel and leaf oils of lemon and lime species. Journal of Agricultural and Food Chemistry, 50(4): 796-805

Lotfy S., Luro F., Carreel F., Froelicher Y., Rist D., Ollitrault P., 2003. Application of cleaved amplified polymorphic sequence method for analysis of cytoplasmic genome among Aurantioideae intergeneric somatic hybrids. Journal of the American Society for Horticultural Science, 128(2): 225-230

Lu Z.H., Zhou Z.Q., Xie R.J., 2011. Molecular phylogeny of the "true citrus trees" group (Aurantioideae, Rutaceae) as inferred from chloroplast DNA sequence. Agricultural Sciences in China, 10(1): 49-57

Luo H., Van Coppenolle B., Seguin M., Boutry M., 1995. Mitochondrial DNA polymorphism and phylogenetic relationships in Hevea brasiliensis. Molecular Breeding, 1(1): 51-63

Luo K., Chen S., Chen K., Song J., Yao H., Ma X., Zhu Y., Pang X., Yu H., Li X., Liu Z., 2010. Assessment of candidate plant DNA barcodes using the Rutaceae family. Science China Life Sciences, 53(6): 701-708

Luro F., Costantino G., Terol J., Argout X., Allario T., Wincker P., Talon M., Ollitrault P., Morillon R., 2008. Transferability of the EST-SSRs developed on Nules clementine (Citrus clementina Hort ex Tan) to other Citrus species and their effectiveness for genetic mapping. BMC genomics, 9(1): 287

Luro F., Rist D., Ollitrault P., 2001. Evaluation of genetic relationships in Citrus genus by means of sequence tagged microsatellites. Acta Horticulturae 546: 237-242

Luro F., Venturini N., Costantino G., Paolini J., Ollitrault P., Costa J., 2012. Genetic and chemical diversity of citron (Citrus medica L.) based on nuclear and cytoplasmic markers and leaf essential oil composition. Phytochemistry, 77: 186-196

Mabberley D.J., 2004. Citrus (Rutaceae): a review of recent advances in etymology, systematics and medical applications. Blumea-Biodiversity, Evolution and Biogeography of Plants, 49(2-3): 481-498

Mahadani P., Ghosh S.K., 2014. Utility of indels for species-level identification of a biologically complex plant group: a study with intergenic spacer in Citrus. Molecular biology reports, 41(11): 7217-7222

Malhotra S., Shukla R., Kulshrestha S., Siddiqui A., 2016. Chemical Composition and Antioxidant Activity of Citrus Medica Var Acidica Essential Oil. Journal of Chemical, Biological and Physical Sciences (JCBPS), 6(2): 574

Malhotra S., Suri S., Tuli R., 2009. Antioxidant activity of citrus cultivars and chemical composition of Citrus karna essential oil. Planta medica, 75(1): 62-64

Malik M.N., Scora R.W., Soost R.K., 1974. Studies on the origin of the lemon. Hilgardia 42(9): 361-405

Manca Dell'Arca A., 1780. Agricoltura in Sardegna. Orsino, Napoli

Mignani I., Mulas M., Mantegazza M., Lovigu N., Spada A., Nicolosi E., Bassi D., 2015. Characterization by molecular markers of "Pompia" a natural Citrus hybrid cultivated in Sardinia. – Acta Hortic. (ISHS) 1065:165-172

Mignani I., Mulas M., Mantegazza R., Lovigu N., Spada A., Nicolosi E., Bassi D., 2004. Caratterizzazione morfologica, biochimica e molecolare di accessioni di "Pompia", agrume della Sardegna. In: Atti delle VII giornate scientifiche SOI, Castel dell'Ovo, Napoli

Milella A., 1960. Le cultivar di arancio diffuse in Sardegna. Riv. Ortoflorfrutt. It., XLIV (7-8): 3-42

Moris G.G., 1837/59. Flora sardoa. Tip. Regia, Torino

Muse S.V., 2000. Examining rates and patterns of nucleotide substitution in plants. Plant molecular biology, 42(1): 25-43

NCBI, 2014. http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi

Nematollahi A., Vahdati K., Golein B., Nabipour A., Danesh M., 2009. Assessment of Genetic Variation in Citrus Germplasm of South of Iran Using SSR Markers. Acta Horticulturae 839: I International Symposium on Biotechnology of Fruit Species: Biotechfruit 2008: 549-554

Newmaster S.G., Fazekas A.J., Ragupathy S., 2006. DNA barcoding in land plants: evaluation of rbcL in a multigene tiered approach. Botany, 84(3): 335-341

Nicolè S., Erickson D.L., Ambrosi D., Bellucci E., Lucchin M., Papa R., Kress W.J., Barcaccia G., 2011. Biodiversity studies in Phaseolus species by DNA barcoding. Genome, 54(7): 529-545

Nicolosi E., Deng Z.N., Gentile A., Malfa S.L., Ciotinella G., Tribulato E., 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor Appl Genet 100:1155–1166

Novelli V.M., Cristofani M., Machado M.A., 2000. Evaluation of microsatellite markers in cultivars of sweet orange (Citrus sinensis [L.] Osbeck). Acta Horticulturae 535: 47-50

Novelli V.M., Cristofani M., Souza A.A., Machado M.A., 2006. Development and characterization of polymorphic microsatellite markers for the sweet orange (Citrus sinensis L. Osbeck). Genetics and Molecular Biology, 29(1): 90-96

Ollitrault F., Terol J., Martin A.A., Pina J.A., Navarro L., Talon M., Ollitrault P., 2012. Development of InDel markers from Citrus clementina (Rutaceae) BAC-end sequences and interspecific transferability in Citrus. American journal of botany, 97(11): e124-9

Ollitrault F., Terol J., Pina J.A., Navarro L., Talon M., Ollitrault P., 2010. Development of SSR markers from Citrus clementina (Rutaceae) BAC end sequences and interspecific transferability in Citrus. American journal of botany, 97(11): e124-e129

Ollitrault P., Jacquemond C., Dubois C., Luro F., 2003. Citrus. In: Hamon P., Seguin M., Perrier X., Glaszmann, J.C. (eds). Genetic diversity of cultivated tropical plants. CIRAD, Montpellier

Ollitrault P., Terol J., Chen C., Federici C.T., Lofty S., Hippolyte I., Ollitrault F., Berard A., Chauveau A., Costantino G., Kacar Y., Mu L., Cuenca J., Garcia-Lor A., Froelicher Y., Aleza P., Boland A., Billot C., Navarro L., Luro F., Roose M.L., Gmitter F.G., Talon M., Brunel D., 2011. A reference linkage map of C. clementina based on SNPs, SSRs and indels. In Plant & Animal Genomes XIX Conference, January 15-19, 2011; San Siego, CA

Ollitrault P., Terol J., Chen C., Federici C.T., Lotfy S., Hippolyte I., Ollitrault F., Berard A., Chaveau A., Cuenca J., Costantino G., Kacar Y., Mu L., Garcia-Lor A., Froelicher Y., Aleza P., Boland A., Billot C., Navarro L., Luro F., Roose M.L., Gmitter F.G., Talon M., Brunel D., 2012b. A reference genetic map of C. clementina hort. ex Tan.; citrus evolution inferences from comparative mapping. BMC genomics, 13(1): 593

Ollitrault P., Terol J., Garcia-Lor A., Bérard A., Chauveau A., Froelicher Y., Belzile C., Morillon R., Navarro L., Brunel D., Talon M., 2012a. SNP mining in C. clementina BAC end sequences; transferability in the Citrus genus (Rutaceae), phylogenetic inferences and perspectives for genetic mapping. BMC genomics, 13(1): 13

Olmstead R.G., Palmer J.D., 1994. Chloroplast DNA systematics: a review of methods and data analysis. American journal of botany, 81: 1205-1224

Palladio R. T. A., (IV sec.). Opus Agricolturae. L. IV, Cap. X

Petretto G.L., Sarais G., Maldini M.T., Foddai M., Tirillini B., Rourke J.P., Chessa M., Pintore G., 2015. Citrus monstruosa discrimination among several Citrus species by multivariate analysis of volatiles: a metabolomic approach. Journal of Food Processing and Preservation

Poiana M., 2009. Gli oli essenziali agrumari. In: Citrus: Trattato di agrumicoltura. (edited by V. Vacante e F. Calabrese). Edagricole, Milano

Polat I., Kacar Y.A., Yesiloglu T., Uzun A., Tuzcu O., Gulsen O., Incesu M., Kafa G., Turgutoglu E., Anil S., 2012. Molecular characterization of sour orange (Citrus aurantium) accessions and their relatives using SSR and SRAP markers. Genetics and Molecular Research, 11(3): 3267-3276

Pozzana M.C., 1990. Il giardino dei frutti. Ponte delle Grazie, Firenze

Praloran J.C., 1971. Les agrumes. G.P. Maisonneuve et Larose, Paris

Prasad D.A., Prasad B.R., Prasad D.K., Shetty P., Kumar K.S., 2016. GC-MS Compositional Analysis of Essential Oil of Leaf and Fruit Rind of Citrus maxima (Burm.) Merr. from Coastal Karnataka, India. Journal of Applied Pharmaceutical Science Vol, 6(05): 068-072

Qiao Y., Xie B.J., Zhang Y., Zhang Y., Fan G., Yao X.L., Pan S.Y., 2008. Characterization of aroma active compounds in fruit juice and peel oil of Jinchen sweet orange fruit (Citrus sinensis (L.) Osbeck) by GC-MS and GC-O. Molecules, 13(6): 1333-1344

Reinhard H., Sager F., Zoller O., 2008. Citrus juice classification by SPME-GC-MS and electronic nose measurements. LWT-Food Science and Technology, 41(10): 1906-1912

Risso J.A., Poiteau A., 1818. Histoire naturelle des oranges. Autod Ed., Paris

Roose M.L., Feng D., Cheng F.S., Tayyar R.I., Federici C.T., Kupper R.S., 2000. Mapping the Citrus genome. Acta Horticulturae 535: 25-32

Şahin F., Güllüce M., Daferera D., Sökmen A., Sökmen M., Polissiou M., Agar G., Özer H., 2004. Biological activities of the essential oils and methanol extract of Origanum vulgare ssp. vulgare in the Eastern Anatolia region of Turkey. Food control, 15(7): 549-557

Sanches-Silva A., Costa D., Albuquerque T.G., Buonocore G.G., Ramos F., Castilho M.C., Machado A.V., Costa H.S., 2014. Trends in the use of natural antioxidants in active food packaging: a review. Food Additives & Contaminants: Part A, 31(3): 374-395

Scora R.W., 1975. On the history and origin of Citrus. Bull Torrey Bot Club 102: 369–375

Scora R.W., Kumamoto J., Soost R.K., Nauer E.M., 1982. Contribution to the origin of the grapefruit, Citrus paradisi (Rutaceae). Systematic Botany: 170-177

Scotland R., Hughes C., Bailey D., Wortley A., 2003. The big machine and the much- maligned taxonomist DNA taxonomy and the web. Systematics and Biodiversity, 1(2): 139-143

Seberg O., Humphries C.J., Knapp S., Stevenson D.W., Petersen G., Scharff N., Andersen N.M., 2003. Shortcuts in systematics? A commentary on DNA-based taxonomy. Trends in Ecology & Evolution, 18(2): 63-65

Singh A., Deverumath R.M., RamaRao S., Singh V.P., Raina S.N., 2008. Assessment of genetic diversity, and phylogenetic relationships based on ribosomal DNA repeat unit length variation and Internal Transcribed Spacer (ITS) sequences in chickpea (Cicer arietinum) cultivars and its wild species. Genet. Resour. Crop. Evol. 55: 65-79

Singh A., Sinha P., Dubey A.K., Srivastav M., 2011. Characterization of citrus germplasm using simple sequence repeat (SSR) markers. The Indian Journal of Genetics and Plant Breeding, 71(4): 349-355

Skoog D.A., West D.M, Holler F.J., 1996. Chimica Analitica, una introduzione, EdiSES S.r.l., Napoli 1996, III ed., Chapt. 26

Smith D.C., Forland S., Bachanos E., Matejka M., Barrett V., 2001. Qualitative analysis of citrus extracts by GC/MS: an undergraduate experiment. The Chemical Educator, 6(1): 28-31

Snoussi H., Duval M.F., Garcia-Lor A., Belfalah Z., Froelicher Y., Risterucci A.M., Perrier X., Jacquemoud-Collet J.P., Navarro L., Harrabi M., Ollitrault, P., 2012. Assessment of the genetic diversity of the Tunisian citrus rootstock germplasm. BMC genetics, 13(1): 16

Song H.S., Sawamura M., Ito T., Kawashimo K., Ukeda H., 2000. Quantitative determination and characteristic flavour of Citrus junos (yuzu) peel oil. Flavour and Fragrance Journal, 15(4): 245-250

Stevens P. F., 2013. http://www.mobot.org/MOBOT/research/APweb/

Swingle W.T., 1943. The botany of Citrus and its wild relatives of the orange family. In: H.J. Webber and L.D. Batchelor (eds.), The Citrus Industry, Vol. I. Berkeley and Los Angeles: Univ.California Press

Swingle W.T., Reece P.C., 1967. The botany of Citrus and its wild relatives. In: Reuther W., Webber H.J., Batchelor L.D. (eds) The Citrus Industry. Vol. I. History, world distribution, botany, and varieties. Univ of California, Div of Agricultural Sci, Berkeley: 190–430

Tanaka C., 1929. Remarks on citrus and citrus relatives in China. Lingnan Science Journal (7): 337-348

Tanaka T., 1954. Species problem in Citrus (Revisio Aurantiacearum IX). Japanase Society for promotion of Science, Tokyo, Japan

Tanaka T., 1969. Taxonomic problem of Citruss in the Orient. Bulletin of the University of Osaka Prefecture. Ser. B, Agriculture and biology, 21: 133-138

Teofrastus (IV a.C.) – Historia Plantarum. Haeredes Iacobi Lunctae, Ludguni, 1552

Tian D., Wang Q., Zhang P., Araki H., Yang S., Kreitman M., Nagylaki T., Hudson R., Bergelson J., Chen J.Q., 2008. Single-nucleotide mutation rate increases close to insertions/deletions in eukaryotes. Nature, 455(7209): 105-108

Tomi F., Barzalona M., Casanova J., Luro F., 2008. Chemical variability of the leaf oil of 113 hybrids from Citrus clementina (commun) x Citrus deliciosa (Willow Leaf). Flavour and fragrance journal, 23(3): 152-163

Tongnuanchan P., Benjakul S., 2014. Essential oils: extraction, bioactivities, and their uses for food preservation. Journal of food science, 79(7): R1231-R1249

Uzun A., Yesiloglu T., Polat I., Aka-Kacar Y., Gulsen O., Yildirim B., Tuzcu O., Tepe S., Canan I., Anil S., 2011. Evaluation of genetic diversity in lemons and some of their relatives based on SRAP and SSR markers. Plant Molecular Biology Reporter, 29(3): 693-701

Vasemägi A., Gross R., Palm D., Paaver T., Primmer C.R., 2010. Discovery and application of insertion-deletion (INDEL) polymorphisms for QTL mapping of early life-history traits in Atlantic salmon. BMC genomics, 11(1): 156

Virdis M., 2002. Il condaghe di Santa Maria di Bonarcado. Cues, Cagliari

Viuda- Martos M., Ruiz Navajas Y., Sánchez Zapata E., Fernández- López J., Pérez- Álvarez J.A., 2010. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. Flavour and Fragrance Journal, 25(1): 13-19

Wali S., Munir F., Mahmood T., 2013. Phylogenetic studies of selected Citrus species based on chloroplast gene, rps14. Int J Agric Biol, 15: 357-361

Webber H.J., 1943. Cultivated varieties of citrus. In: The citrus industry (1): 475-668

Webber H.J., Reuther W., Lawton H.W., 1967. History and development of the Citrus industry p. 1-39. In: W. Reuther et al. (eds), The Citrus Industry, (1) University of California, Division of Agricoltural Sciences, Berkeley

Will K.W., Rubinoff D., 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics, 20(1): 47-55

Wu G.A., Prochnik S., Jenkins J., Salse J., Hellsten U., Murat F., Perrier X., Ruiz M., Scalabrin S., Terol J., Takita M.A., Labadie K., Poulain J., Couloux A., Jabbari K., Cattonaro F., Del Fabbro C., Pinosio S., Zuccolo A., Chapman J., Grimwood J., Tadeo F.R., Estornell L.H., Muñoz-Sanz J.V., Ibanez V., Herrero-Ortega A., Aleza P., Pérez-Pérez J., Ramón D., Brunel D., Luro F., Chen C., Farmerie W.G., Desany B., Kodira C., Mohiuddin M., Harkins T., Fredrikson K., Burns P., Lomsadze A., Borodovsky M., Reforgiato G., Freitas-Astúa J., Quetier F., Navarro L., Roose M., Wincker P., Schmutz J., Morgante M., Machado M.A., Talon M., Jaillon O., Ollitrault P., Gmitter F., Rokhsar D., 2014. Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. Nat Biotechnol 32: 656-662 doi:10.1038/nbt.2906

Yamamoto M., Kobayashi S., Nakamura Y., Yamada Y., 1993. Phylogenetic relationships of citrus revealed by RFLP analysis of mitochondrial and cloroplastic DNA. Japanese Journal of Breeding 43(3): 355-365

Yamamoto M., Tsuchimochi Y., Ninomiya T., Koga T., Kitajima A., Yamasaki A., Inafuku-Teramoto S., Yang X., Yang X., Zhong G., Nasir N., Kubo T, Tominaga S., 2013. Diversity of chloroplast DNA in various mandarins (Citrus spp.) and other citrus demonstrated by CAPS analysis. Journal of the Japanese Society for Horticultural Science, 82(2): 106-113

Yildiz E., Kaplankiran M., Demirkeser T.H., Aydin U.Z., Toplu C., 2013. Identification of zygotic and nucellar individuals produced from several citrus crosses using SSRs markers. Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 41(2): 478

# CHAPTER I

## Karyological analysis and DNA barcoding of Pompia, a first step towards the identification of its relatives.

### Introduction

The DNA barcoding is a system useful to identify species and to assess the distinctiveness of genotypes and relatedness among genotypes (Pallottini et al., 2004) compared to traditional molecular markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and insertion-deletion polymorphisms (InDels). It was developed to resolve the taxonomic problems especially at the species level such as missing morphological features of a particular life stage (Velzen et al., 2007), missing body parts (Wong and Hanner, 2008), or homoplasy of some characters (Vences et al., 2005). The concept has been very efficient in resolving and deciphering true species status for many organisms (Laskar et al., 2013).

After evaluating the performance of seven leading candidate plastid DNA regions (atpF-atpH, psbK-psbI, psbA-trnH spacers and matK, rbcL, rpoB, rpoC1 genes), in 2009 the Plant Working Group of the Consortium of Barcode of Life (CBOL) identified the two-marker combination rbcL/matK as the standard DNA barcodes for plants and, later on, added psbA-trnH and the nuclear DNA markers internal transcript spacers (ITS1, ITS2) of 5.8S rRNA as supplementary barcode loci. Yet, the screening for single or multiple regions from plastid and nucleus, appropriate for DNA barcoding in different plant groups, has been an important research focus around the globe (Tribathi et al., 2013) .

The two chloroplast regions rbcL and matK are found to be useful to discriminate at the species level in many cases, while in biologically complex plant group like *Citrus*, *Berberis*, *Bromeliaceae* and *Potatoes*, they provide completely ineffective results (Maia et al., 2012; Luo et al., 2010; Roy et al., 2010; Spooner, 2009). Recently, Luo et al. (2010) and Mahadani and Ghosh (2014), investigated the identification efficiency of candidate DNA barcode sequences among many related species of genus *Citrus*, reporting conflicting conclusions. The first study used 7 regions (psbA-trnH, matK, ycf5, rpoC1, rbcL, ITS2 and ITS), reporting that ITS2 locus had the highest identification efficiency among other regions analyzed and psbA-trnH locus shows higher inter-specific divergence than other chloroplast loci investigated. Therefore, they suggest that psbA-trnH can be used as complementary barcode

together with ITS2 for a broad range of plant taxa (Luo et al., 2010). The second study used 3 regions: matK, psbA-trnH and trnL-trnF which were previously reported to be highly polymorphic due to the presence of numerous informative indels (Liu et al., 2012). Hence one of the most relevant finding of this research was the confirmation that indels of cpDNA are an important character for species identification of complex plant group as *Citrus* (Mahadani and Gosh, 2014).

Citrus species have very small mitotic chromosomes (1.0-4.0 μm) and most of them are similar in morphology (Frost, 1925a; Krug, 1943; Sharma and Bal, 1957). The chromosome number was established by Frost (1925a) as n = 9 or 2n = 18. It is constant in the genus, with the exception of some cultivated polyploids (Krug, 1943; Esen and Soost, 1971; Hutchinson and Barrett, 1981) such as 2n=18 or 2n=27 for *C. aurantifolia* and *C. latifolia* respectively (Longley, 1925; Krug and Bacchi, 1943) and 2n=18,27,36 in *C. limonia* (Frost, 1925a,b). The origin of such polyploids may be chromosome complement duplication of nucellar cells before somatic embryogenesis in the case of tetraploidization (Frost, 1925b) or during megasporogenesis by conducing to an unreduced gamete giving after fertilization with a regular a gamete to a triploid hybrid (Esen et al., 1979). Some other factors, such as interspecific hybridization, ploidy level, atmospheric temperature during flowering period and the mono/polyembryonic nature of the citrus may also contribute to the frequency of polyploidy progenies (Cameron and Soost, 1969; Oiyama et al., 1981; Wakana et al., 1981; Aleza et al. 2011).

This work focuses on the application of DNA barcoding to a *Citrus* species of unknown origin called Pompia. To this end, two DNA barcode regions, psbA-trnH intergenic spacer region and trnL intron and one nuclear-originated locus ITS, were selected and their sequence analysed to investigate the origin of Pompia along with its phylogenetic relationship with other species belonging to the same genus. A karyology and ploidy analysis by flow cytometry completed this characterization.

## Materials and Methods

### *Germplasm sampling*

In total, 8 species belonging to the *Citrus* genus, presents in germplasm banks of CRB Citrus INRA-CIRAD, San Giuliano, Corsica (France) were selected as representative of the most likely ancestors of Pompia, based on morphological traits, plant descriptors, and molecular markers (D'Aquino et al., 2005; Camarda et al., 2013; Mignani et al., 2015). The two accessions Poncire Commun and Diamante were used to represent the species *C. medica*. One accession identified as Rhobs el arsa (Acc. No ICVN0110244) which appeared to be morphologically similar to citrus was also investigatedto better understand hits relationship with Pompia and other citrus species adopted in this study.In addition, 10 accessions of *Citrus* spp. var. Pompia, were obtained from Sardinia (Bitti, Milis, Oliena and Siniscola). A list of varieties and landraces with information on their origins can be found in Table 1.

**Table 1:** List of accessions with Swingle and Reece (1967) classification names adopted in this study.

| Species | Variety | Origin | Accession No |
|---|---|---|---|
| *C. micrantha* | Micrantha- | CRB Citrus[1] | SRA1115 |
| *C. maxima* | Sans pèpin pummelo | CRB Citrus | SRA710 |
| *C. reticulata* | Cleopatra mandarin | CRB Citrus | ICVN0110273 |
| *C. medica* | Diamante citron | CRB Citrus | SRA540 |
| *C. medica* | Poncire commun citron | CRB Citrus | SRA701 |
| *C. sinensis* | Olinda Valencia sweet orange | CRB Citrus | SRA18 |
| *C. x aurantium* | Maroc sour orange | CRB Citrus | ICVN0110033 |
| *C. x bergamia* | Castagnaro bergamot | CRB Citrus | SRA 612 |
| *C. limon* | Femminello | CRB Citrus | SRA180 |
| *Citrus* sp. | Rhobs el arsa citron | CRB Citrus | ICVN0110244 |
| *Citrus* sp. | Pompia | Milis | M1 |
| *Citrus* sp. | Pompia | Milis | M3 |
| *Citrus* sp. | Pompia | Bitti | B |
| *Citrus* sp. | Pompia | Oliena | O150 |
| *Citrus* sp. | Pompia | Oliena | O8 |
| *Citrus* sp. | Pompia | Siniscola | ME1 |
| *Citrus* sp. | Pompia | Siniscola | S3 |
| *Citrus* sp. | Pompia | Siniscola | T1 |
| *Citrus* sp. | Pompia | Siniscola | T4 |
| *Citrus* sp. | Pompia | Siniscola | T5 |

[1]CRB Citrus INRA-CIRAD San Giuliano Corsica, France

### *Flow Cytometry screening*

Nuclei were prepared from leaf tissues, by gentle chopping with a razor blade of 0.1g fresh weights in 0.4 ml of Cystain UV Precise P nuclei extraction buffer (SysmexPartec GmbH, Gorlitz, Germany) with 1% w/v PVP. For each considered sample, 3 replicates were analysed.

Following nuclei extraction, the suspension was filtered through nylon tissue of 30 mm mesh width as recommended by the manufacturer. After filtration, 1.6 ml of staining buffer was added to the lysate and the tubes were stored in the dark on ice for 1 h before measurement. The fluorescence intensity of DAPI-stained nuclei was determined using the flow cytometer CyFlow® Cube Ploidy Analyser (SysmexPartec GmbH, Gorlitz, Germany) equipped with an UV-Light Emitting Diode (l = 355nm - 375 nm). Data were plotted on a logarithmic scale and calibration of C values was made with nuclei extracted from *C. limon*. Ploidy histograms were quantitatively analysed with the FCS Express 5 Flow software (SysmexPartec GmbH), after manual treatment to exclude noise.

***Chromosome count***

Pompia seeds were collected from ripe fruit yielded in November in Siniscola. Seeds were pretreated in 20% NaClO for 20 min, and germinated on Petri dishes on tissue paper, incubated at 24 $\pm$1°C. Root tips long about 0.5-1 cm were excised, treated with 0.3% colchicine (alkaloid cytostatic) for 4 h at room temperature, then fixed ethanol/acetic acid solution (v/v, 3:1) at 4°C overnight. After 3 washes with distilled water, the root tips were hydrolysed in 1N HCl for 8 min at 60°C, stained in Shiff's reagent, and observed under a microscope with a drop of 50% $CH_3COOH$. Permanent slides were prepared by dehydration in alcohols progressive series, and then analyzed for karyotype.

The evaluation was done with the help of an AxiophotZeisse microscope, equipped with an Infinity Analyze Lumenera Camera. The output was analyzed trough the KaryoType software. The classification of chromosomes in metacentric (m), sub-metacentric (sm), sub-telocentric (st) and telocentric (t) was made following Levan et al. (1964).

***Genomic DNA extraction***

Genomic DNA was isolated from 0.1 g of powdered, frozen, young leaf tissue using the MATAB DNA extraction protocol, described by Cabasson et al. (2001). DNA integrity was estimated by electrophoresis on a 0.8% agarose/TAE gel using the 1 kb Plus DNA ladder (Invitrogen, Carlsbad, California) as size standards. The purity and quantity of the DNA extracts were assessed with a NanoDrop 3300 spectrophotometer (Thermo Scientific, USA).

*DNA barcode markers and PCR assays*

Preliminary investigations aimed at selecting the optimal chloroplast regions for DNA barcoding were done by downloading from Genbank the available sequences of chloroplast

barcode regions: psbA-trnH, trnL-intron, rbcL and matK for the *Citrus* species reported on table 1. Nucleotide alignments were performed with MEGA7.

Molecular investigations were carried out by amplifying two chloroplast markers (the trnL gene intron and the psbA-trnH intergenic spacer) and the two nuclear internal transcribed spacers (ITS1 and ITS2). The primers pairs adopted to amplify both chloroplast and nuclear regions, along with the relative nucleotide sequences and the corresponding references, are supplied in Table 2.

**Table 2:** Primer list. For each nuclear and chloroplast marker, the table reports on the amplicon length, primer names, primer sequences, annealing temperature and reference source.

| | Ampliconlength (bp) | | | | | |
|---|---|---|---|---|---|---|
| Marker | *Citrus* spp. | Pompia | Primername | Primersequence (5'-3') | Ta (°C) | References |
| ITS-5.8S rRNA | 533-564 | 534-563 | ITS1 | TCCGTWRGTGAACCWGCGG | 54 | *White et al. 1990 |
| | | | ITS4 | TCCTCYRMTTAKYGATATGC | 54 | *White et al. 1990 |
| psbA-trnH IGS | 451-468 | 452 | psbA3'f | GTTATGCATGAACGTAATGCTC | 56 | Sang et al. 1997 |
| | | | trnHf | CGCATGGTGGATTCACAATCC | 54 | Tate and Simpson 2003 |
| trnL intron | 530-542 | 536 | trnL_F | GGATAGGTGCAGAGACTCRATGGAAG | 56 | Nicolé et al. 2011 |
| | | | trnL_R | TGACATGTAGAATGGGACTCTATCTTTAT | 56 | Nicolé et al. 2011 |

* these primers were modified from White et al., 1990.

For each chloroplast and nuclear marker, PCR amplifications were conducted in a volume of 20 µl, containing 15 ng of genomic DNA as a template, 1× 10X AccuPrime™ Pfx Reaction Mix (Invitrogen, Thermo Fisher Scientific), primers to a final concentration of 0.2 µM each and 0.25 U of AccuPrime™ Pfx DNA Polymerase (Invitrogen, Thermo Fisher Scientific). All PCR amplifications were performed in the GeneAmp 9700 PCR System (Applied Biosystems, USA). The experimental conditions for PCR amplification were as follows: 2 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 55°C and 1min at 68°C. Positive and negative controls were used as reference standards. The PCR-derived fragments were resolved in 2% agarose/TAE gels and visualized under UV light via Sybr Safe DNA staining (Life Technologies, USA). Amplification products originated with plastidial primer combinations (trnL intron and psbA-trnK IGS) were subjected to EXOI/FAP (Thermo Scientific, USA) treatment and then directly sequenced on an ABI3100 automated sequencer. For the ITS 5.8S rRNA region, amplification products were purified by using the QIAquick PCR Purification Kit. Purified PCR products were adenylated in reaction volume of 10µl containing 1X PCR buffer (100mMTris-HCl pH 9.0, 15 mM $MgCl_2$ and 500 mMKCl), 0.2 mM dNTPs, and 0.5 U of *Taq* DNA polymerase (BIOLINE, USA). Adenylated amplicons were sub-cloned by using the kit StrataClone PCR Cloning Kit (Agilent) and transformed into

chemically competent StrataCloneSoloPack Competent Cells (Agilent). Clones were plated on LB plates (1.5% agar, 50 µg/ml ampicillin, 40 µg/ml X-Gal), and transformed colonies were selected by Colony-PCR. Amplification reactions were performed in a total volume of 20 µl including 2 µl of 10X reaction buffer, 1.5 mM MgCl2, 300 µM dNTPs, 1.5 U of BIO*Taq* DNA polymerase (BIOLINE), 0.2 µM of T3 and T7 primers. Positive colonies were grown over night on LB media. For each PCR product, 5 positive clones were selected by colony PCR. Plasmid were purified from positive clones by using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich), by following the manufacturer's instructions. The sequencing of PCR products (trnL intron and psbA-trnH IGS) and plasmids (ITS 5.8S rRNA regions) was done by using an ABI3100 automated sequencer (Applied Biosystems).

The obtained sequences were visualized and manually edited with Geneious 5.4 to minimize any possible error during sequencing. Sequence similarity searches were performed using the GenBankBLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST) against the NCBI nucleotide databases to check the correspondence between the sequences of the obtained amplicons and the expected sequences.

All of the nuclear and chloroplast DNA sequences of generated in this study have been deposited in the NCBI databases under the GenBank accession numbers: KY656107-KY656138.

***Character- and tree-based analyses***

The character-based technique was employed to look for unique sets of diagnostic characters related to single species of *Citrus*. Rather than using hierarchies or distance trees, character-based analysis classifies taxonomic groups based on shared specific informative character states, SNPs or InDels, at either one or multiple nucleotide positions (DeSalle et al., 2005). The BLASTn algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to perform sequence similarity searches against the nr nucleotide databases of NCBI and assess the specificity of PCR amplifications. Separate data analyses were performed for each individual sequence and for the combined chloroplast datasets. Analysis of polymorphism distribution was performed using the DnaSP v.4 software (Rozas et al., 2003) to generate a map containing haplotype data without considering sites with alignment gaps. This program detects positions characterized by the presence of specific character states that are limited to a particular *Citrus* accession and shared by all the members of that cluster.

For a tree-based analysis, multiple sequence alignments were performed with the software MEGA 7 (Tamura et al., 2007). The same software was used to calculate interspecific genetic divergences according to the Kimura 2-parameter distance model (Kimura, 1980). Based on the pairwise nucleotide sequence divergences (Tamura Nei), the Maximum –Likelihood (ML) tree was estimated starting from the haplotype sequences of each plant accession. A bootstrap analysis was conducted to measure the stability of the computed branches with 1000 resampling replicates.

***Population structure analysis***

The population structure of the *Citrus* collection investigated in this study was addressed by using the Bayesian model-based clustering algorithm implemented in the STRUCTURE software (Pritchard et al., 2000; Falush et al., 2003), which identifies subgroups according to combination and distribution of molecular markers. All simulations were executed assuming the admixture model, with no a priori population information. Analyses of SNP data were performed with 500 000 iterations and 500 000 burn-ins by assuming the allele frequencies among populations to be correlated (Falush et al., 2003). Ten replicate runs were performed, with each run exploring a range of K spanning from 1 to 9. The most likely value of K was estimated according to Evanno et al. (2005). Individuals with membership coefficients of $qi \geq 0.7$ were assigned to a specific group, whereas individuals with $qi < 0.7$ were identified as admixed.

## Results and discussion

Flow cytometric analysis was performed to evaluate the DNA content of all Pompia accessions. As internal reference, we adopted *C. limon*, whose DNA content has been already estimated by Curk et al. (2016). No significant difference in our estimates of the DNA content was detected between Pompia accessions and with respect to *C. limon* (Figure 1). These findings provided preliminary evidences for a diploid nature of Pompia. All the citrus genotypes included in this study were diploid.

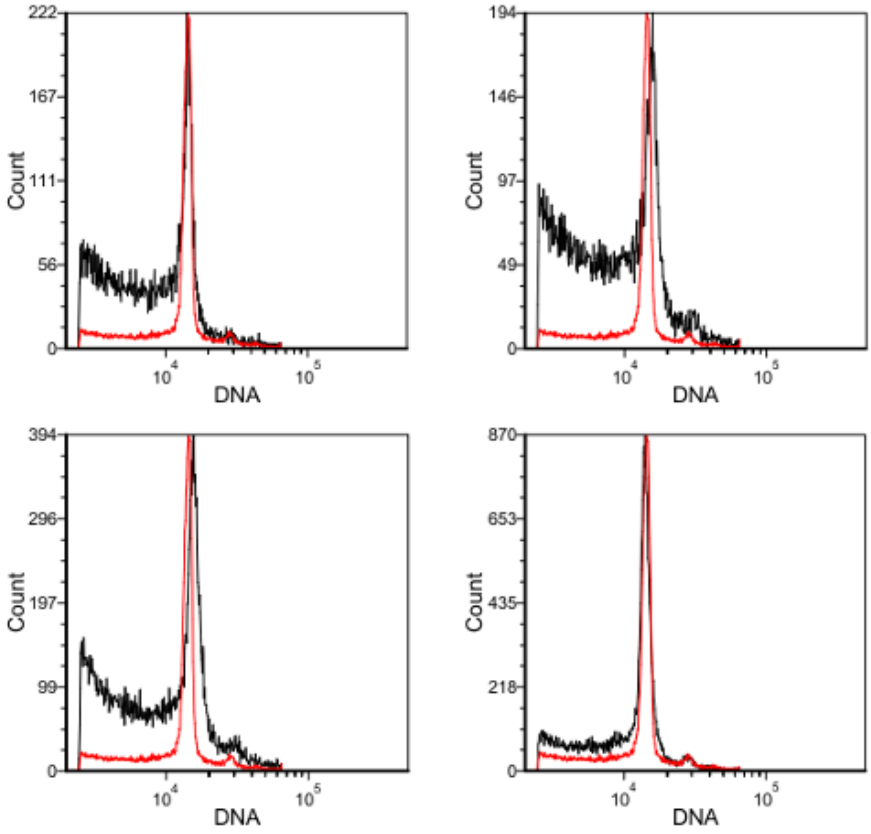



**Figure 1:** Flow cytometry

The data regarding the Pompia chromosome number and karyotype are showed in Figure 2 and 3. This data confirms the diploidy of Pompia 2n=2x=18, like the greater part of *Citrus* spp. (Hynniewta et al., 2011). In fact, the diploidy is the general rule in *Citrus*, with a basic chromosome number x=9 (Krug, 1943). The karyotypic formula for Pompia, 2n = 2x = 16m + 2sm, indicates the presence of meta-centric and sub-metacentric chromosomes, while sub-telocentric and telocentric chromosomes were absent.

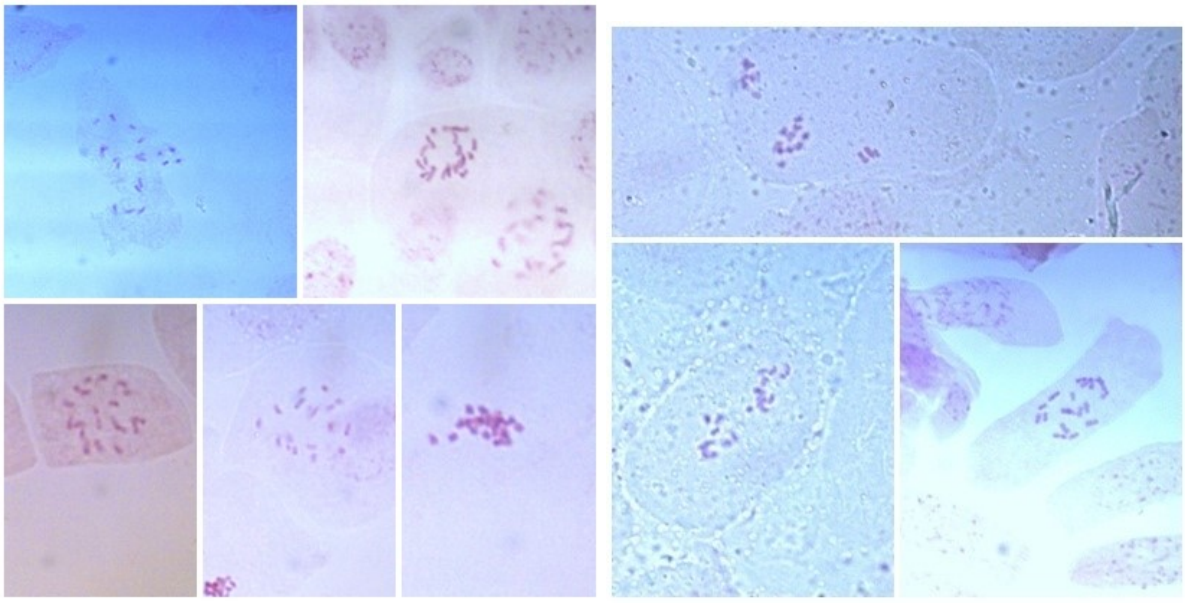


**Figure 2:** Chromosomes of Pompia (2x=2n=18)

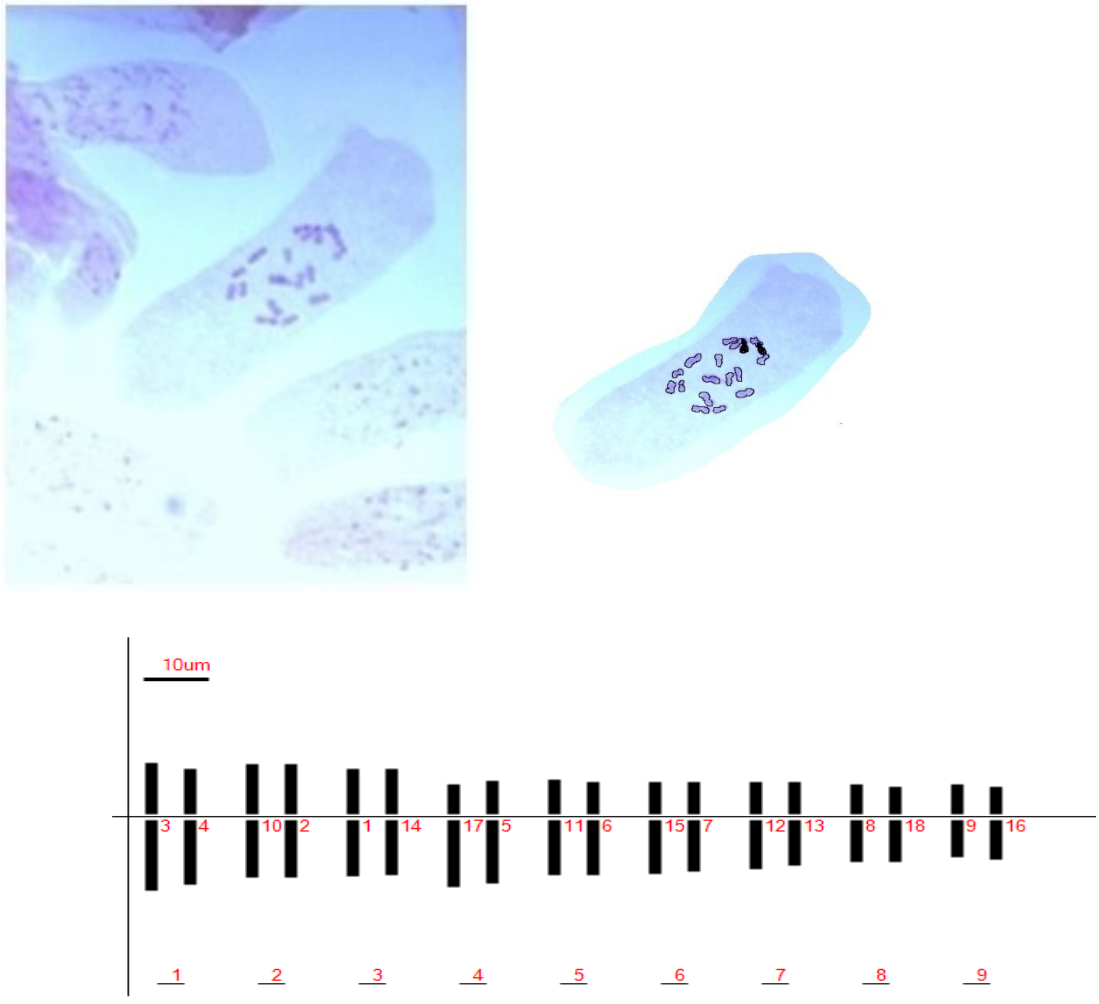



**Figure 3:** Pompia karyograms (chromosome information was containing in Supplementary file 1)

Preliminary investigations aimed at selecting the most suitable chloroplast regions for cpDNA barcoding were done by aligning all available cpDNA sequences for the following regions: psbA-trnH, trnL intron, rbcL and matK. The alignment of sequences related to rbcL and matK did not display polymorphisms between our selected references and were not considered for further investigations. Contrariwise, the sequences of psbA-trnH and trnL did produce several Single Nucleotide Variants (SNV), either SNPs or InDels, among the selected accessions and were therefore considered as suitable for following investigations.

PCR amplification of the selected chloroplast and nuclear markers was successful in all examined accessions of Citrus spp.. Primer pairs designed for the amplification of the regions trnL intron, psbA-trnH IGS and ITS 5.8S rRNA provided a 100% amplification success rate. As expected, the noncoding cpDNA sequences differed for presence of SNPs and InDels, and required manual editing of the alignments. The same was true for the two ITS regions flanking the 5.8S rRNA. The size of nuclear (i.e. ITS) sequences ranged from 533bp to 564bp, whereas the cpDNA sequences ranged from 451bp to 468bp and from 530bp to 542bp, for the psbA-trnH IGS and the trnL intron, respectively (Table 2). Consequently, the cpDNA region considered in this study covered a total length of 1015 bp (Table 2). The alignments of ITS and cpDNA regions are provided in the supplementary files 2 and 3, respectively.

Estimates of evolutionary divergence between sequences were performed with MEGA7, by computing the average number of base substitutions per site from between sequences (Table 3). These statistics were performed by taking into account only single nucleotide polymorphisms, without taking into account insertions and deletions eventually present in our sequences. If compared to the chloroplast sequences, nuclear sequences displayed the highest values of average number of base substitution per site. Preliminary investigations were performed to verify weather samples of known origin and kinship were characterized by low sequence divergence. Hence, *C.reticulata* and *C. maxima* showed a very low level of sequence polymorphisms with *C. sinensis* and *C. aurantium* (Table 3). At the same time, the number of base substitutions per site in the comparison between the sequence obtained from *C. bergamia* and those found in *C. limon* or *C. aurantium,* was equal to 0.000 and 0.016, respectively. As for *C. limon*, its nuclear sequence displayed no differentiation at all with that of its male ancestor, *C. medica*, and one of its descendant, *C. bergamia* (Table 3). In a following approach, the evolutionary divergence among *Citrus* accessions was investigated by adopting Pompia's sequences as reference in each comparison. In this case, the nuclear sequences that displayed the highest average number of

base substitutions per site were sequenced from *C. reticulata* (0.0226), followed by the ITS sequences obtained by *C. micrantha* (0.0168) and *C. sinensis* (0.0132). This finding indicates that two of the basic *Citrus* taxa, *C. reticulata* and *C. micrantha* did not participate directly in the genesis of Pompia. Based on the same dataset, the ITS sequences that displayed the lower level of differentiation from Pompia were *C. medica* (0.0048), *C. limon* (0.0048), *C. bergamia* (0.0048) and Rhobs el arsa (0.008). At this level of investigation, without considering InDels eventually included in target sequences, cpDNA sequences appeared to be less informative as values of average number of base substitutions per site among all pairwise comparisons with Pompia cpDNA sequences ranged from complete identity (Rhobs el arsa, *C. bergamia*, *C. aurantium*, *C. limon*) to a maximum level of 0.009 (*C. medica*).

**Table 3:** Estimates of Evolutionary Divergence between Sequences. The table reports on the average number of base substitutions per site from between sequences. Chloroplast data are shown above the diagonal, while nuclear data are displayed below the diagonal

| | *C. micrantha* | *C. reticulata* | *C. maxima* | *C. medica* | *C. limon* | *C. sinensis* | *C. aurantium* | *C bergamia* | Rhobsel arsa | pompia |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. micrantha* | - | 0.006 | 0.008 | 0.009 | 0.006 | 0.007 | 0.006 | 0.006 | 0.006 | 0.006 |
| *C. reticulata* | 0.023 | - | 0.006 | 0.007 | 0.006 | 0.005 | 0.006 | 0.006 | 0.006 | 0.006 |
| *C. maxima* | 0.014 | 0.017 | - | 0.009 | 0.002 | 0.001 | 0.002 | 0.002 | 0.002 | 0.002 |
| *C. medica* | 0.018 | 0.025 | 0.016 | - | 0.009 | 0.008 | 0.009 | 0.009 | 0.009 | 0.009 |
| *C. limon* | 0.018 | 0.025 | 0.016 | 0.000 | - | 0.001 | 0.000 | 0.000 | 0.000 | 0.000 |
| *C. sinensis* | 0.016 | 0.019 | 0.002 | 0.018 | 0.018 | - | 0.001 | 0.001 | 0.001 | 0.001 |
| *C. aurantium* | 0.014 | 0.017 | 0.000 | 0.016 | 0.016 | 0.002 | - | 0.000 | 0.000 | 0.000 |
| *C. bergamia* | 0.018 | 0.025 | 0.016 | 0.000 | 0.000 | 0.018 | 0.016 | - | 0.000 | 0.000 |
| Rhobsel arsa | 0.016 | 0.021 | 0.008 | 0.008 | 0.008 | 0.010 | 0.008 | 0.008 | - | 0.000 |
| pompia | 0.0168 | 0.0226 | 0.0112 | 0.0048 | 0.0048 | 0.0132 | 0.0112 | 0.0048 | 0.008 | - |

Next, we adopted the NJ tree method to analyse the genetic distinctiveness of Pompia, by using both nuclear and cpDNA markers. The NJ tree allows the conversion of sequence polymorphisms into genetic distances using nucleotide substitution models (Wiemers and Fiedler 2007). According to this, the NJ tree built for the nuclear sequences (Supplementary file 4) grouped most sequences in two highly supported clusters (bootstrap ≥ 80%). The first cluster included several sequences identified in Pompia, with sequences retrieved from *C. medica* and *C. limon*, while a second well supported node included *C. aurantium*, *C. maxima* and *C. sinensis* together with a second set of sequences identified from Pompia. As expected, the NJ tree constructed from the whole set of cpDNA polymorphisms (Supplementary file 5) provided a slightly different scenario. In this case, all Pompia sequences were clustered together with *C. aurantium*, *C. bergamia* and *C. limon,* even though the bootstrap support of this node appeared to be relatively low (bootstrap ≥ 60%). Interestingly *C. maxima* and *C. sinensis* that were grouped a part from the fore mentioned node, clustered in its close proximity.

The alignment of ITS sequences displayed a 29bp long insertion from position 213 and position 241 of the alignment, within the ITS1 region (Supplementary file 2). Remarkably, our accessions of Pompia proved to contain both alleles and were therefore considered heterozygous at this locus. As displayed in Figure 4 (panels A-B), the of two distinct alleles in the genomic locus encoding for the ribosomal RNAs is supported by the presence of 8 SNPs. This finding provides evidence supporting the commonly accepted idea that Pompia originated very recently. Furthermore, this finding provided a useful tool for the identification of the two parental species originally adopted for the constitution of Pompia. In a following approach, all ITS sequences were grouped according to the presence of the afore mentioned deletion and used for pairwise sequence comparisons by using the two alleles found in Pompia as references (Figure 4, panels A-B). As displayed in Figure 4 (panel A), sequences of Pompia carrying the deletion showed the highest number of polymorphic sites in the comparison with *C. micrantha* (n:9), *C. reticulata* (n:13), *C. maxima* (n:8), *C. sinensis* (n:9) and *C. aurantium* (n:8). Conversely, sequences of Pompia not carrying the deletion displayed the lowest number of polymorphic sites in the comparison with *C. aurantium* (n:0), *C. maxima* (n:1) and *C. sinensis* (n:2) (Figure 4, panel B). Based on the present estimation on the number of polymorphic sites in each pairwise comparison, it seems to likely that Pompia inherited the allele carrying the 29bp long insertion from *C. aurantium* (Figure 4, panel B), while the other allele likely derived from one of the following species: *C. medica*, *C. limon* and *C. bergamia*. It is worth noting that, based on current references (Curk et al., 2015), *C. medica* is one of the parental lines that originated *C. limon*, which in turns was adopted as parental line, together with *C. aurantium*, to originate *C. bergamia*.

To better discriminate between maternal and paternal contributions, we decided to perform the same statistics by using all cpDNA sequences (Figure 4, panel C), to integrate the nuclear data and better define the maternal contribution to the Pompia genotype. As displayed in Figure 4, the number of polymorphic sites between Pompia cpDNA sequences and those obtained from other *Citrus* accessions ranged from a maximum of nine SNPs (*C. medica*), to minimum level corresponding to complete identity for sequences obtained from the following species: *C. aurantium*, *C. limon*, *C. bergamia*, Rhobs el arsa (Figure 4, panel C). Accordingly, it is very likely that the maternal parental line of Pompia is represented by one of these latter species.

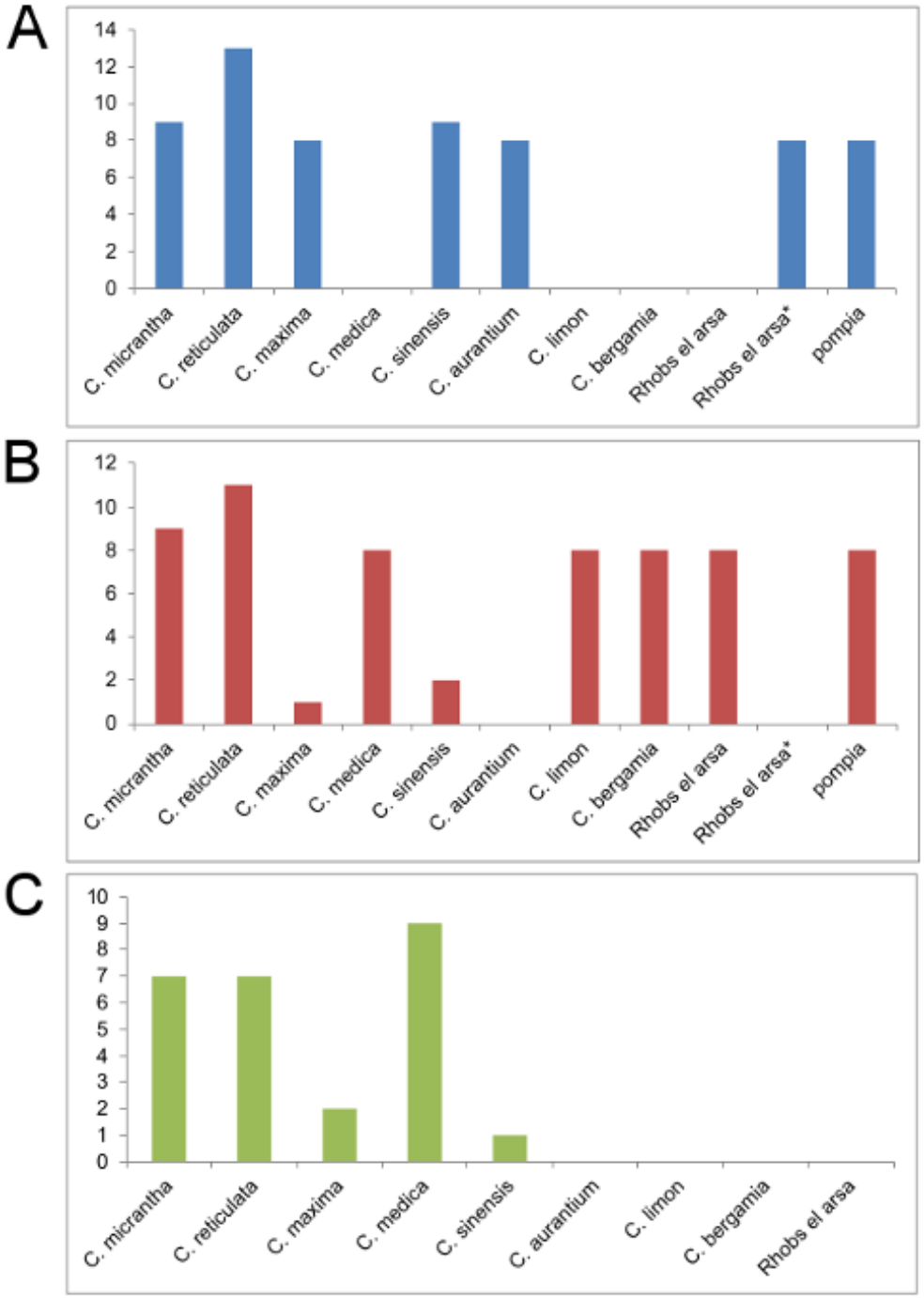



**Figure 4:** Number of polymorphic sites between sequences of all investigated *Citrus* accessions. (A) ITS1-ITS2 region, by adopting Pompia sequences carrying the deletion in position 213 as reference. (B) ITS1-ITS2 region, by adopting the Pompia sequences not carrying the deletion in position 213 as reference. (C) Number of polymorphic sites detected by using the cpDNA sequences

Next, a character-based approach was employed to identify diagnostic attributes shared between the members of a given taxonomic group but absent from a different clade that descends from

the same node (Rach et al., 2008). In this method we adopted all available Single Nucleotide Variants (SNV), either SNPs or InDels, to increase the resolution of our investigations and better discriminate the citrus species that most likely were adopted as parental lines to generate the Pompia.

Among all *Citrus* accessions considered in this study, the occurrence of SNVs proved to be highly variable in the different marker regions. Hence, while the region corresponding to the ITS1 provided 44 SNVs, 29 of which were InDels, the region corresponding to the ITS2 underlined 5 SNPs and a single InDel in position 448 of the alignment. As for the chloroplast markers, the analysis of the intergenic region psbA-trnH revealed 51 SNVs, 40 of which were InDels, while the trnL intron appeared to be less informative as it provided 4 SNPs and 12 InDels (for a total of 16 SNVs). Hence, among the cpDNA markers, psbA-trnH showed the highest number of SNVs, proving to be the most suitable regions for discrimination of our *Citrus* accessions, along with the nuclear ITS1.

SNVs analysis for the nuclear region revealed 6 haplotypes out of the 10 accessions of *Citrus* (Figure 5). Our data indicate that two distinct haplotypes were the most common and accounted for six accessions out of 10. The first haplotype, Hap_1, included Pompia, *C. aurantium* and Rhobs el arsa. A second well-represented haplotype, Hap_2, included the nuclear sequences retrieved from *C. bergamia*, *C. limon*, *C. medica*, together with Pompia and Rhobs el arsa. Unique haplotypes were detected for the following accessions: *C. maxima*, *C. sinensis*, *C. reticulata* and *C.micrantha*. The alignment position 239 was the only one able to discriminate all accessions included in Hap_1 (genotype: 239T) from those included in Hap_2 (genotype: 239delY) and the remaining haplotypes (genotype: 239C). It is worth noting that the four taxa from which all cultivated *Citrus* species originated by interspecific hybridization, namely *C. medica*, *C. maxima*, *C. reticulata* and *C. micrantha,* were represented by single and distinctive haplotypes.

Interestingly, the SNVs analysis of the entire chloroplast data set revealed the same number of haplotype identified by using the nuclear markers (n:6), out of the 10 accessions of *Citrus* considered in this study (Table 4). It is worth noting that a single haplotype, namely Hap_1, embraced the sequences found in Pompia, together with Rhobs el arsa, *C. limon*, *C. bergamia* and *C. aurantium*. Unique haplotypes were found for the remaining species: *C. maxima*, *C. sinensis*, *C. medica*, *C. reticulata* and *C. micrantha*. These results indicated that *C. medica*, among others, could not be considered the maternal progenitor of Pompia. Beside the

identification of diagnostic SNPs and InDels wich, alone or in combination, could be useful for the discrimination of several *Citrus* species investigated in this research, the nuclear and chloroplast haplotypes were used to compute the two ML trees displayed in Figures 6 and 7.

In agreement with the previous results, the analysis of our data with the software STRUCTURE allowed to clearly distinguish *C. medica*, *C. maxima*, *C. reticulata* and *C. micrantha* as the four ancestral taxa (Figure 8) and confirmed the genetic relationships reported in literature for all investigated *Citrus* hybrids. However, STRUCTURE did not allow to distinguish between *C. aurantium* and *C. maxima* genotypes and between Pompia and Rhobs el arsa, *C. bergamia* and *C. limon*. Our data suggest that Pompia is a hybrid between *C. medica* x *C.aurantium* or *C. medica* x *C. maxima*. Furthermore, Pompia has a very similar genetic structure and probably has the same origin as *C. limon*, *C. bergamia* and Rhobs el arsa. Given the marked morphological and genotypic similarity shared by Pompia and Rhobs el arsa, we cannot rule out the possibility that the two accessions shared the same ancestors and referred to the same species.

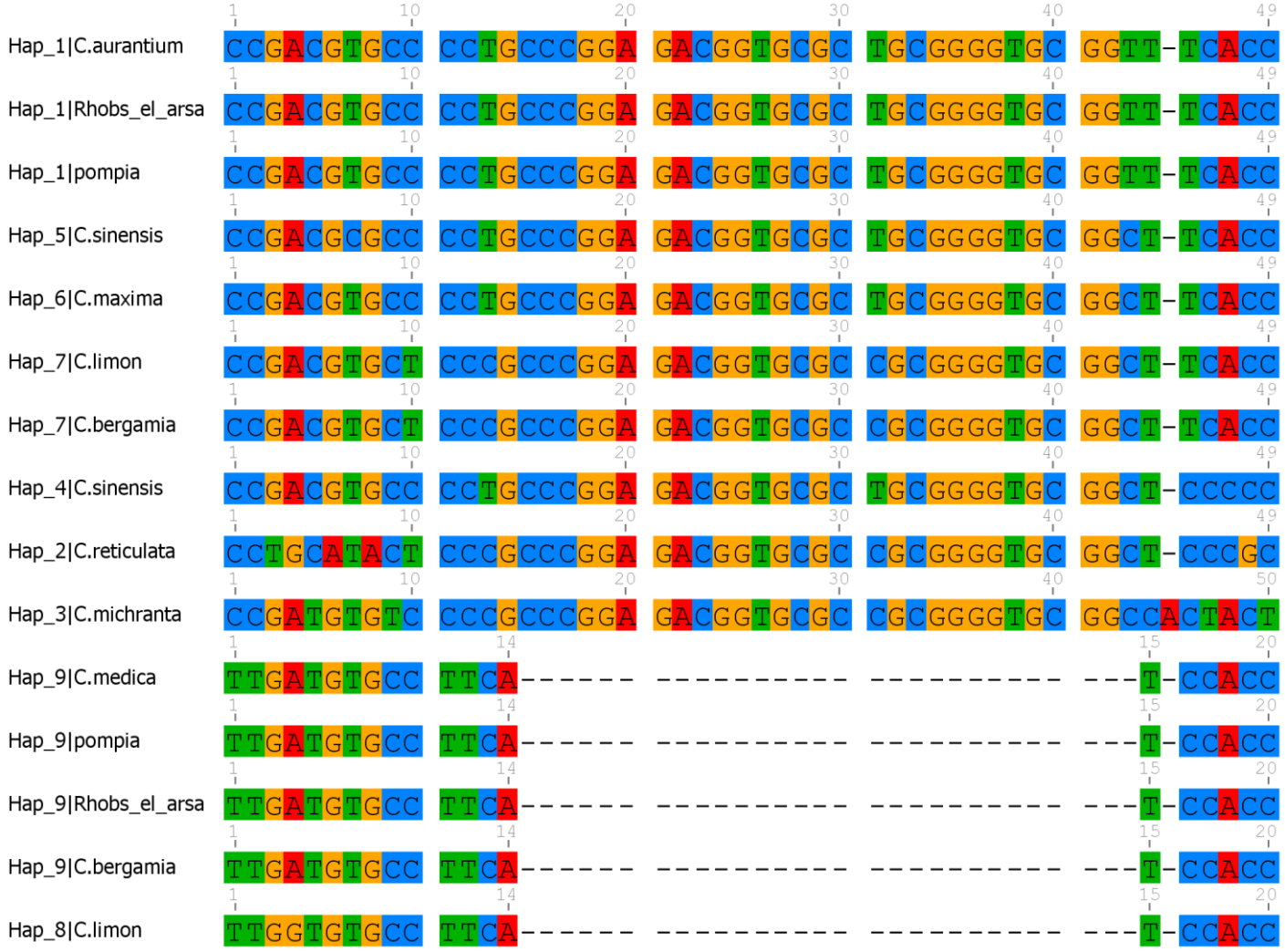



**Figure 5:** Consensus sequence related to the 50 SNV, either SNPs or InDels, detected in the ITS1 and ITS2 regions

**Table 4:** Consensus sequence related to the 67SNV, either SNPs or InDels, detected in the psbA-trnH intergenic spacer (n:51) and trnL intron (n:16). For each haplotype, the table reports on the SNP position and nucleotide composition with respect to the consensus sequence

| | | psbA-trnHintergenicregion | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 44 | 52 | 93 | 103 | 111 | 113 | 137 | 155 | 172 | 177 | 178 | 179 | 180 | 181 | 182 | 183 | 184 | 186 | 219 | 221 | 222 | 223 | 224 | 225 | 226 | 227 |
| | Consensus | A | A | G | G | A | C | T | A | A | ID | ID | ID | ID | ID | ID | ID | ID | T | T | A | A | T | T | T | A | G |
| Hap_1 | Pompia | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | T | . | . | . | . | . | . | . |
| Hap_1 | Rhobsel arsa | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | T | . | . | . | . | . | . | . |
| Hap_1 | *C. limon* | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | T | . | . | . | . | . | . | . |
| Hap_1 | *C. bergamia* | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | T | . | . | . | . | . | . | . |
| Hap_1 | *C. aurantium* | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | T | . | . | . | . | . | . | . |
| Hap_5 | *C. maxima* | . | . | . | . | A | . | G | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | G | . | . | . | . | . | . | . |
| Hap_8 | *C. sinensis* | . | . | . | . | A | . | . | . | . | ID | ID | ID | ID | ID | ID | ID | ID | . | G | . | . | . | . | . | . | . |
| Hap_4 | *C. medica* | C | . | A | A | G | . | . | C | . | A | T | G | C | G | A | C | T | G | G | ID | ID | ID | ID | ID | ID | ID |
| Hap_6 | *C. reticulata* | C | . | . | . | G | . | . | . | . | A | T | G | C | G | A | C | T | . | G | . | . | . | . | . | . | T |
| Hap_7 | *C. micrantha* | C | . | . | . | G | A | . | . | C | A | T | G | C | G | A | C | T | . | T | . | . | . | . | . | . | C |

| | | psbA-trnHintergenicregion | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 228 | 258 | 270 | 271 | 272 | 273 | 274 | 275 | 276 | 277 | 278 | 279 | 280 | 281 | 282 | 314 | 315 | 316 | 317 | 318 | 319 | 320 | 321 | 322 | 369 | |
| | Consensus | T | C | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | A | A | A | C | A | A | A | A | A | C | |
| Hap_1 | Pompia | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_1 | Rhobsel arsa | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_1 | *C. limon* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_1 | *C. bergamia* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_1 | *C. aurantium* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_5 | *C. maxima* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | A | . | . | . | . | . | |
| Hap_8 | *C. sinensis* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | . | . | . | ID | . | . | . | . | . | |
| Hap_4 | *C. medica* | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | |
| Hap_6 | *C. reticulata* | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | . | ID | ID | ID | ID | . | . | . | . | . | |
| Hap_7 | *C. micrantha* | . | T | A | A | A | T | C | A | A | A | G | A | A | A | G | ID | ID | ID | ID | ID | . | . | . | . | . | |

| | | trnL intron | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 163 | 223 | 230 | 277 | 286 | 287 | 288 | 289 | 290 | 291 | 305 | 306 | 307 | 308 | 309 | 310 |
| | Consensus | C | G | T | T | ID | ID | ID | ID | ID | ID | A | T | T | G | T | T |
| Hap_1 | Pompia | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_1 | Rhobsel arsa | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_1 | *C. limon* | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_1 | *C. bergamia* | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_1 | *C. aurantium* | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_5 | *C. maxima* | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_8 | *C. sinensis* | . | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |
| Hap_4 | *C. medica* | A | A | . | . | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID | ID |
| Hap_6 | *C. reticulata* | A | . | G | C | G | A | A | A | A | A | . | . | . | . | . | . |
| Hap_7 | C. micrantha | A | . | . | . | ID | ID | ID | ID | ID | ID | . | . | . | . | . | . |

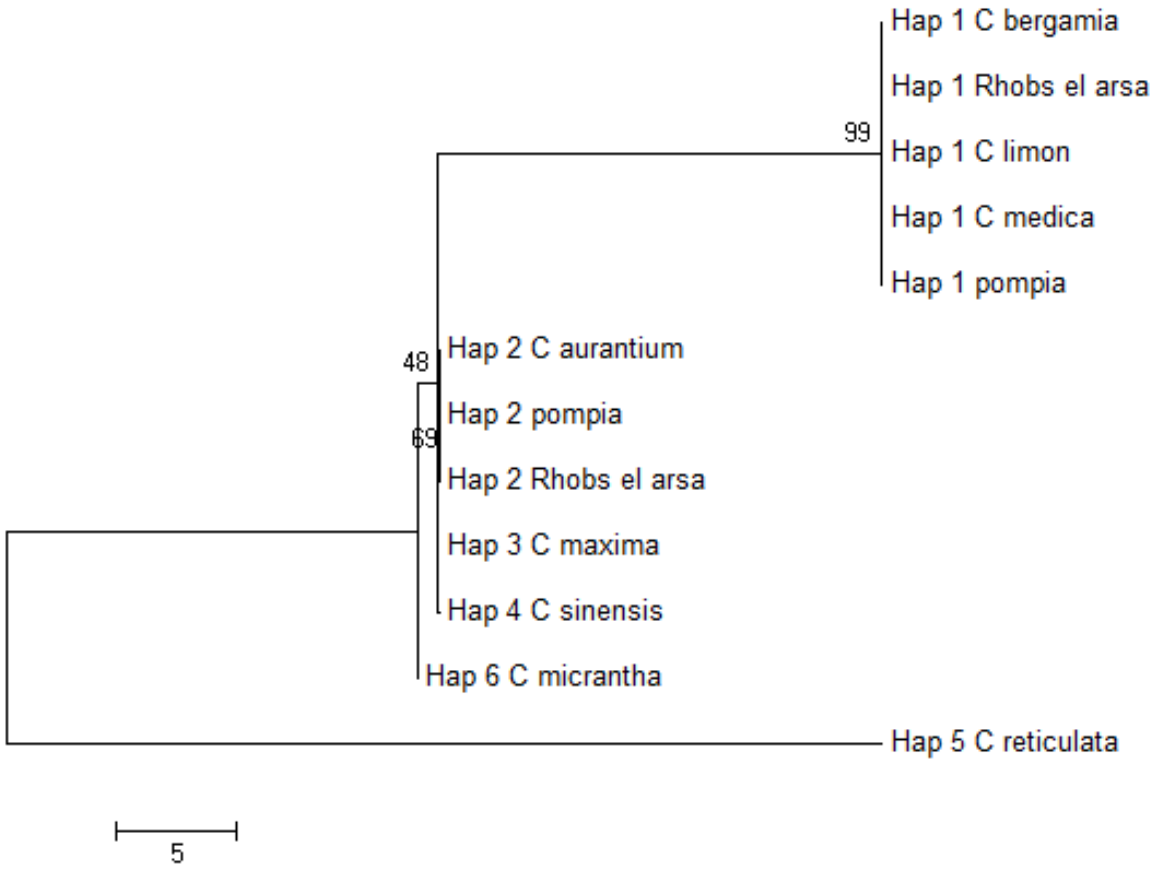



**Figure 6:** Haplotypes ITS

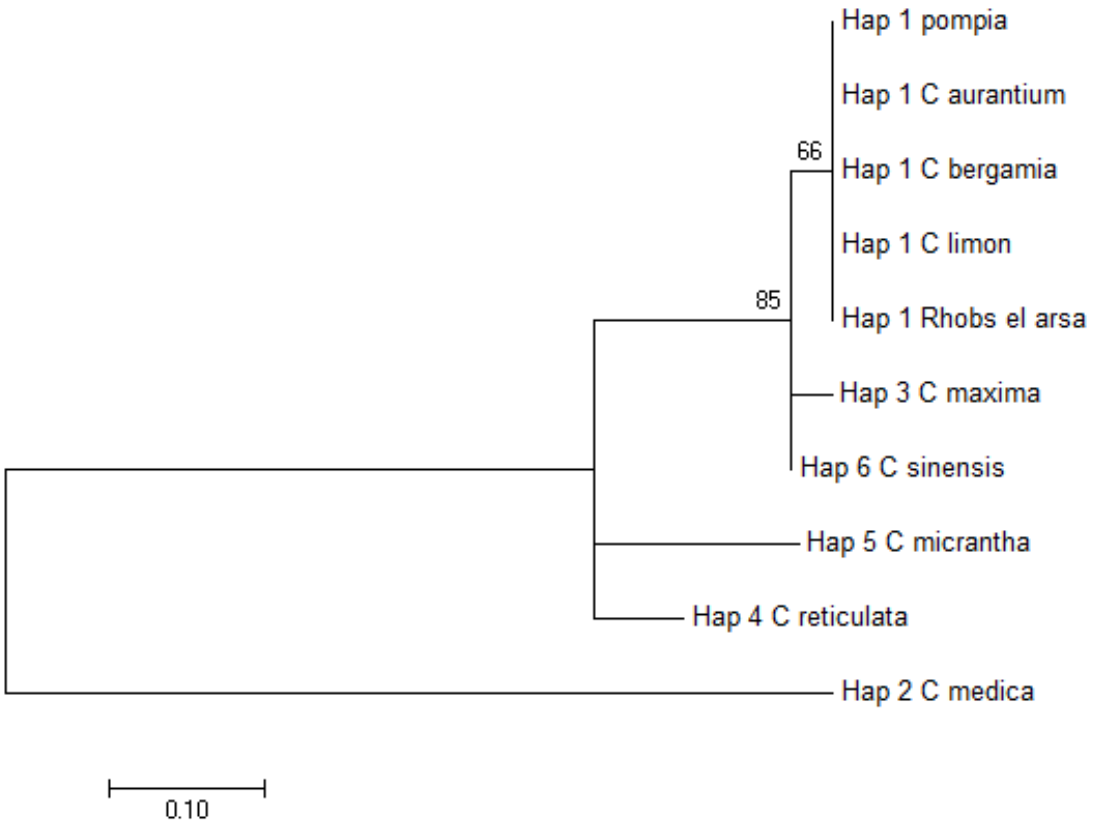



**Figure 7:** Haplotypes cpDNA

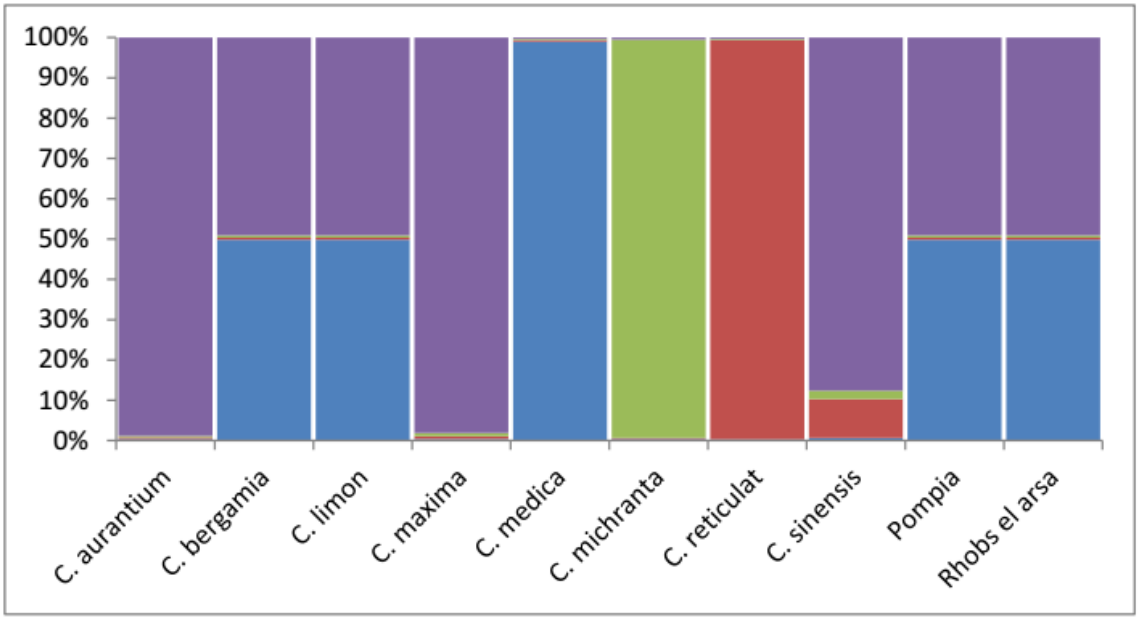



**Figure 8:** Genetic relationships of the *Citrus* accessions investigated in this study as estimated by STRUCTURE by using the ITS data set. Each sample is represented by a vertical histogram partitioned into K=4 coloured segments that represent the estimated membership. The proportion of ancestry (%) is reported on the ordinate axis and the name of each accession is reported below each histogram. Violet, blue, green and red correspond to the inferred contributions of *C. maxima*, *C. medica*, *C. micrantha* and *C. reticulata*, respectively

**Conclusions**

The present study confirms that Pompia species is diploid and shows its karyotype. The results support the commonly accepted idea that Pompia is a very recent hybrid and confirm that phylogenetic origin is closely related to *C. aurantium*, *C. maxima* as maternal parent and *C. medica* as paternal ancestor. Accordingly, genomic regions investigated in this study do not allow to distinguish between Pompia and other closely related hybrids such as *C. limon*, *C. bergamia* and Rhobs el arsa. As for the polymorphic content of each assessed region, our data indicated that the barcode region trnL-intron is far less informative than the other assessed regions. Moreover, psbA-trnH intergenic spacer proved to be most suitable region for the discrimination and traceability of our *Citrus* accessions, together with the nuclear ITS1. Nevertheless, the analysis of the afore mentioned markers, either alone or in combinations, does not provide polymorphisms useful for precise and univocal determination of the genetic origin of Pompia. Additional investigations based on a higher number of loci and/or different marker types will be needed to identify the most probable ancestors of Pompia.

## References


Aleza P., Froelicher Y., Schwarz S., Agust M., Hernandez M., Juarez J.,Luro F., Morillon R., Navarro L. and Ollitrault P., 2011. Tetraploidization events by chromosome doubling of nucellar cells are frequent in apomictic citrus and are dependent on genotype and environment. *Annals of Botany*, pp 1-14. doi:10.1093/aob/mcr099

Cabasson C., Luro F., Ollitrault P., Grosser J., 2001. Non-random inheritance of mitochondrial genomes in Citrus hybrids produced by protoplast fusion. Plant Cell Reports, 20(7): 604-609

Camarda I., Mazzola P., Brunu A., Fenu G., Lombardo G., Palla F., 2013. Un agrume nella storia della Sardegna: Citrus limónvar. Pompia Camarda var. nova. Quad. Bot. Amb. Appl. 24: 109-118

Cameron J.W., Soost R.K., 1969. Characters of new populations of Citrus polyploids, and the relation between tetraploidy in the pollen parent and hybrid tetraploid progeny. Proc. First Int. Citrus Symp. 1: 199-205

CBOL Plant Working Group, 2009. A DNA barcode for land plants. ProcNatlAcad Sci USA 106: 12794–12797

Curk F., Ancillo G., Ollitrault F., Perrier X., Jacquemoud-Collet J.P., Garcia-Lor A., Navarro L., Ollitrault P., 2015. Nuclear species-diagnostic SNP markers mined from 454 amplicon sequencing reveal admixture genomic structure of modern citrus varieties. PloSone, 10(5): e0125628

Curk F., Ollitrault F., Garcia-Lor A., Luro F., Navarro L., Ollitrault P., 2016. Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. Annals of botany, 117(4): 565-583

D'Aquino S., Fronteddu F., Usai M., Palma A., 2005. Qualitative and physiological properties of 'Pompia', a citron-like fruit. Plant Genetic Resources Newsletter 143: 40-45

DeSalle R., Egan M.G., Siddall M., 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462): 1905-1916

Esen A., Soost R.K., 1971. Unexpected triploids in Citrus: their origin, identification, and possible use. Journal of Heredity, 62(6): 329-333

Esen A., Soost R.K., Geraci G., 1979. Genetic evidence for the origin of diploid megagametophytes in Citrus. Journal of Heredity, 70(1): 5-8

Evanno G., Regnaut S., Goudet J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular ecology, 14(8): 2611-2620

Falush D., Stephens M., Pritchard J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics, 164(4): 1567-1587

Frost H.B., 1925a. The chromosomes of Citrus. J. Washington Acad. Sri., 15: 1 – 3

Frost H.B., 1925b. Tetraploidy in citrus. Proceedings of the National Academy of Sciences, 11(9): 535-537

Hutchison D.J., Barrett H.C., 1981. Tetraploid frequency in nucellar seedlings from single trees of Carrizo and Troyer Citrus hybrids, Proceedings of 4th International Citrus Congress , Tokyo International Society of Citriculture pp 27-29

Hynniewta M., Malik S.K., Rao S.R., 2011. Karyological studies in ten species of Citrus (Linnaeus, 1753)(Rutaceae) of North-East India. Comparative cytogenetics, 5(4): 277

Kimura M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution, 16(2): 111-120

Krug C.A., 1943. Chromosome numbers in the subfamily Aurantioideae with special reference to the genus Citrus. Bot. Gaz., 104: 602 - 611

Krug C.A., Bacchi O., 1943. Triploid varieties of Citrus. Journal of Heredity, 34: 277-283

Laskar B.A., Bhattacharjee M.J., Dhar B., Mahadani P., Kundu S., Ghosh S.K., 2013. The species dilemma of northeast Indian mahseer (Actinopterygii: Cyprinidae): DNA barcoding in clarifying the riddle. PloS one, 8(1): e53704

Levan A., Fredga K., Sandberg A.A., 1964. Nomenclature for centromeric position on chromosomes. Hereditas, 52(2): 201-220

Liu J., Provan J., Gao L.M., Li D.Z., 2012. Sampling strategy and potential utility of indels for DNA barcoding of closely related plant species: a case study in Taxus. International journal of molecular sciences, 13(7): 8740-8751

Longley A.E., 1925. Polycary, polyspory and polyploidy in Citrus and Citrus relatives. Journal of the Washington Academy of Science 15: 347-351

Luo K., Chen S., Chen K., Song J., Yao H., Ma X., Zhu Y., Pang X., Yu H., Li X., Liu Z., 2010. Assessment of candidate plant DNA barcodes using the Rutaceae family. Science China Life Sciences, 53(6): 701-708

Mahadani P., Ghosh S.K., 2014. Utility of indels for species-level identification of a biologically complex plant group: a study with intergenic spacer in Citrus. Molecular biology reports, 41(11): 7217-7222

Maia V.H., Da Mata C.S., Franco L.O., Cardoso M.A., Cardoso S.R.S., Hemerly A.S., Ferreira P.C.G., 2012. DNA barcoding Bromeliaceae: achievements and pitfalls. PLoS One, 7(1): e29877

Mignani I., Mulas M., Mantegazza M., Lovigu N., Spada A., Nicolosi E., Bassi D., 2015. Characterization by molecular markers of “Pompia” a natural Citrus hybrid cultivated in Sardinia. – ActaHortic. (ISHS) 1065:165-172

Nicolè S., Erickson D.L., Ambrosi D., Bellucci E., Lucchin M., Papa R., Kress W.J., Barcaccia G., 2011. Biodiversity studies in Phaseolus species by DNA barcoding. Genome, 54(7): 529-545

Oiyama I., Okudai N., Takahara T., 1981. Ploidy levels of seedlings obtained from 2x X 4x crosses in Citrus. Proc. Int. Soc. Citric. 1: 32-34

Pallottini L., Garcia E., Kami J., Barcaccia G., Gepts P., 2004. The genetic anatomy of a patented yellow bean. Crop science, 44(3): 968-977

Pritchard J.K., Stephens M., Rosenberg N.A., Donnelly P., 2000. Association mapping in structured populations. The American Journal of Human Genetics, 67(1): 170-181

Rach J., DeSalle R., Sarkar I.N., Schierwater B., Hadrys H., 2008. Character-based DNA barcoding allows discrimination of genera, species and populations in Odonata. Proceedings of the Royal Society of London B: Biological Sciences, 275(1632): 237-247

Roy S., Tyagi A., Shukla V., Kumar A., Singh U.M., Chaudhary L.B., Datt B., Bag S.K., Singh P.K., Nair N.K., Husain T., Tuli R., 2010. Universal plant DNA barcode loci may not work in complex groups: a case study with Indian Berberis species. PloS one, 5(10): e13674

Rozas J., Sánchez-DelBarrio J.C., Messeguer X., Rozas R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics, 19(18): 2496-2497

Sang T., Crawford D., Stuessy T., 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). American Journal of Botany, 84(8): 1120-1120

Sharma A.K., Bal A.K., 1957. Chromosomes studies in Citrus.i. Agron. Lusitana, 19: 101-126

Spooner D.M., 2009. DNA barcoding will frequently fail in complicated groups: an example in wild potatoes. American Journal of Botany, 96(6): 1177-1189

Tamura K., Dudley J., Nei M., Kumar S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular biology and evolution, 24(8): 1596-1599

Tate J.A., Simpson B.B., 2003. Paraphyly of Tarasa (Malvaceae) and diverse origins of the polyploid species. Systematic Botany, 28(4): 723-737

Tripathi A.M., Tyagi A., Kumar A., Singh A., Singh S., Chaudhary L.B., Roy S., 2013. The Internal Transcribed Spacer (ITS) Region and trnhH-psbA Are Suitable Candidate Loci for DNA Barcoding of Tropical Tree Species of India. PLoS ONE, 8(2): e57934 http://doi.org/10.1371/journal.pone.0057934

Velzen R., Bakker F.T., Loon J.J., 2007. DNA barcoding reveals hidden species diversity in Cymothoe (Nymphalidae). Proc. Neth. Entomol. Soc. Meet. 18: 95-103

Vences M., Thomas M., Bonett R.M., Vieites D.R., 2005. Deciphering amphibian diversity through DNA barcoding: chances and challenges. Philosophical Transactions of the Royal Society of London Biological Sciences, 360(1462): 1859-1868

Wakana A., Iwamasa M., Uemoto S., 1981. Seed development in relation to ploidy of zygotic embryo and endosperm in polyembryonic Citrus. Proc. Int. Soc. Citric. 1: 35-39

White T.J., Bruns T., Lee S.J.W.T., Taylor J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1): 315-322

Wiemers M., Fiedler K., 2007. Does the DNA barcoding gap exist?–a case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in zoology, 4(1): 8

Wong E.H.K., Hanner R.H., 2008. DNA barcoding detects market substitution in North American seafood. Food Research International, 41(8): 828-837

## Supplemental material

### **Supplementary File 1:** Output Karyotype software

Karyotype asymmetry degree (Stebbins, 1971): 2A

Karyotype formula (Levan et al., 1964): 2n = 2x = 16m + 2sm

Original Table (L: long arms; S: short arms; 0: no satellite; 1: on the long arm; 2: on the short arm; 3: intercalary satellite)

| ID | L | S | L+S | L-S | L/S | Group ID | Satellites |
|---|---|---|---|---|---|---|---|
| 1 | 11.34 | 9.16 | 20.50 | 2.18 | 1.24 | 3 | 0 |
| 2 | 11.54 | 10.02 | 21.56 | 1.52 | 1.15 | 2 | 0 |
| 3 | 14.04 | 10.24 | 24.28 | 3.80 | 1.37 | 1 | 0 |
| 4 | 12.93 | 9.16 | 22.09 | 3.77 | 1.41 | 1 | 0 |
| 5 | 12.61 | 6.68 | 19.29 | 5.92 | 1.89 | 4 | 0 |
| 6 | 10.90 | 6.53 | 17.43 | 4.37 | 1.67 | 5 | 0 |
| 7 | 10.26 | 6.43 | 16.69 | 3.83 | 1.60 | 6 | 0 |
| 8 | 8.34 | 5.90 | 14.25 | 2.44 | 1.41 | 8 | 0 |
| 9 | 7.48 | 6.04 | 13.52 | 1.44 | 1.24 | 9 | 0 |
| 10 | 11.54 | 10.12 | 21.66 | 1.42 | 1.14 | 2 | 0 |
| 11 | 10.90 | 6.92 | 17.82 | 3.98 | 1.57 | 5 | 0 |
| 12 | 9.79 | 6.53 | 16.32 | 3.26 | 1.50 | 7 | 0 |
| 13 | 9.10 | 6.34 | 15.43 | 2.76 | 1.44 | 7 | 0 |
| 14 | 10.96 | 8.98 | 19.94 | 1.97 | 1.22 | 3 | 0 |
| 15 | 10.65 | 6.53 | 17.18 | 4.12 | 1.63 | 6 | 0 |
| 16 | 7.81 | 5.51 | 13.32 | 2.31 | 1.42 | 9 | 0 |
| 17 | 13.32 | 6.07 | 19.39 | 7.25 | 2.19 | 4 | 0 |
| 18 | 8.42 | 5.43 | 13.85 | 2.99 | 1.55 | 8 | 0 |

**HaploidTable**

| Group ID | L | S | L+S | L-S | S/L+S(%) | L/S |
|---|---|---|---|---|---|---|
| 1 | 13.48 | 9.70 | 23.18 | 3.78 | 41.85 | 1.39 |
| 2 | 11.54 | 10.07 | 21.61 | 1.47 | 46.60 | 1.15 |
| 3 | 11.15 | 9.07 | 20.22 | 2.07 | 44.87 | 1.23 |
| 4 | 12.96 | 6.38 | 19.34 | 6.59 | 32.97 | 2.03 |
| 5 | 10.90 | 6.72 | 17.62 | 4.17 | 38.16 | 1.62 |
| 6 | 10.46 | 6.48 | 16.94 | 3.98 | 38.26 | 1.61 |
| 7 | 9.44 | 6.43 | 15.87 | 3.01 | 40.51 | 1.47 |
| 8 | 8.38 | 5.67 | 14.05 | 2.72 | 40.33 | 1.48 |
| 9 | 7.64 | 5.77 | 13.42 | 1.87 | 43.02 | 1.32 |

Karyotypeparameterstable

| x | 2n | THL | CVCI | CVCL | MCA |
|---|---|---|---|---|---|
| 9 | 18 | 162.25 | 9.94 | 18.51 | 18.54 |

The relative length

| Group | L(%) | S(%) | L+S (%) | L/S | Type |
|---|---|---|---|---|---|
| 1 | 8.31 | 5.98 | 8.31+5.98=14.29 | 1.39 | m |

| | | | | | |
|---|---|---|---|---|---|
| 2 | 7.11 | 6.21 | 7.11+6.21=13.32 | 1.15 | m |
| 3 | 6.87 | 5.59 | 6.87+5.59=12.46 | 1.23 | m |
| 4 | 7.99 | 3.93 | 7.99+3.93=11.92 | 2.03 | m |
| 5 | 6.72 | 4.14 | 6.72+4.14=10.86 | 1.62 | m |
| 6 | 6.44 | 3.99 | 6.44+3.99=10.44 | 1.61 | m |
| 7 | 5.82 | 3.96 | 5.82+3.96=9.78 | 1.47 | m |
| 8 | 5.17 | 3.49 | 5.17+3.49=8.66 | 1.48 | m |
| 9 | 4.71 | 3.56 | 4.71+3.56=8.27 | 1.32 | m |

| ID | L(%) | S(%) | L+S (%) | L/S | Type |
|---|---|---|---|---|---|
| 1 | 3.49 | 2.82 | 3.49+2.82=6.32 | 1.24 | m |
| 2 | 3.56 | 3.09 | 3.56+3.09=6.64 | 1.15 | m |
| 3 | 4.33 | 3.16 | 4.33+3.16=7.48 | 1.37 | m |
| 4 | 3.98 | 2.82 | 3.98+2.82=6.81 | 1.41 | m |
| 5 | 3.88 | 2.06 | 3.88+2.06=5.94 | 1.89 | sm |
| 6 | 3.36 | 2.01 | 3.36+2.01=5.37 | 1.67 | m |
| 7 | 3.16 | 1.98 | 3.16+1.98=5.14 | 1.60 | m |
| 8 | 2.57 | 1.82 | 2.57+1.82=4.39 | 1.41 | m |
| 9 | 2.30 | 1.86 | 2.30+1.86=4.16 | 1.24 | m |
| 10 | 3.56 | 3.12 | 3.56+3.12=6.67 | 1.14 | m |
| 11 | 3.36 | 2.13 | 3.36+2.13=5.49 | 1.57 | m |
| 12 | 3.02 | 2.01 | 3.02+2.01=5.03 | 1.50 | m |
| 13 | 2.80 | 1.95 | 2.80+1.95=4.76 | 1.44 | m |
| 14 | 3.38 | 2.77 | 3.38+2.77=6.14 | 1.22 | m |
| 15 | 3.28 | 2.01 | 3.28+2.01=5.29 | 1.63 | m |
| 16 | 2.41 | 1.70 | 2.41+1.70=4.10 | 1.42 | m |
| 17 | 4.10 | 1.87 | 4.10+1.87=5.98 | 2.19 | sm |
| 18 | 2.59 | 1.67 | 2.59+1.67=4.27 | 1.55 | m |

Other Information

longest/shortest = 1.82

The total haploid length of the chromosome set (Peruzzi et al., 2009), THL = 162.25

Coefficient of Variation of Centromeric Index (Paszko, 2006), CVCI = 9.94

Coefficient of Variation of Chromosome Length (Paszko, 2006), CVCL = 18.51

Mean Centromeric Asymmetry (Peruzzi and Eroglu, 2013), MCA = 18.54

---

Number of chromosome which (long arm/short arm) > 2: 1 (5.56%)

The Karyotype asymmetry index (Arano, 1963), AsK% = 59.14%

The total form percent (Huziwara, 1962), TF% = 40.86%

The index of Karyotype symmetry (Greilhuber and Speta, 1976), Syi = 69.09%

The index of chromosomal size resemblance (Greilhuber and Speta, 1976), Rec = 74.26%

The intra chromosomalasymmetryindex (Romero Zarco, 1986), A1 = 0.31

The inter chromosomal asymmetry index( Romero Zarco, 1986), A2 = 0.19

The degree of asymmetry of Karyotype (Watanabe et al., 1999), A = 0.19

The dispersionindex (Lavania and Srivastava, 1992), DI = 6.80

The asymmetryindex (Paszko, 2006), AI = 1.84

**Supplementary File 2:** CLUSTAL multiple sequence alignment by MUSCLE (3.8)

```
C_reticulata
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
M14_Pompia_milis
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
B3_Pompia_bitti
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
O1506II_Pompia_oliena
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
O81_Pompia_oliena
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
ME12III_Pompia_siniscola
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
T41III_Pompia_siniscola
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
S31_Pompia_siniscola
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
C_medica_var_diamante
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
C_medica_var_PC
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
C_limon
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
2Rhobs_el_arsa
GGATCATTGTTGAAACCTGCCCAGCAGAACGACCCGTGAACCAGTTGATATCACCGGCGG
C_micrantha
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
C_aurantium
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
O1503II_Pompia_oliena
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
M31II_Pompia_milis
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
O86_Pompia_oliena
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
1Rhobs_el_arsa
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
C_sinensis
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
C_maxima
GGATCATTGTCGAAACCTGCCCAGCAGAACGACCCGCGAACCAGTTGATATCACCGGCGG
********** ************************ **********************

C_reticulata
CGGGAGGGGGTGCGCATCCGCAACGGGCGCTCCTCCTTCCCGCCCCATGCCGCGGGGAGA
M14_Pompia_milis
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
B3_Pompia_bitti
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
O1506II_Pompia_oliena
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
O81_Pompia_oliena
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
ME12III_Pompia_siniscola
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
```

```
T41III_Pompia_siniscola
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
S31_Pompia_siniscola
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_medica_var_diamante
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_medica_var_PC
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_limon
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
2Rhobs_el_arsa
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_micrantha
CGGGAGGGGGGATGCGTCCGCAGCGGGCGCTCCTCCTTCTCGCCCCACGCCGCGGGGAGA
C_aurantium
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
O1503II_Pompia_oliena
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
M31II_Pompia_milis
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
O86_Pompia_oliena
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
1Rhobs_el_arsa
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_sinensis
CGGGAGGGGGGACGCGCCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
C_maxima
CGGGAGGGGGGACGCGTCCGCAGCGGGCGCTCCTCCTTCCCGCCCCACGCCGCGGGGAGA
**********   **  ***** ********************* ******* ************

C_reticulata
GGGACTCGTCCCGCTCCCGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
M14_Pompia_milis
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
B3_Pompia_bitti
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
O1506II_Pompia_oliena
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
O81_Pompia_oliena
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
ME12III_Pompia_siniscola
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
T41III_Pompia_siniscola
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
S31_Pompia_siniscola
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
C_medica_var_diamante
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
C_medica_var_PC
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
C_limon
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
2Rhobs_el_arsa
GGGACTCGTTCTGCTCCCGGCTGGCGAAACAACAAACCCCCGGCGCGGACTGCGCCAAGG
C_micrantha
GGGACTCGTCCCGCTCCCGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
C_aurantium
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
```

```
O1503II_Pompia_oliena
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
M31II_Pompia_milis
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
O86_Pompia_oliena
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
1Rhobs_el_arsa
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
C_sinensis
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
C_maxima
GGGACTCGTCCCGCTCCTGGCTGGCGAAACAACGAACCCCCGGCGCGGACTGCGCCAAGG
********* * ***** *************** *************************

C_reticulata
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCCGCGGGGGTGCGGCG
M14_Pompia_milis
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
B3_Pompia_bitti
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
O1506II_Pompia_oliena
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
O81_Pompia_oliena
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
ME12III_Pompia_siniscola
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
T41III_Pompia_siniscola
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
S31_Pompia_siniscola
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
C_medica_var_diamante
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
C_medica_var_PC
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
C_limon
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
2Rhobs_el_arsa
AAATCTAACGAGAGAGCACGCTCCCGCGGC----------------------------G
C_micrantha
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCCGCGGGGGTGCGGCG
C_aurantium
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGTG
O1503II_Pompia_oliena
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGTG
M31II_Pompia_milis
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGTG
O86_Pompia_oliena
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGTG
1Rhobs_el_arsa
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGTG
C_sinensis
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGCG
C_maxima
AAATCTAACGAGAGAGCACGCTCCCGCGGCCCCGGAGACGGTGCGCTGCGGGGGTGCGGCG
**************************                                  *

C_reticulata
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
```

```
M14_Pompia_milis
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
B3_Pompia_bitti
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
O1506II_Pompia_oliena
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
O81_Pompia_oliena
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
ME12III_Pompia_siniscola
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
T41III_Pompia_siniscola
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
S31_Pompia_siniscola
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_medica_var_diamante
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_medica_var_PC
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_limon
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
2Rhobs_el_arsa
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_micrantha
CCTTCTTTCACATGCATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_aurantium
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
O1503II_Pompia_oliena
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
M31II_Pompia_milis
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
O86_Pompia_oliena
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
1Rhobs_el_arsa
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_sinensis
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
C_maxima
CCTTCTTTCACATGTATCCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
************** ******************************************

C_reticulata
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
M14_Pompia_milis
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
B3_Pompia_bitti
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
O1506II_Pompia_oliena
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
O81_Pompia_oliena
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
ME12III_Pompia_siniscola
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
T41III_Pompia_siniscola
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
S31_Pompia_siniscola
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_medica_var_diamante
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
```

```
C_medica_var_PC
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_limon
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
2Rhobs_el_arsa
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_micrantha
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_aurantium
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
O1503II_Pompia_oliena
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
M31II_Pompia_milis
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
O86_Pompia_oliena
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
1Rhobs_el_arsa
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_sinensis
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
C_maxima
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG

************************************************************

C_reticulata
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
M14_Pompia_milis
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
B3_Pompia_bitti
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
O1506II_Pompia_oliena
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
O81_Pompia_oliena
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
ME12III_Pompia_siniscola
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
T41III_Pompia_siniscola
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
S31_Pompia_siniscola
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_medica_var_diamante
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_medica_var_PC
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_limon
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
2Rhobs_el_arsa
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_micrantha
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_aurantium
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
O1503II_Pompia_oliena
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
M31II_Pompia_milis
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
```

```
O86_Pompia_oliena
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
1Rhobs_el_arsa
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_sinensis
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
C_maxima
TCTTTGAACGCAAGTTGCGCCCCAAGCCATTAGGCCGAGGGCACGTCTGCCTGGGTGTCA
************************************************************

C_reticulata
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
M14_Pompia_milis
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
B3_Pompia_bitti
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
O1506II_Pompia_oliena
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
O81_Pompia_oliena
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
ME12III_Pompia_siniscola
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
T41III_Pompia_siniscola
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
S31_Pompia_siniscola
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
C_medica_var_diamante
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
C_medica_var_PC
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
C_limon
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
2Rhobs_el_arsa
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCCGGGGTGCGGGCGG
C_micrantha
CGCATCGTTGCCCCACCCCACCCCCCCAAAACCAAGGCGGGGGCCCCGGGGTGTGGGCGG
C_aurantium
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
O1503II_Pompia_oliena
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
M31II_Pompia_milis
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
O86_Pompia_oliena
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
1Rhobs_el_arsa
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
C_sinensis
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
C_maxima
CGCATCGTTGCCCCACCCCACCCCCCC-AAACCAAGGCGGGGGCCCTGGGGTGCGGGCGG
*************************** ****************** ****** ******

C_reticulata
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATCTGAGTCCTCGGCGAGC
M14_Pompia_milis
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
B3_Pompia_bitti
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
```

```
O1506II_Pompia_oliena
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
O81_Pompia_oliena
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
ME12III_Pompia_siniscola
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
T41III_Pompia_siniscola
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
S31_Pompia_siniscola
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_medica_var_diamante
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_medica_var_PC
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_limon
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
2Rhobs_el_arsa
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_micrantha
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_aurantium
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
O1503II_Pompia_oliena
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
M31II_Pompia_milis
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
O86_Pompia_oliena
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
1Rhobs_el_arsa
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_sinensis
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
C_maxima
AGATTGGCCTCCCGTGCGCTGACCGCTCGCGGTTGGCCCAAATATGAGTCCTCGGCGACC
****************************************** ************** *

C_reticulata                GAAGCCGCGGCGATCGGTGGTGAA
M14_Pompia_milis            GAAGCCGCGGCGATCGGTGGTGAA
B3_Pompia_bitti             GAAGCCGCGGCGATCGGTGGTGAA
O1506II_Pompia_oliena       GAAGCCGCGGCGATCGGTGGTGAA
O81_Pompia_oliena           GAAGCCGCGGCGATCGGTGGTGAA
ME12III_Pompia_siniscola    GAAGCCGCGGCGATCGGTGGTGAA
T41III_Pompia_siniscola     GAAGCCGCGGCGATCGGTGGTGAA
S31_Pompia_siniscola        GAAGCCGCGGCGATCGGTGGTGAA
C_medica_var_diamante       GAAGCCGCGGCGATCGGTGGTGAA
C_medica_var_PC             GAAGCCGCGGCGATCGGTGGTGAA
C_limon                     GAAGCCGCGGCGATCGGTGGTGAA
2Rhobs_el_arsa              GAAGCCGCGGCGATCGGTGGTGAA
C_micrantha                 GAAGCCGCGGTGATCGGTGGTGAA
C_aurantium                 GAAGCCGCGGCGATCGGTGGTGAA
O1503II_Pompia_oliena       GAAGCCGCGGCGATCGGTGGTGAA
M31II_Pompia_milis          GAAGCCGCGGCGATCGGTGGTGAA
O86_Pompia_oliena           GAAGCCGCGGCGATCGGTGGTGAA
1Rhobs_el_arsa              GAAGCCGCGGCGATCGGTGGTGAA
C_sinensis                  GAAGCCGCGGCGATCGGTGGTGAA
C_maxima                    GAAGCCGCGGCGATCGGTGGTGAA
                            ********** **************
```

**Supplementary File 3:** CLUSTAL multiple sequence alignment by MUSCLE (3.8)

```
C_medica_var_Diamante
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTCAAATTCAACCATTGAT
C_medica_var_PC
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTCAAATTCAACCATTGAT
C_reticulata
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTCAAATTCAACCATTGAT
C_micrantha
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTCAAATTCAACCATTGAT
T5_Pompia_Siniscola
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
T1_Pompia_Siniscola
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
T4_Pompia_Siniscola
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
ME1_Pompia_Siniscola
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
M1_Pompia_Milis
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
BPompia_Bitti
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
Rhobs_el_arsa
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
C_aurantium
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
C_bergamia
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
C_limon
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
C_maxima_6
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
C_sinensis
TCCGCCCCTCCGCTATTTACACAATTTTGAAATACAAAGATCTAAAATTCAACCATTGAT
****************************************** ***************

C_medica_var_Diamante
TATTTTTGTTTATCTTATCTTACTTATGAAGAACCAAATGAAAATCGAAGGGCAGAAAAC
C_medica_var_PC
TATTTTTGTTTATCTTATCTTACTTATGAAGAACCAAATGAAAATCGAAGGGCAGAAAAC
C_reticulata
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGGGCAGAAAAC
C_micrantha
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGGGAAGAAAAC
T5_Pompia_Siniscola
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
T1_Pompia_Siniscola
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
T4_Pompia_Siniscola
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
ME1_Pompia_Siniscola
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
M1_Pompia_Milis
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
BPompia_Bitti
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
Rhobs_el_arsa
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
```

```
C_aurantium
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
C_bergamia
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
C_limon
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
C_maxima_6
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
C_sinensis
TATTTTTGTTTATCTTATCTTACTTATGAAGAGCCAAATGAAGATCGAAGAGCAGAAAAC
******************************** ********* ******* * *******

C_medica_var_Diamante
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGCAATTAACTGTAAAATTAGAAAATGC
C_medica_var_PC
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGCAATTAACTGTAAAATTAGAAAATGC
C_reticulata
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAAATGC
C_micrantha
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTCGAAAATGC
T5_Pompia_Siniscola
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
T1_Pompia_Siniscola
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
T4_Pompia_Siniscola
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
ME1_Pompia_Siniscola
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
M1_Pompia_Milis
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
BPompia_Bitti
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
Rhobs_el_arsa
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
C_aurantium
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
C_bergamia
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
C_limon
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
C_maxima_6
TACAACCTTTCTATTGGCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
C_sinensis
TACAACCTTTCTATTGTCTTTTTTCTTTGCTATGAAATTAACTGTAAAATTAGAAA----
*************** ***************** ***************** ****

C_medica_var_Diamante
GACTCGAATTTCTAATTAATAATCTAATAATAAAATTAGA--------AATTTATTAGTA
C_medica_var_PC
GACTCGAATTTCTAATTAATAATCTAATAATAAAATTAGA--------AATTTATTAGTA
C_reticulata
GACTCTAATTTCTAATTAATAATCTAATAATAAAATTAGAAATTTATTAATTTATTAGTA
C_micrantha
GACTCTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTACTAATTTATTAGTA
T5_Pompia_Siniscola
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
T1_Pompia_Siniscola
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
```

```
T4_Pompia_Siniscola
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
ME1_Pompia_Siniscola
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
M1_Pompia_Milis
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
BPompia_Bitti
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
Rhobs_el_arsa
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
C_aurantium
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
C_bergamia
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
C_limon
----CTAATTTCTAATTAATAATCTAATAATAAAATTATAAATTTAGTAATTTATTAGTA
C_maxima_6
----CTAATTTCTAATTAATAATCTAATAATAAAATTAGAAATTTAGTAATTTATTAGTA
C_sinensis
----CTAATTTCTAATTAATAATCTAATAATAAAATTAGAAATTTAGTAATTTATTAGTA
    * ******************************** *        ************

C_medica_var_Diamante
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_medica_var_PC
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_reticulata
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_micrantha
GTATTAGCGCATACCAATAATATCATACTAAATCAAAGAAAGAAATCAAAGAAAGAAAAG
T5_Pompia_Siniscola
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
T1_Pompia_Siniscola
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
T4_Pompia_Siniscola
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
ME1_Pompia_Siniscola
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
M1_Pompia_Milis
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
BPompia_Bitti
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
Rhobs_el_arsa
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_aurantium
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_bergamia
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_limon
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_maxima_6
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
C_sinensis
GTATTAGCGCATACCAACAATATCATACT-------------AAATCAAAGAAAGAAAAG
**************** ***********             ******************
```

```
C_medica_var_Diamante
CATAAAAATACTT---------AAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_medica_var_PC
CATAAAAATACTT---------AAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_reticulata
CATAAAAATACTTA----AAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_micrantha
CATAAAAATACTT-----AAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
T5_Pompia_Siniscola
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
T1_Pompia_Siniscola
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
T4_Pompia_Siniscola
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
ME1_Pompia_Siniscola
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
M1_Pompia_Milis
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
BPompia_Bitti
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
Rhobs_el_arsa
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_aurantium
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_bergamia
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_limon
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_maxima_6
CATAAAAATACTTAAACAAAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
C_sinensis
CATAAAAATACTTAAAC-AAAAAAAAAAAAAATGAACTAAAAACTAATAAAGAACCCCGA
*************         ************************************

C_medica_var_Diamante
TAAAGAAA-CCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_medica_var_PC
TAAAGAAA-CCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_reticulata
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_micrantha
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
T5_Pompia_Siniscola
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
T1_Pompia_Siniscola
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
T4_Pompia_Siniscola
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
ME1_Pompia_Siniscola
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
M1_Pompia_Milis
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
BPompia_Bitti
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
Rhobs_el_arsa
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_aurantium
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
```

```
C_bergamia
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_limon
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_maxima_6
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
C_sinensis
TAAAGAAACCCGACTAAATAACGGATCAATACTGACCCCCAGCTGGGGGTCAGTATTGAT
******** **************************************************

C_medica_var_Diamante
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_medica_var_PC
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_reticulata
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_micrantha
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
T5_Pompia_Siniscola
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
T1_Pompia_Siniscola
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
T4_Pompia_Siniscola
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
ME1_Pompia_Siniscola
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
M1_Pompia_Milis
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
BPompia_Bitti
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
Rhobs_el_arsa
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_aurantium
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_bergamia
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_limon
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_maxima_6
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
C_sinensis
CCGTTATTTTCAAAAACCCGCCTACACAAAGACCAAAATCTTACCCATTTGTACCCAGGA
************************************************************

C_medica_var_Diamante
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_medica_var_PC
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_reticulata
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_micrantha
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
T5_Pompia_Siniscola
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
T1_Pompia_Siniscola
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
T4_Pompia_Siniscola
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
```

```
ME1_Pompia_Siniscola
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
M1_Pompia_Milis
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
BPompia_Bitti
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
Rhobs_el_arsa
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_aurantium
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_bergamia
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_limon
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_maxima_6
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
C_sinensis
ATTAAAAATGGGTAATCCTGAGCCAAATCCTCTTCTCTTTTCCAAGAACAAACAGGGGTT
************************************************************

C_medica_var_Diamante
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_medica_var_PC
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_reticulata
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_micrantha
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
T5_Pompia_Siniscola
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
T1_Pompia_Siniscola
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
T4_Pompia_Siniscola
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
ME1_Pompia_Siniscola
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
M1_Pompia_Milis
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
BPompia_Bitti
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
Rhobs_el_arsa
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_aurantium
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_bergamia
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_limon
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_maxima_6
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
C_sinensis
CAGAAAGCGAAAAAGGGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGA
************************************************************
```

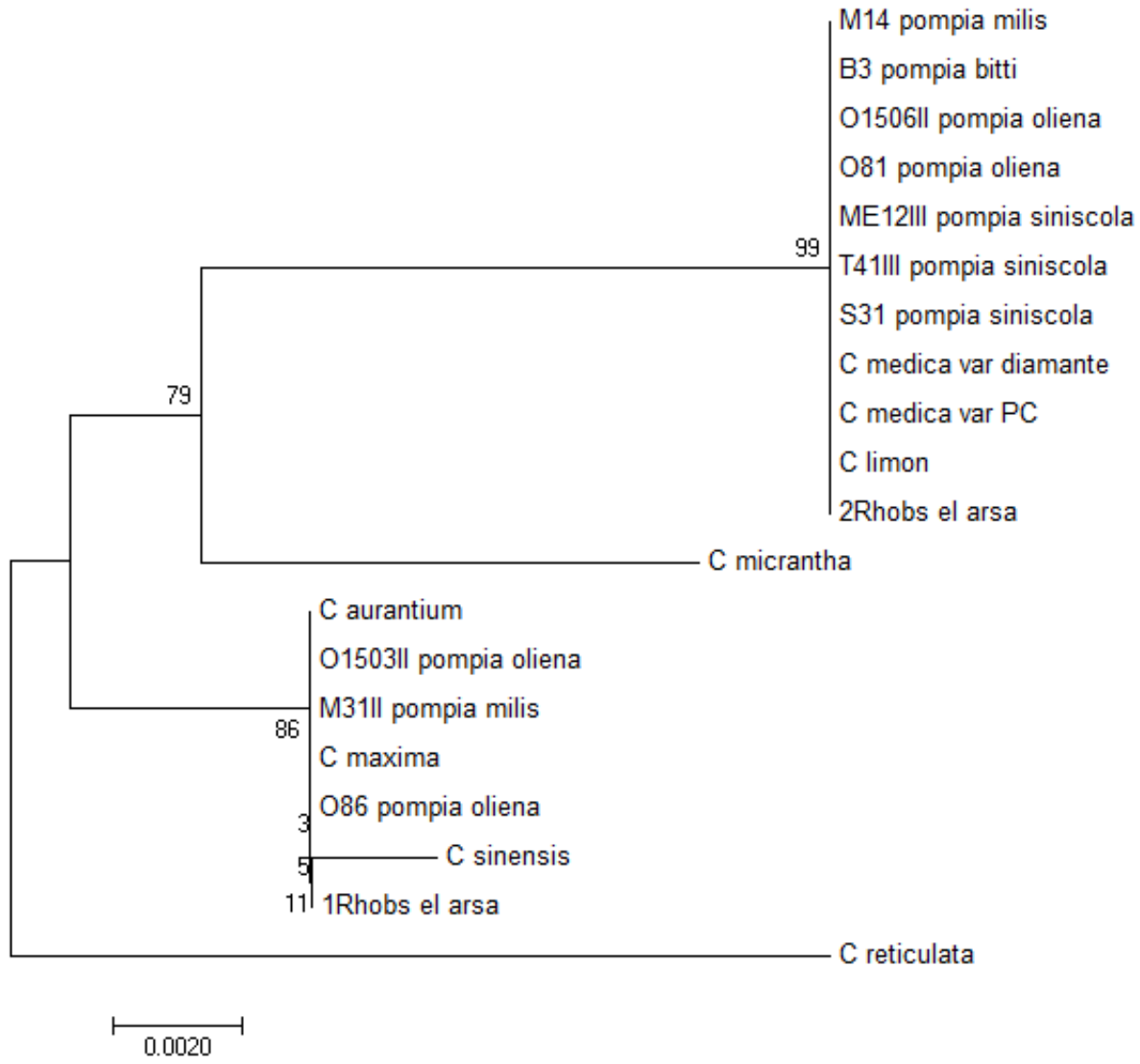
```
C_medica_var_Diamante
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTAAAATGAATGCTTCTATCGAATATC
C_medica_var_PC
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTAAAATGAATGCTTCTATCGAATATC
C_reticulata
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTAAAATGAATGCTTCTATCGAATATC
C_micrantha
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTAAAATGAATGCTTCTATCGAATATC
T5_Pompia_Siniscola
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
T1_Pompia_Siniscola
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
T4_Pompia_Siniscola
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
ME1_Pompia_Siniscola
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
M1_Pompia_Milis
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
BPompia_Bitti
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
Rhobs_el_arsa
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
C_aurantium
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
C_bergamia
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
C_limon
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
C_maxima_6
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
C_sinensis
GTTGACTGCCCTTTTTGGTAAAGAAAAAAGAAAGTCAAATGAATGCTTCTATCGAATATC
********************************** ************************

C_medica_var_Diamante
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAAACTATGTATACGCAGCGAAAAACT
C_medica_var_PC
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAAACTATGTATACGCAGCGAAAAACT
C_reticulata
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGGATACGCAGCGAAAAACT
C_micrantha
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
T5_Pompia_Siniscola
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
T1_Pompia_Siniscola
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
T4_Pompia_Siniscola
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
ME1_Pompia_Siniscola
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
M1_Pompia_Milis
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
BPompia_Bitti
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
Rhobs_el_arsa
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
C_aurantium
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
```

```
C_bergamia
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
C_limon
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
C_maxima_6
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
C_sinensis
GAAACTCGATAAAGGATGAAGGATAAGGGTATATAGACTATGTATACGCAGCGAAAAACT
********************************** ****** ****************

C_medica_var_Diamante
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGA---
C_medica_var_PC
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGA---
C_reticulata
AACTCAAAAATCACAACCAAATACGTATTCCTTTTTATGAAAAAGAAAAAGAAAAGAATT
C_micrantha
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
T5_Pompia_Siniscola
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
T1_Pompia_Siniscola
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
T4_Pompia_Siniscola
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
ME1_Pompia_Siniscola
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
M1_Pompia_Milis
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
BPompia_Bitti
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
Rhobs_el_arsa
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
C_aurantium
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
C_bergamia
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
C_limon
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
C_maxima_6
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
C_sinensis
AACTCAAAAATCACAACCAAATACGTATTTCTTTTTAT------GAAAAAGAAAAGAATT
**************************** ********      *************

C_medica_var_Diamante
---ATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_medica_var_PC
---ATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_reticulata
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_micrantha
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
T5_Pompia_Siniscola
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
T1_Pompia_Siniscola
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
T4_Pompia_Siniscola
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
```

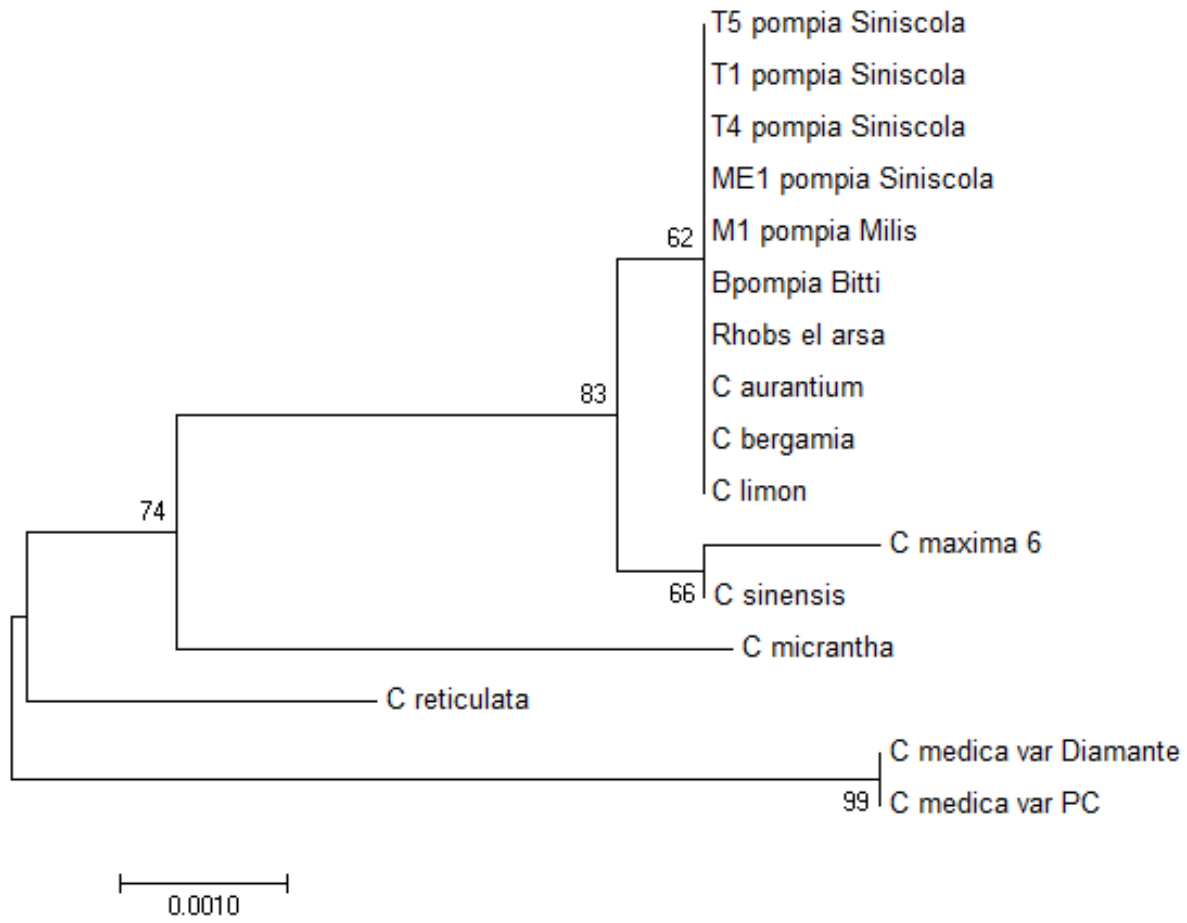
```
ME1_Pompia_Siniscola
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
M1_Pompia_Milis
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
BPompia_Bitti
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
Rhobs_el_arsa
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_aurantium
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_bergamia
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_limon
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_maxima_6
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
C_sinensis
GTTATTGTTATGAATCGATTCTAAGTTGAAGAAAGAATCGAATATTCCCGGCTCAAATCA
**********************************************************

C_medica_var_Diamante
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_medica_var_PC
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_reticulata
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_micrantha
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
T5_Pompia_Siniscola
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
T1_Pompia_Siniscola
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
T4_Pompia_Siniscola
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
ME1_Pompia_Siniscola
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
M1_Pompia_Milis
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
BPompia_Bitti
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
Rhobs_el_arsa
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_aurantium
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_bergamia
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_limon
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_maxima_6
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
C_sinensis
TTCACTCCACCTCCATGGTCTGATCGATCCTTTCTTTTGACTTTTGAAGAACTGATTAAT
**********************************************************

C_medica_var_Diamante
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_medica_var_PC
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
```

```
C_reticulata
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_micrantha
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
T5_Pompia_Siniscola
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
T1_Pompia_Siniscola
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
T4_Pompia_Siniscola
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
ME1_Pompia_Siniscola
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
M1_Pompia_Milis
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
BPompia_Bitti
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
Rhobs_el_arsa
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_aurantium
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_bergamia
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_limon
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_maxima_6
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
C_sinensis
CGGACGAGAATAAAGATAGAGTCCCATTCTACATGTCAATATCAATACGGGCAACAATGA
************************************************************

C_medica_var_Diamante
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_medica_var_PC
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_reticulata
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_micrantha
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
T5_Pompia_Siniscola
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
T1_Pompia_Siniscola
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
T4_Pompia_Siniscola
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
ME1_Pompia_Siniscola
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
M1_Pompia_Milis
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
BPompia_Bitti
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
Rhobs_el_arsa
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_aurantium
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_bergamia
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_limon
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
```

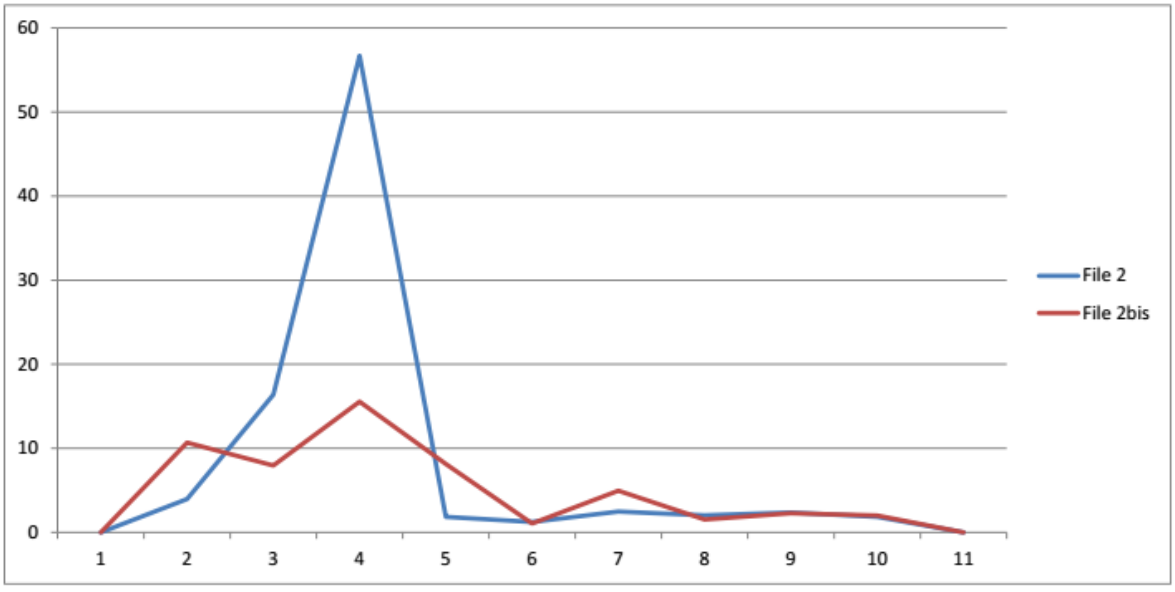
```
C_maxima_6
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
C_sinensis
AATTTAGAGTAAAAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCC
**********************************************************
```




**Supplementary File 4:** NJ ITS




**Supplementary File 5:** NJ cpDNA




**Supplementary File 6:** deltaK

# CHAPTER II

## Genetic characterization of Pompia based on SSR and InDel molecular markers

### Introduction

Due to their high variability, codominant inheritance, reproducibility and genomic distribution (Barkley et al., 2006; Gulsen and Roose, 2001a,b), Simple Sequence Repeat markers (SSRs) are widely used for *Citrus* genotyping (Kijas et al., 1997; Luro et al., 2008; Ollitrault et al., 2010; Biswas et al., 2012; Liu et al., 2013). However, these markers can be subjected to homoplasy and thus may introduce a phylogenetic bias in pedigree reconstructions (Barkley et al., 2009). Recently, the insertion/deletion (InDel) markers have been increasingly used in genetic studies of citrus (Garcìa-Lor et al., 2012; Ollitrault et al., 2014). To date, pedigree reconstructions of Pompia genotype have been based mainly on morphological markers. Camarda et al. (2013) proposed the first classification for Pompia in *C. limon* var. *Pompia*, because of its similarity to the lemon for two characters: the acidity of the pulp and the hilly aspect of the apex of the fruit. However, such a conclusion can be counteracted by other phenotypic traits differentiating Pompia from the lemon such as the surface of the very bumpy rind, the rounded shape flattened at the poles, the different fragrance and flavor. It could be eventually a lemon hybrid. Such view was supported by studies that employed molecular markers. Mignani et al (2015), proposed a hybrid origin between Etrog citron and Zagara Bianca lemon. In agreement with such proposal another study that employed both genetic and morphological markers concluded that Pompia is a hybrid between lemon and citron (Camarda et al., 2013). Both studies were based on the use of dominant multilocus markers such as AFLP, RAPD (Mignani et al., 2015) and ISSR (Camarda et al., 2013). These markers counterbalance the lower informativity on allelic status of loci with the high number of loci investigated. However, these two studies considered only few species: citron, lemon, sour orange, grapefruit and Volkamer lemon (Mignani et al., 2015) or three species of lemon and a citron (Camarda et al., 2013). The aims of our study were to gain insigths on the origin of Pompia and the diversity in Pompia germplasm. To this end we studied the polimorphysm of nuclear SSR and of six maternally inherited markers.

## Materials and Methods

### Plant material

#### *Citrus species to be compared*

15 citrus varieties were chosen to represent the genetic diversity of *Citrus* (Table 1). All the accessions studied are available at Center for Biological Resources (CRB) Citrus INRA-CIRAD of San Giuliano, Corsica (France).

*C. micrantha* is represented by a single variety. Two Italian varieties, Diamante and Poncire commun and a Corsican variety (Corsican) represented the genetic basis of *C. medica*. Willowleaf (or Avana in Italian), a typical Mediterranean mandarin and the maternal parent of clementine, Sunki and Cleopatra mandarins, were chosen to represent the *C. reticulata*. The pummelo (*C. maxima*) was also represented by three accessions, Chandler, Seedless and Deep red. In addition to these 10 varieties representing the ancestral species, 5 citrus crops were chosen because of their presence in the Mediterranean area for several centuries: the Salustiana sweet orange (*C. sinensis*), Granito sour orange(*C. aurantium*), the Eureka lemon (*C. limon*), Mexican lime (*C. aurantifolia*) and Castagnaro bergamot (*C. bergamia*). Only one accession was used for these varieties because their diversity is based only on somatic mutations not detectable with SSR or InDel markers (Luro et al., 2000; Ollitrault et al., 2003; Barkley et al., 2006; García-Lor et al., 2013; Curk et al., 2016). Note that the Mexican variety (*C. aurantifolia*) was chosen, as it is a direct product of an ancient cross between *C. micrantha* and *C. medica* (Nicolosi et al., 2000) and as progenitor of many of lime varieties (Curk et al., 2016). Finally, one Citrus spp. registered under the name of Rhobs el arsa and present in the CRB Citrus INRA-CIRAD collection, is added to investigated his relationship with *Citrus* species analyzed.

#### *Pompia accessions*

The sampling of Pompia plants was carried out in different sites in Sardinia: Milis, Bitti, Oliena and Siniscola (Table 2, Figure 1). Sampling sites were chosen to represent the geographic distribution of Pompia in Sardinia.

Sampled trees were found in private gardens and were highly differentiated for age and growing conditions. Some trees were grafted on a sour orange rootstock and other not grafted. One Pompia accession originating from the nursery Oscar Tintori (Pescia, PT, Italy) was also added as geographical control.

**Table 1:** List of accessions with Swingle and Reece (1967) classification names adopted in this study

| Species | Variety | Origin | Accession No |
|---|---|---|---|
| *C. micrantha* | Micrantha- | CRB Citrus[1] | SRA1115 |
| *C. medica* | Diamante citron | CRB Citrus | SRA540 |
| *C. medica* | Poncire commun citron | CRB Citrus | SRA701 |
| *C. medica* | Corsican citron | CRB Citrus | SRA613 |
| *C. reticulata* | Willowleaf mandarin | CRB Citrus | SRA133 |
| *C.reticulata* | Sunki mandarin | CRB Citrus | SRA705 |
| *C. reticulata* | Cleopatra mandarin | CRB Citrus | ICVN0110273 |
| *C. maxima* | Chandler pummelo | CRB Citrus | SRA608 |
| *C. maxima* | Seedless pummelo | CRB Citrus | SRA710 |
| *C. maxima* | Deep red pummelo | CRB Citrus | SRA757 |
| *C. sinensis* | Salustiana sweet orange | CRB Citrus | SRA508 |
| *C. x aurantium* | Granito sour orange | CRB Citrus | ICVN0110015 |
| *C. limon* | Eureka lemon | CRB Citrus | SRA289 |
| *C. aurantifolia* | Mexican lime | CRB Citrus | SRA140 |
| *C. x bergamia* | Castagnaro bergamot | CRB Citrus | SRA 612 |
| *Citrus* sp. | Rhobs el arsa citron | CRB Citrus | ICVN0110244 |

[1]CRB Citrus INRA-CIRAD San Giuliano Corsica, France

**Table 2:** Geographical origin of Pompia specimens

| Origin | Sample Code | Origin | Sample Code | Origin | Sample Code |
|---|---|---|---|---|---|
| BITTI | B | SINISCOLA | COOP2 | SINISCOLA | S5 |
| MILIS | M1 | SINISCOLA | ME1 | SINISCOLA | S6 |
| MILIS | M3 | SINISCOLA | ME2 | SINISCOLA | S7 |
| OLIENA | O3 | SINISCOLA | T1 | SINISCOLA | S8 |
| OLIENA | O4 | SINISCOLA | T2 | SINISCOLA | S9 |
| OLIENA | O5 | SINISCOLA | T3 | SINISCOLA | S10 |
| OLIENA | O6 | SINISCOLA | T4 | SINISCOLA | S11 |
| OLIENA | O7 | SINISCOLA | T5 | SINISCOLA | S12 |
| OLIENA | O8 | SINISCOLA | T6 | SINISCOLA | S13 |
| OLIENA | O20 | SINISCOLA | T7 | SINISCOLA | S14 |
| OLIENA | O150 | SINISCOLA | T8 | SINISCOLA | S15 |
| SINISCOLA | CON1 | SINISCOLA | T11 | SINISCOLA | S16 |
| SINISCOLA | CON2 | SINISCOLA | S1 | SINISCOLA | S17 |
| SINISCOLA | CON3 | SINISCOLA | S2 | SINISCOLA | S18 |
| SINISCOLA | COR2 | SINISCOLA | S3 | SINISCOLA | S19 |
| SINISCOLA | COOP1 | SINISCOLA | S4 | SINISCOLA | S20 |


**Figure 1:** Geographical origins of Pompia specimens (represented by red circles with inside the number of samples)

### *Molecular markers*

Ccmp5, Ccmp6 (Weising and Gardner, 1999) and Ntcp9 (Bryan at al., 1999) plastidial markers along with 3 mitochondrial (mtDNA) markers (nad7 1 / 2, 4/3 and nad2 rrn 5 / 18-1) developed by Froelicher et al. (2011) were used to analyze DNA sequence variation of cytoplasmatic genomes (Table 3). The cytoplasmic genomes of citrus have a maternal inheritance.

**Table 3:** cpDNA and mtDNA markers (primer sequence and annealing temperature)

| Marker | Marker Id | Forward primer sequence | Reverse primer sequence | Tm °C |
|---|---|---|---|---|
| Chloroplasticgenome | ccmp5 | TGTTCCAATATCTTCTTGTCATTT | AGGTTCCATCGGAACAATTAT | 55 |
| | ccmp6 | CGATGCATATGTAGAAAGCC | CATTACGTGCGACTATCTCC | 55 |
| | ntcp9 | CTTCCAAGCTAACGATGC | CTGTCCTATCCATTAAGACAATG | 55 |
| Mithochondrialgenome | nad2 4/3 | GACCTTCACCTCAAATCA | TTCAGATAACACGCACC | 55 |
| | rrn5/rrn18-1 | GAGGTCGGAATGGGATCGGG | GGGTGAAGTCGTAACAAGGT | 55 |
| | nad7 1/2 | TTTGATATAGGCTCGCT | GGAACATAGCATAGGG | 55 |

Nuclear SSR markers were chosen based on their chromosomal location with a large genome dispersion and on their heterozygosity detected in Pompia genotyping. For a complete list of SSR markers and primers see Table 4.

**Table 4:** Identity, primer sequence, annealing temperature (Tm) and position of the nuclear markers on clementine genome sequence

| Marker | Marker Id | Reference | Forward primer sequence | Reverse primer sequence | Tm °C | Scafold | Genome position (in nucleotides) |
|---|---|---|---|---|---|---|---|
| genomic SSR | Ci01C06 | FR692356 | GGACCACAACAAAGACAG | TGGAGACACAAAGAAGAA | 50 | 6 | 24790953..24791517 (- strand) |
| | Ci01H05 | AJ567401 | AAAACAACCAAAAGGACAAGATT | TTCAAACTAAACAAACCAACTCGA | 55 | 7 | 2490945..2491244 (+ strand) |
| | Ci02D11 | FR754319 | GAGTTGACCGAGAAGATT | TGAGTTTCAGTAAGTGTATGAG | 55 | 3 | 6873219..6873706 (- strand) |
| | Ci03C08 | FR677576 | CAGAGACAGCCAAGAGA | GCTTCTTACATTCCTCAAA | 55 | 2 | 27339948..27340389 (- strand) |
| | Ci06B07 | AM489745 | CGGAACAACTAAAACAAT | TGGGCTTGTAGACAGTTA | 50 | 2 | 35138766..35138894 (- strand) |
| | Ci07D05 | FR677574 | TCGTTCTTGCTTTTCCAC | GAATCAAACTACCCTCCAAT | 55 | 2 | 26516983..26517235 (+ strand) |
| | Ci01F04 | AM489736 | AAGCATTTAGGGAGGGTCACT | TGCTGCTGCTGTTGTTGTTCT | 55 | 8 | 1063542..1063894 (+ strand) |
| EST-SSR | MEST0001 | DY262452 | CAAGCCTCTCTCTTTAGTCCCA | AGTTCTTTGGTGCTTCAGGC | 55 | 1 | 20039980..20041301 (+ strand) |
| | MEST0016 | DY264179 | ACCTGAGCCCTTTTTGGTTT | GCCAGATCAAGGCTCAAATC | 55 | 9 | 2507093..2508266 (- strand) |
| | MEST0072 | DY268828 | CCAGCCTAACAATGGCTGTT | GATGATGAAGGTCTGCGGAT | 55 | 3 | 44928038..44929235 (- strand) |
| | MEST0109 | DY274239 | CCAGTCACCACAACCATCAC | TCAAAAACCCAGATCCCAAC | 55 | 4 | 3916444..3918149 (+ strand) |
| | MEST0110 | DY274244 | CTGGCTCAGCTCTGCTCATT | ATGACATAATCGTGCCCTGC | 55 | 2 | 36212502..36216776 (- strand) |
| | MEST0146 | DY278930 | AATCACCACACCCACATGTTT | CAACTCCCACCAAATCCATC | 55 | 4 | 22392031..22394239 (- strand) |
| | MEST0149 | DY279121 | TGCAGCTACCTCGGTAACAC | GGCCATCTTGGTTCAGAGAG | 55 | 9 | 25713508..25714711 (+ strand) |
| | MEST0154 | DY279967 | AAGCCTCAAGTCAAGGCAAA | GCCCCATTTTGTATGGAGTG | 55 | 5 | 3964891..3971102 (- strand) |
| | MEST0244 | DY289007 | AACCACCACTTAAACGCACC | TCCGGTGATGGTCTTACACA | 55 | 3 | 2378570..2379463 (- strand) |
| | MEST0268 | DY291937 | AATTAGGAGCAGCGTTGGAA | TCTCCGAGATCGTTTCGTTC | 55 | 2 | 9941256..9943064 (- strand) |
| | MEST0307 | DY296153 | GGAAGGAAGTTCAGGGGTTC | GCATTGTCCAATGAGCAGAA | 55 | 3 | 40117999..40119042 (+ strand) |
| | MEST0321 | DY297854 | ATAACCGTTTCTCTCAATTTTCA | AAGAAAGAAGAGATCGCTGGC | 55 | 1 | 28146736..28149541 (- strand) |
| | MEST0369 | DY272147 | GAACGGCGAACTCTTCCTC | GATCCATTGTCGGACGTTTT | 55 | 3 | 3587345..3588833 (+ strand) |
| | MEST0431 | DY291553 | GAGCTCAAAACAATAGCCGC | CATACCTCCCCGTCCATCTA | 55 | 1 | 27168718..27171055 (- strand) |
| | MEST0488 | DY297637 | CACGCTCTTGACTTTCTCCC | CTTTGCGTGTTTGTGCTGTT | 55 | 6 | 21253670..21258035 (+ strand) |
| | MEST0543 | DY278907 | CCTCCTAAAGTGAGCCCTCC | AGCCCACAAAGAAAAAGCAA | 55 | 2 | 4137732..4140258 (- strand) |
| | MEST0940 | XM_006450173 | GAGTCGCAGGACGAAAATCT | GTGGATTGATTCGGTGATCC | 55 | 1 | 758862..760017 (+ strand) |
| | MEST1118 | XM_006434638 | CAACAAGTGGGAAGGGAGAC | GACGTTTTCCGGACTTCAAA | 55 | 5 | 39862762..39864726 (- strand) |
| geneINDELs | IDAPV | Garcia Lor *et al*. 2013 | CAGCTATTGGAAAGGTTTGT | GGAGACAGGCATAAAACATC | 55 | 1 | 25990860..25991013 (+ strand) |
| | IDCAX | | TAAGCTGCATTTAACCCTTT | GCAATTGGGAGATAGTCAAT | 55 | 1 | 431697..431934 (+ strand) |
| | IDHYB1 | | AAAAACAAAGCACCCAGAT | GCCACCAGAACCTGTAATAA | 55 | 9 | 29490589..29490789 (- strand) |
| | IDPEPC2 | | TTGGAGTCTCTCTTCCAGCAA | GTGAGAGCCACAATGCAAAA | 55 | 2 | 11091393..11091543 (- strand) |
| | IDEMA | | CTCTTTCTGCTTCCTGACATC | GCCGGTGAATAAAACACAAC | 55 | 1 | 7406793..7407057 (+ strand) |

***DNA extraction, amplification and marker scoring***

DNA was extracted from young leaves using the "MATAB-chloroform" method as adapted by Cabasson et al. (2001). About 100 mg of leaves were ground in liquid nitrogen by a pestle and mortar containing a pinch of Fontainebleau sand. To the ground powder 750 µl of extraction buffer (0.1 M Tris pH 8; 1.4 M NaCl; 20 mM EDTA; MTAB 2%; PVP 1%; sodium bisulfite 0.5%) preheated at 65°C were added. The ground material was transferred to a microtube and incubated in water bath at 65°C for 30 min. Followed a deproteinization, by adding 750 µL of chloroform / isoamyl-alcohol mixture (24:1, v:v) and stirring at room temperature for 10 min. After a centrifugation of 10 min at 10,000 g, the aqueous phase was removed and the nucleic acids were precipitated by adding 500 µl of isopropanol (centrifugation of 3 min at 10,000 g). After a wash with 1 ml of 70% ethanol the pellet of nucleic acids was resuspended in 250 µl of ultra pure water. DNA purity and integrity was checked by electrophoresis on a 0.8% agarose gel in TAE buffer. DNA was quantified specrophotometrically by measuring UV absorbance with a NanoDrop 2000.

PCR reactions were carried out on a mixture comprising 10 ng of DNA, 1X PCR Buffer Eurogenetec®; 2 mM $MgCl_2$; 0.2 mM dNTPs; 0.2 µM of each primer; 1 unit of *Taq* polymerase (Goldstar Eurogenetec®). All primers were designed to be used at an annealing temperature of 55°C (Luro et al., 2008). The PCR thermal profile consisted of three stages at different temperatures: 45 X [30 sec at 94°C; 1 min at 50 or 55°C depending on the primer pair; 1 min at 72°C]. An additional stage of 4 minutes at 72°C was added at the end of the cycle. The PCR amplified fragments were electrophoresed on a 5% polyacrylamide gel (7M Urea; 0.5X TBE; acrylamide: bis-acrylamide (19:1)) in denaturing conditions. A pre-migration of the gel for 30 minutes in 0.5X TBE buffer, at constant power (80 W) was performed in order to orient the gel mesh.

Samples and marker (10 bp ladder "Invitrogen" to 0.1 µg/µl) were added to denaturing loading buffer (98% v/v formamide, 20 mM EDTA, 0.025% w/v xylene cyanol and bromophenol blue) in equivalent quantities and heated at 94°C for 10 minutes. 5 µl of the denatured samples were loaded in the gel. The electrophoresis was performed in 0.5X TBE buffer, at constant power (80 W), for a variable duration between 1:30 h for the shorter fragments (~ 100 bases) and 2:30 h for longer fragments (~ 300 bases).

Size fractionated DNA fragments were visualized by staining the gels with a silver nitrate solution (Chalhoub et al., 1997). Glass sheets were separated; the one in which is located the gel was placed in a 10% ethanol bath for 30 min to fix the DNA fragments on the gel. The gel was then washed

with pure water, and transferred into a bath of nitric acid at 1% for 5 minutes the DNA is oxidized. After rinsing two times for 5 minutes in water, the gel was stained in a bath of silver nitrate ($AgNO_3$) 0.1% for 30 minutes. Two rinse in sodium carbonate (30 g/L + 600 µL of formaldehyde) were done: the first to remove the surplus of silver nitrate, the second to visualize the DNA fragments by precipitation of silver salts.The duration of this stage depended on rate of appearance of the bands and was regulated by the operator. The staining reaction on the gel was stopped by a rinse in acetic acid at 10% for few minutes. Following a wash with water the gel was dried and analyzed. Band scoring was done manually and repeated at least twice to minimize scoring errors.

***Data analysis***

Diversity of banding patterns was elaborated based on the Nei genetic distance (1978) and an index of similarity (Simple matching) which took into account the percentage of common alleles between two citrus divided by the totality of the observed alleles. DARwin Software (Perrier et al., 2003) was used to calculate a distance matrix and represent the diversity in the form of a tree constructed according to the Weighted Neighbor-Joining method.

We performed two indices to infer the most probable Pompia parents: LAP index is for each genotype the proportion of loci sharing at least one Pompia allel; LGP index is calculated for each pair of genotypes and represents the proportion of loci available for the reconstitution of the Pompia genotype, with the condition of the presence of one allel of Pompia in each genotype. A pair of genotypes are considered as possible parents if Pompia genotype is present in their summed allelelic constitution at all loci. Each Pompia's parents had a LAP index of 100% and the pair had a LGP index of 100%. The efficiency of the parent detection of Pompia depends on the species discrimination power of the used markers.

**Results and Discussion**

***Genetic diversity inferred by maternally inherithed markers.***

The banding profiles of the chloroplast and mitochondrial markers of Pompia accessions were indistinguishable from those of sour orange, bergamot and lemon (Supplementary file 1, Figure 2). Lemon is the hybrid between sour orange and citron (Nicolosi et al., 2000) while bergamot derives from sour orange and lemon (Curk et al., 2016). Thus it is expected that these two hybrids share the same genetic profile of cpDNA and mtDNA of sour orange, as the inheritance of the cytoplasmic organelles genomes is maternal. The sour orange was obtained from a cross between a mandarin and a pummelo (Nicolosi et al., 2000), as demonstrated by genome sequences (Wu et al., 2014). The three pummelo plants included in our study have a cpDNA and mtDNA profiles different from

sour orange, as previously noted by Garcìa-Lor et al. (2013). This difference was attributable to the fact that the diversity of pummelos in our germplasm do not represent the broad diversity of C. maxima and then pummelo ancestor of sour orange is absent. The relationships between Citrus spp. as revealed by plastidial and mitochondrial markers were summarized in a NJ tree (Figure 2). Based on these finding we can conclude that one of the three citrus: sour orange, bergamot or lemon, could be the Pompia female parent, or that the true female parent of Pompia is highly similar to these species at cytoplasmatic genomic level.

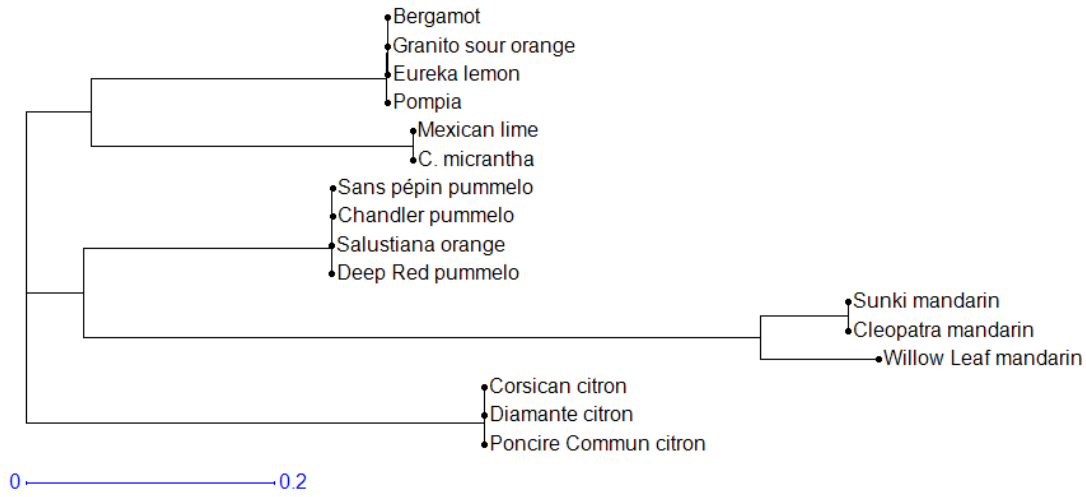



**Figure 2:** Tree representation of the cytoplasmic genomes diversity of the 16 citrus varieties (3 cpDNA markers + 3 mtDNA markers) by the "neighbor joining" method based on the genetic distances of each citrus couple calculated with the Dissimilarity index of "simple matching"

### *Diversity of the citrus data set at nuclear DNA level*

No polymorphisms among samples collected in Sardinia or among them, Oscar Tintori Pompia accession and Rhobs el arsa accession were observed. Our results suggest that Rhobs el arsa is Pompia and that this Citrus specimen is registred under false name in collection. Furthermore this finding confirms the vegetative propagation of Pompia trees in the different locations.

The allelic profiles (genotypes) of 16 citrus as revealed by the 23 nuclear markers, is reported in the Supplementary file 2 and the genetic relationships among the 16 citrus and the diversity of the citrus data set were represented by the NJ-tree (Figure 3). Two main clusters were revealed one including citrons-micrantha and their hybrid, Mexican lime, and the other, the mandarins-pummelos and their hybrids (orange and sour orange). Close to the first one, a subgroup includes Pompia, lemon and bergamot. Pompia is genetically close to lemon. This subgroup was more similar to the cluster of citrons-micrantha, than to mandarin-pummelo cluster. This finding seems in agreement with a

citron-lemon-bergamot origin and incongruent with a participation as parent of sour orange or mandarin or pummelo. A possible explanation could be that citrons are highly homozygous (20% of heterozygous loci), and thus only a few alleles are not found in lemon and in Pompia (21% and 18% respectively). Therefore, the genetic distance that separates the citrons from their descendants is smaller (greater similarity) than with the other parent, if it was a member of the mandarin-pummelo group.The sour orange, is an interspecific hybrid, pummelo x mandarin, and thus has a higher heterozygosity compared to the citron (Nicolosi et al., 2000; Wu et al., 2014). Therefore, a higher percentage of alleles are expected not to match Pompia or lemon alleles (38% and 36% respectively), which results in higher genetic distances.

Nevertheless sour orange is not totally included in the pummelo-mandarin group and it seems to have an intermediate position between the two main groups whereas it's a direct hybrid between mandarin and grapefruit (Wu et al., 2014). This apparent bias results from the selection of heterozygous Pompia markers. Markers differentiating citron from sour orange where indirectly selected and among them 3 (MEST 488, 530 and 56) with alleles not present in the three accessions of pummelos and mandarins used in this study. This bias is due to the under representation of mandarin and pummelo diversity of the present citrus set.

The citron diversity is very low represented by very short distances between citron accessions while the diversity among mandarins and pummelos are much higher. This finding is in agreement with other studies based on molecular markers (Barkley et al., 2005; Garcia-Lor et al., 2012).

***The parental origin of Pompia***

Calculated values for the LAP and LGP indices are presented in Table 5. Three citrus scored a LAP index of 100%: the Poncire citron, sour orange and lemon. Taking account of putative errors of genotyping all the varieties with at least a LAP index of 90% were considered as putative Pompia's parents. For LGP index the self fertilization was also considered as possible origin of Pompia (Table 5). Some pairs scored high LGP index (over 90%), but only the combination [citron Poncire Commun - Sour orange] scored an LGP of 100%, meaning that for the all 23 loci studied, the two Pompia alleles may be sexually inherited from the two parents. The combination [Lemon – Sour Orange] was rejected as possible parents by the MEST 431 marker (Figure 4). The LGP of the two varieties of citron, Corsican and Diamante associated with sour orange is about 96%. The combination [Lemon - Citron] scored an LGP of only 52% of markers.

The affinity between the Pompia and lemon has already been observed by Mignani et al. (2015) through the use of dominant markers AFLP and RAPD and some co-dominant markers SCAR. The

study reported a high genetic similarity of Pompia and the Etrog citron and proposed a possible parent-offspring relationship between Etrog citron and Pompia. Another recent study, also based on the use of dominant markers (ISSR) proposed citron and lemon as Pompia parents (Camarda et al., 2013).The use of dominant multilocus markers, however, did not allow a complete allelic reconstruction of each locus, neither the discrimination between homozygotes and null alleles. Our conclusion were based on co-dominant and monolocus SSR markers which were scored with a stringent protocol: each banding profile was verified by performing 3 repetitions and independent scoring.

The refusal for parental origin lemon x sour orange and the choice for the cross sour orange x citron is only based on the analysis of the locus MEST 431. At this locus one allele of Pompia is present only in the citron and the Mexican lime. The low percentage of discriminating markers is due to the low number of heterozygous markers in citrons: only 5 out of 23 loci of the citron Poncire Commun and only 3 in Corsican citron. The strong homozygosity in citron has already been described (Ollitrault et al., 2003; Barkley et al., 2006; Luro et al., 2012). It would be based on the high rate of self-fertilization during evolution.

The Poncire citron originated in Italy and would generate the variety Corsican (Luro et al., 2012). Its culture was quite common in Italy and Sardinia. The sour orange, introduced by the Moors in the eighth century (Calabrese, 1990), was present in many Mediterranean regions, used for ornamental purposes for food and perfumery (candied fruits, jams, floral water, essential oils). Hybrids of sour orange x citron were to be quite frequent as the trees of both species were present in the same places.

**Conclusions**

Pompia is the product of a cross between a sour orange as ovule parent and citron as a pollinator parent. We propose a new taxonomic denomination for Pompia as *C. aurantium* x *C. medica* var. *Pompia*, until a more rigourous taxonomical identification will be available.

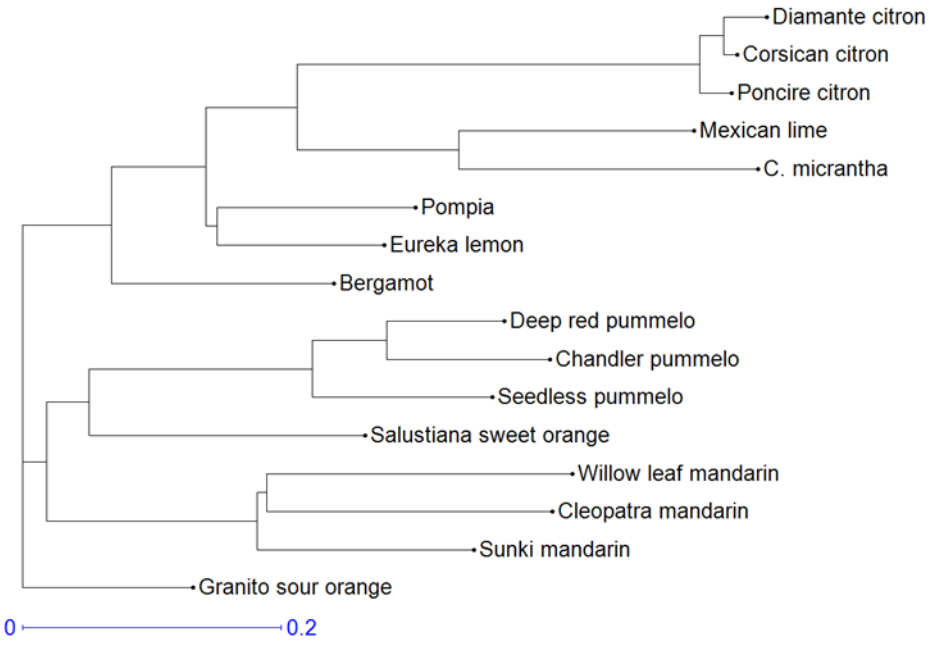



**Figure 3:** Tree representing the diversity and the relationships between the 16 citrus varieties based on the genetic distance of simple matching index calculated with the allelic genotypes of 23 nuclear markers

**Table 5:** Values (%) of LAP and LGP indices for the 15 citrus candidates for the parental origin of Pompia

| Code | Candidate varieties | **LAP**[a] | **LGP**[b] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | C13 | C14 | C15 |
| C1 | Seedlesspummelo | 65 | 13 | | | | | | | | | | | | | | |
| C2 | Chandler pummelo | 61 | 13 | 4 | | | | | | | | | | | | | |
| C3 | Deepredpummelo | 70 | 13 | 4 | 4 | | | | | | | | | | | | |
| C4 | Cleopatra mandarin | 57 | 22 | 22 | 22 | 0 | | | | | | | | | | | |
| C5 | Sunki mandarin | 61 | 26 | 22 | 26 | 4 | 4 | | | | | | | | | | |
| C6 | Willowleaf mandarin | 52 | 22 | 13 | 17 | 0 | 4 | 0 | | | | | | | | | |
| C7 | Corsican citron | **96** | 48 | 52 | 57 | 43 | 48 | 43 | 4 | | | | | | | | |
| C8 | Poncire citron | **100** | 48 | 52 | 57 | 48 | 52 | 48 | 4 | 4 | | | | | | | |
| C9 | Diamante citron | **96** | 48 | 52 | 57 | 43 | 48 | 43 | 4 | 4 | 0 | | | | | | |
| C10 | C. micrantha | 43 | 22 | 13 | 13 | 9 | 13 | 4 | 30 | 30 | 30 | 0 | | | | | |
| C11 | Salustianaorange | 74 | 22 | 22 | 17 | 13 | 17 | 13 | 52 | 57 | 52 | 17 | 9 | | | | |
| C12 | Granito sour orange | **100** | 30 | 26 | 26 | 17 | 17 | 17 | **96** | **100** | **96** | 26 | 17 | 17 | | | |
| C13 | Mexican lime | 87 | 48 | 43 | 48 | 43 | 39 | 39 | 17 | 17 | 17 | 9 | 57 | 83 | 9 | | |
| C14 | Eureka lemon | **100** | 74 | 70 | 70 | 61 | 61 | 61 | 48 | 52 | 48 | 48 | 70 | **96** | 48 | 43 | |
| C15 | Bergamot | 78 | 52 | 48 | 43 | 39 | 39 | 39 | 57 | 57 | 57 | 39 | 39 | 52 | 52 | 61 | 35 |

[a] : Proportion of locus sharing at least a same allele than Pompia ; [b] : Proportion of locus where the combined genotypes of each pair of candidates sharing the alleles of Pompia.

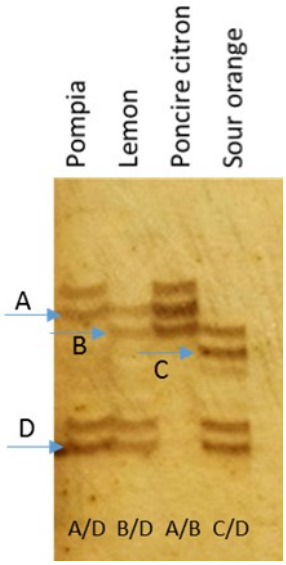



**Figure 4:** Electrophoretic and genotypic profiles of SSR marker MEST 431 for Pompia, lemon, Poncire citron and sour orange (alphabetic letters represent the different detected alleles. Allele A is only present in Pompia and citron and not in lemon and sour orange)

## References


Barkley N.A., Krueger R.R., Federici C.T., Roose M.L., 2009. What phylogeny and gene genealogy analyses reveal about homoplasy in citrus microsatellite alleles. Plant Syst.Evol. 282(1-2): 71-86

Barkley N.A., Roose M.L., Krueger R.R., Federici C.T., 2006. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theor.Appl.Genet. 112(8): 1519-1531

Biswas M.K., Chai L., Mayer C., Xu Q., Guo W., Deng X., 2012. Exploiting BAC-end sequences for the mining, characterization and utility of new short sequences repeat (SSR) markers in Citrus. Molecular biology reports, 39(5): 5373-5386

Bryan, G.J., McNicoll J., Ramsay G., Meyer R.C., De Jong W.S., 1999. Polymorphic simple sequence repeat markers in chloroplast genomes of Solanaceous plants. Theor. Appl. Genet. 99(5): 859-867

Cabasson C., Luro F., Ollitrault P., Grosser J., 2001. Non-random inheritance of mitochondrial genomes in Citrus hybrids produced by protoplast fusion. Plant Cell Reports, 20(7): 604-609

Calabrese F., 1990. La favolosa storia degli agrumi. Agricoltura 208: 83-128

Camarda I., Mazzola P., Brunu A., Fenu G., Lombardo G., Palla F., 2013. Un agrume nella storia della Sardegna: Citrus limón var. Pompia Camarda var. nova. Quad. Bot. Amb. Appl. 24: 109-118

Chalhoub B.A., Thibault S., Laucou V., Rameau C., Höfte H., Cousin R., 1997. Silver staining and recovery of AFLP amplification products on large denaturing polyacrylamide gels. Bio.Tech. 22(2): 216-220

Curk F., Ollitrault F., Garcia-Lor A., Luro F., Navarro L., Ollitrault P., 2016. Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers. Annals of Botany 117 (4): 565-583. Doi:10.1093/aob/mcw005

Froelicher Y., Mouhaya W., Bassene J.B., Costantino G., Kamiri M., Luro F., Morillon R. and Ollitrault P., 2011. New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. Tree Genetics & Genomes 7: 49-61

García-Lor A., Curk F., Snoussi-Trifa H., Morillon R., Ancillo G., Luro F., Navarro L., Ollitrault P., 2013. A nuclear phylogenetic analysis: SNPs, indels and SSRs deliver new insights into the relationships in the "true citrus fruit trees" group (Citrinae, Rutaceae) and the origin of cultivated species. Annals of Botany 111(1): 1-19. Doi: 10.1093/aob/mcs22

García-Lor A., Luro F., Navarro L., Ollitrault P., 2012. Comparative use of InDel and SSR markers in deciphering the interspecific structure of cultivated citrus genetic diversity: a perspective for genetic association studies. Mol Genet Genomics, 287: 77-94. Doi 10.1007//s00438-011-0658-4

Gulsen O., Roose M. L., 2001b. Determination of genetic diversity and phylogenetic relations to citrus ancestors in lemons by DNA markers. Bahce, 30 (1-2): 53-63

Gulsen O., Roose M.L., 2001a. Lemons: diversity and relationships with selected Citrus genotypes as measured with nuclear genome markers. Journal of the American Society for Horticultural Science, 126(3): 309-317

Kijas J.M.H., Thomas M.R., Fowler J.C.S., Roose, M.L., 1997. Integration of trinucleotide microsatellites into a linkage map of Citrus. Theoretical and Applied Genetics, 94(5): 701-706

Liu S.R., Li W.Y., Long D., Hu C.G., Zhang J.Z., 2013. Development and characterization of genomic and expressed SSRs in citrus by genome-wide analysis. PloS one, 8(10): e75149

Luro F., Costantino G., Terol J., Argout X., Allario T., Wincker P., Talon M., Ollitrault P., Morillon R., 2008. Transferability of the EST-SSRs developed on Nules clementine (Citrus clementina Hort ex Tan) to other Citrus species and their effectiveness for genetic mapping. BMC Genomics, 9: 287

Luro F., Rist D., Ollitrault P., 2000. Evaluation of genetic relationships in Citrus genus by means of sequence tagged microsatellites. In International Symposium on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture. Acta Horticulturae 546: 237-242

Luro F., Venturini N., Costantino G., Paolini J., Ollitrault P., Costa J., 2012. Genetic and chemical diversity of citron (Citrus medica L.) based on nuclear and cytoplasmic markers and leaf essential oil composition. Phytochemistry 77:186-196

Mignani I., Mulas M., Mantegazza M., Lovigu N., Spada A., Nicolosi E., Bassi D., 2015. Characterization by molecular markers of "Pompia" a natural Citrus hybrid cultivated in Sardinia. – Acta Hortic. (ISHS) 1065:165-172

Nei M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, 89(3), 583-590

Nicolosi E., Deng Z.N., Gentile A., La Malfa S., Continella G., Tribulato E., 2000. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor Appl Genet 100:1155-1166

Ollitrault F., Terol J., Pina J.A., Navarro L., Talon M., Ollitrault P., 2010. Development of SSR markers from Citrus clementina (Rutaceae) BAC end sequences and interspecific transferability in Citrus. American journal of botany, 97(11): e124-e129

Ollitrault P., Garcia-Lor A., Terol J., Curk F., Ollitrault F., Talon M., Navarro L., 2014. Comparative values of SSRs, SNPs and InDels for citrus genetic diversity analysis. Acta Horticulturae

Ollitrault P.; Jacquemond C.; Dubois C., Luro F., 2003. Citrus. In :Hamon, P. ; Seguin, M. ; Perrier, X.; Glaszmann, X., Genetic diversity of cultivated plants. CIRAD, Montpellier: 193-197

Perrier X., Flori A., Bonnot F., 2003. Methods for data analysis. In: Hamon, Seguin, Perrier and Glaszmann (Eds.), Genetic diversity of cultivated tropical plants. Science Publishers, Inc. and CIRAD, Montpellier, 31-63

Weising K., Gardner R.C., 1999. A set of conserved PCR primers for the analysis of simple sequence repeat polymorphisms in chloroplast genomes of dicotyledonous angiosperms. Genome. 42(1): 9-19

Wu G.A., Prochnik S., Jenkins J., Salse J., Hellsten U., Murat F., Perrier X., Ruiz M., Scalabrin S., Terol J., Takita M.A., Labadie K., Poulain J., Couloux A., Jabbari K., Cattonaro F., Del Fabbro C., Pinosio S., Zuccolo A., Chapman J., Grimwood J., Tadeo F.R., Estornell L.H., Muñoz-Sanz J.V., Ibanez V., Herrero-Ortega A., Aleza P., Pérez-Pérez J., Ramón D., Brunel D., Luro F., Chen C., Farmerie W.G., Desany B., Kodira C., Mohiuddin M., Harkins T., Fredrikson K., Burns P., Lomsadze A., Borodovsky M., Reforgiato G., Freitas-Astúa J., Quetier F., Navarro L., Roose M., Wincker P., Schmutz J., Morgante M., Machado M.A., Talon M., Jaillon O., Ollitrault P., Gmitter F., Rokhsar D., 2014. Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. Nat Biotechnol 32: 656-662 doi:10.1038/nbt.2906

## Supplemental material

**Supplementary File 1:** Size of amplified DNA fragments (in nucleotides) from Pompia and 15 other citrus varieties with 3 pairs of primers from cpDNA and 3 pairs from mtDNA

| | Chloroplastic genome | | | Mitochondrial genome | | |
|---|---|---|---|---|---|---|
| **Variety** | **ccmp5** | **ccmp6** | **ntcp9** | **nad2 4/3** | **rrn5/ rrn18-1** | **nad7 1/2** |
| **Pompia** | **86** | **126** | **270** | **260** | **280** | **180** |
| Chandler pummelo | 95 | 128 | 273 | 260 | 280 | 180 |
| Sans pépin pummelo | 95 | 128 | 273 | 260 | 280 | 180 |
| DeepRedpummelo | 95 | 128 | 273 | 260 | 280 | 180 |
| Cleopatra mandarin | 95 | 127 | 263 | 250 | 274 | 170 |
| Sunki mandarin | 95 | 127 | 263 | 250 | 274 | 170 |
| Willowleafmandarin | 95 | 127 | 263 | 243 | 274 | 170 |
| Diamante citron | 100 | 125 | 265 | 240 | 280 | 180 |
| Corsican citron | 100 | 125 | 265 | 240 | 280 | 180 |
| Poncire Commun citron | 100 | 125 | 265 | 240 | 280 | 180 |
| *C. micrantha* | 96 | 133 | 270 | 250 | 280 | 180 |
| Salustiana sweetorange | 95 | 128 | 273 | 260 | 280 | 180 |
| Granito sour orange | 86 | 126 | 270 | 260 | 280 | 180 |
| Mexican lime | 96 | 133 | 270 | 250 | 280 | 180 |
| Eureka lemon | 86 | 126 | 270 | 260 | 280 | 180 |
| Bergamot | 86 | 126 | 270 | 260 | 280 | 180 |

**Supplementary File 2:** Genotype of the 16 citrus varieties with the allelic size (in nucleotides) of the 23 nuclear markers(when allelic sizes are upperthan the ladder each different allele is idendified by a lower alphabetic letter; N: nul allele)

| | Seedlesspummelo | Chandler pummelo | Deepredpummelo | Cleopatra mandarin | Sunki mandarin | Willowleaf mandarin | Corsican citron | Poncire citron | Diamante citron | C. micrantha | Salustiana orange | Granito sour orange | Mexican lime | Eureka lemon | Bergamot | Pompia |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MEST 268 | 202 | 202 | 194 | 200 | 202 | 198 | 202 | 202 | 202 | 188 | 200 | 202 | 188 | 202 | 202 | 202 |
| | 202 | 202 | 202 | 200 | 210 | 204 | 202 | 202 | 202 | 188 | 202 | 210 | 202 | 210 | 210 | 210 |
| MEST 110 | 218 | 218 | 218 | 234 | 234 | 244 | 218 | 218 | 218 | 214 | 222 | 222 | 214 | 218 | 218 | 218 |
| | 222 | 222 | 222 | 244 | 244 | 244 | 218 | 218 | 218 | 226 | 226 | 244 | 218 | 222 | 222 | 222 |
| MEST 72 | 284 | 284 | 284 | 290 | 296 | 290 | 278 | 278 | 278 | 284 | 287 | 284 | 278 | 278 | 278 | 278 |
| | 284 | 284 | 284 | 296 | 296 | 290 | 278 | 278 | 278 | 284 | 290 | 296 | 284 | 296 | 296 | 284 |
| MEST 146 | 259 | 259 | 259 | 256 | 256 | 250 | 262 | 262 | 262 | 262 | 256 | 256 | 262 | 256 | 259 | 259 |
| | 259 | 259 | 259 | 256 | 256 | 256 | 262 | 262 | 262 | 265 | 259 | 259 | 265 | 262 | 262 | 262 |
| MEST 244 | 238 | 244 | 238 | 224 | 224 | 224 | 250 | 244 | 250 | 250 | 224 | 224 | 250 | 238 | 238 | 224 |
| | 244 | 250 | 250 | 224 | 224 | 244 | 250 | 250 | 250 | 250 | 238 | 238 | 250 | 250 | 238 | 250 |
| MEST 149 | d | c | c | a | a | a | b | b | b | d | a | a | e | d | a | d |
| | f | f | f | d | d | d | e | e | e | f | d | d | f | e | d | e |
| MEST 307 | 274 | 274 | 274 | 298 | 280 | 284 | 278 | 278 | 278 | 280 | 274 | 274 | 278 | 278 | 278 | 274 |
| | 274 | 274 | 274 | 298 | 284 | 284 | 278 | 278 | 278 | 280 | 280 | 280 | 280 | 280 | 280 | 278 |
| 01F04a | 188 | 192 | 200 | 200 | 214 | 196 | 188 | 188 | 188 | 168 | 188 | 192 | 172 | 188 | 188 | 188 |
| | 192 | 200 | 200 | 222 | 218 | 204 | 188 | 188 | 188 | 172 | 196 | 200 | 188 | 200 | 200 | 192 |
| MEST 154 | 134 | 137 | 137 | 131 | 131 | 134 | 131 | 131 | 131 | 134 | 131 | 131 | 134 | 131 | 131 | 131 |
| | 134 | 137 | 137 | 134 | 134 | 134 | 134 | 134 | 134 | 137 | 137 | 137 | 134 | 137 | 137 | 137 |
| MEST 431 | b | b | b | a | a | a | d | d | d | d | a | a | e | a | c | a |
| | b | c | b | a | a | a | d | e | d | f | b | c | f | d | d | e |
| MEST 109 | 162 | 164 | 162 | 166 | 162 | 164 | 160 | 160 | 160 | 160 | 162 | 162 | 160 | 162 | 162 | 166 |
| | 164 | 164 | 164 | 166 | 166 | 166 | N | N | N | 162 | 166 | 166 | 160 | N | 166 | N |
| MEST 369 | 174 | 180 | 180 | 168 | 170 | 170 | 176 | 176 | 176 | 170 | 174 | 170 | 182 | 176 | 188 | 170 |
| | 188 | 182 | 182 | 186 | 170 | 186 | 176 | 176 | 176 | 184 | 188 | 188 | 184 | 188 | 188 | 176 |
| IDAPV | 156 | 156 | 156 | 156 | 156 | 156 | 156 | 156 | 163 | 156 | 156 | 156 | 156 | 156 | 156 | 156 |
| | 156 | 156 | 156 | 156 | 156 | 156 | 163 | 163 | 163 | 156 | 156 | 156 | 156 | 156 | 156 | 163 |
| IDCAX | 233 | 233 | 233 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 233 | 233 | 237 | 237 | 233 | 233 |
| | 233 | 233 | 233 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 | 237 |
| IDHYB1 | 203 | 203 | 203 | 203 | 203 | 203 | 206 | 206 | 206 | 197 | 203 | 203 | 197 | 203 | 203 | 203 |
| | 206 | 203 | 203 | 213 | 203 | 203 | 206 | 206 | 206 | 209 | 203 | 203 | 206 | 206 | 203 | 206 |
| IDEMA | 267 | 263 | 267 | 263 | 275 | 267 | 277 | 277 | 273 | 269 | 263 | 267 | 269 | 267 | 267 | 267 |
| | 267 | 267 | 267 | 267 | 275 | 275 | 277 | 277 | 277 | 269 | 267 | 267 | 277 | 277 | 277 | 277 |
| IDEPC2 | 153 | 153 | 153 | 153 | 153 | 153 | 128 | 128 | 128 | 153 | 153 | 153 | 128 | 128 | 128 | 128 |
| | 153 | 153 | 153 | 153 | 153 | 153 | 128 | 128 | 128 | 153 | 153 | 153 | 153 | 153 | 153 | 153 |
| MEST 488 | 145 | 145 | 145 | 145 | 145 | 145 | 139 | 139 | 139 | 147 | 145 | 145 | 139 | 139 | 153 | 139 |
| | 151 | 145 | 157 | 159 | 151 | 149 | 139 | 139 | 139 | 153 | 157 | 153 | 147 | 153 | 153 | 145 |
| MEST 16 | 155 | 155 | 155 | 153 | 153 | 151 | 155 | 155 | 155 | 163 | 154 | 153 | 155 | 153 | 155 | 153 |
| | 155 | 155 | 155 | 153 | 153 | 153 | 155 | 155 | 155 | 165 | 155 | 155 | 165 | 155 | 155 | 155 |
| MEST 321 | 132 | 127 | 132 | 125 | 125 | 125 | 137 | 137 | 137 | 129 | 127 | 125 | 129 | 135 | 135 | 135 |
| | 132 | 132 | 135 | 125 | 125 | 125 | 137 | 137 | 137 | 129 | 132 | 135 | 137 | 137 | 137 | 137 |
| MEST 530 | 278 | 278 | 278 | 292 | 292 | 292 | 280 | 280 | 280 | 288 | 292 | 282 | 280 | 280 | 296 | 280 |
| | 290 | 290 | 290 | 292 | 292 | 292 | 280 | 280 | 280 | 288 | 290 | 296 | 288 | 296 | 296 | 282 |
| MEST 56 | 162 | 174 | 162 | 160 | 154 | 154 | N | N | N | 158 | 154 | 156 | 158 | 156 | 156 | 162 |
| | 174 | 184 | 174 | 162 | 174 | 158 | N | N | N | 174 | 166 | 162 | N | N | 162 | N |
| MEST 458 | a | i | i | c | i | d | b | b | b | h | f | i | e | b | i | b |
| | i | i | j | c | j | f | b | b | b | j | g | j | j | j | j | j |

# CHAPTER III

## Analysis of Pompia phenotype and composition of the essential oil

### Introduction

Published studies concerning the morphological description and composition of the essential oils of the fruit rind and leaves of Pompia have so far not clarified its phylogenetic origin (Chessa et al., 1994; Mignani et al., 2004; D'Aquino et al., 2005; Fenu et al., 2010; Camarda et al., 2013; Petretto et al., 2015; Fancello et al., 2016). The most common hypothesis outlines a kinship with lemon and citron but not approaches the Pompia to sour orange. Moreover, no comparison was made of the phenotype of these different species if not regarding the outward appearance of the fruit (Camarda et al., 2013), its acidity and the polyembryony of the seeds (D'Aquino et al., 2005). The Pompia tree appears medium size, assurgent, vigorous, the branches are thorny, the leaves are large, egg-shaped with a convex apex and may have stipules. The flowers are usually grouped in inflorescences with white petals. Fruit ripening occurs from November to March. They shaped sub-globular, flattened at the poles and higher dimensions. The base is flattened and deeply furrowed with small regularly split calyx, stalk hardly detachable. The top is flat, slightly hollow surrounded by a very marked aureole; the blossom scar is small (Chessa et al., 1994). The epicarp color turns from green to yellow, depending on the degree of maturity of the fruit, is wrinkled, with longitudinal ribs prominent and thick, rich in essential oils. Albedo is very thick; the lodges are usually 13-14, light yellow, with large and stubby vesicles. The amount of juice is low as well as its sugar content, while the acidity is high. The seeds are usually 10-14, medium-sized (Mignani et al, 2004).

A peculiarity of citrus is the presence of glands specific to essential oil storage, in the exocarp and in the leaves. This oil, composed of a complex mixture of different volatile substances for the chemical nature and quantity, is very "aromatizers". The characterization and quantification of these substances result in description of the chemical profiles of essential oils. These compounds are specific to varieties and taxonomic groups and are often used for comparative studies enabling to assess the genetic diversity of a species, to quantify the relationships between varieties or species and to classify the unknown varieties on the basis of the discriminating compounds. For example, as regards the essential oil composition of the rind, inside the mandarins there is a group of varieties where limonene represents the majority compound (83.8-96.2%) and a second group characterized by a higher rate of γ-terpinene (11.2-36.7%) and lowest limonene (52.2-81.3%) (Lota, 1999). The

mandarins classified in the taxonomic group *C. reticulata* differ from other citrus by the presence of N-methyl anthranilate methyl, which can be predominantly (46.6-58%) in the essential oil of the leaves (Fanciullino et al., 2006). The profiles of volatile compounds of essential oil of the 4 species of Kumquat, *Fortunella margarita*, *F. crassifolia*, *F.obovata* and F. *hindsii*, are characterized by having considerable levels of α-pinene (5.6-7.4%), myrcene (2.1-5.4%), γ-terpinene (2.1-3.6%) and β-phellandrene (0.1-3.9%) (Guney et al, 2015; Sutour et al., 2016). The essential oil of the leaves of grapefruit (*C. paradisi*) contains mainly monoterpene, such sabinene (more than 60.2%), (E)-β-ocimene (more than 15%) and p-cymene (up to 12.5%) (Paoli et al., 2016). The typical profile of an essential oil of citron leaf is limonene (12% to 47%), neral (6.5% to 17.4%), geranial (3.8% to 30.6%), nerol (3% to 10.8%) and geraniol (2.6% to 24.6%) (Luro et al., 2012).

This work aims to investigate the Pompia phenotype and its essential oil composition in leaves and fruits, comparatively with its ancestors and lemon, a same interspecific hybrid origin, to estimate the inheritance of parental characters. Moreover, we tested if the chemotaxonomy of essential oil profiles, it's corrobated the genetic origin of Pompia. In second step, the variability of Pompia phenotype was investigated by the sampling of different orchards and locations in Sardinia.

## Materials and Methods

### *Phenotypic analysis*

#### *Plant material*

For each of the four species used in the evaluation, *C. limon* var. Santa Teresa (ICVN010626), *C. medica* var. Poncire Commun (ICVN010701), *C. aurantium* var. Florida (ICVN010852), *C. aurantium* x *C. medica* var. Pompia (ICVN0110244), grown in the collection of CBR Citrus INRA-CIRAD of San Giuliano (Corsica) (latitude 42°27'N - longitude 9°32'E) under the same conditions (watering, fertilizing, size, phytosanitary treatments) 15 fruit spread over 3 trees were collected. All the trees are more than 15 years, all grafted on *C. volkameriana* and grown under the same cultivation techniques. The fruits were collected randomly in the periphery of the tree at eye level and different branches, provided that symptoms of illness, injury or deficiencies were absent. The fruits were collected in the month of January, to full maturity in all species. The same procedure of choice was applied to the leaves.

The sampling covered the collection of the material from 4 locations in Sardinia Milis, Bitti, Oliena and Siniscola, located in two different provinces: Oristano and Nuoro (Table 2, Figure 1, Chap. II). As explained in the previous chapter, the geographical distribution of the samples is unbalanced

favor of the town of Siniscola. This dispersion must be considered in assessing the environmental effect on variation in phenotypic and organoleptic traits of the fruits.

In January 3 ripe fruits from each tree were picked, randomly distributed in the canopy, from only 22 of the 47 samples total samples selected for analysis, coming entirely from private gardens.

***Measurements on plants and fruits***

The observed characters are: the fruit weight (g), the diameter and the height of the fruit (mm), the fruit shape (height / width), the color of the peel (Cartesian coordinates L *, a * and b), the thickness of the pericarp (exocarp + albedo) and endocarp (mm), the diameter (mm) of the fruit axis and the columel, the number of sectors, the juice content (g / 100g of fruit), total soluble solids (TSS) expressed in °Brix and tritatable acidity (TA) expressed as mg of citric acid / 100 mg of juice, the number of seed per fruit, seed weight (mg), the shape of the seed (length / width), the surface of the leaf blade ($cm^2$) and the shape of the leaf (length /width). Also qualitative characters were evaluated such as the presence of an integumentary beak at the level of chalazae of the seed, the presence of aureole at the apex of the fruit and the presence of wrinkles on the surface of the seeds. Even in this case, the weight and thickness of the fruit tissue were measured respectively with a precision balance and a caliper. The proportion (percentage by weight) of the juice was measured after manual pressure by extract the juice on juicer. The juice was filtered through a sieve of 1 mm mesh before being weighed. For TA 1 ml of juice was collected, the assay was performed by measuring the volume of 0.1N NaOH needed to balance the acidity of the juice (pH = 8.1) with the help of Mettler Toledo DL50 titrator. The TSS was estimated on 1 ml of juice with the aid of a refractometer.

The color of the skin was measured since the L * a * b * indices of CIE 1976 color space (CIELab) through a Minolta CR300 colorimeter. The staining parameter CCI (Citrus Color Index), created to measure the color change (degreening) of the peel of the clementines (Jiménez et al., 1981), is a combination of the three indices, ICC = (1000 x a) / (L * x b *). Three measurements were made at three different points on equatorial axis of each fruit. The average of the 3 values represented the color of the fruit. Length and width of the seeds and the leaves, and the leaf area were estimated with the help of the ImageJ software starting from a photo of the organs and comparing it with a reference of 20 mm and 4 $cm^2$, respectively.

On plants were recorded: poise, vigor, medium height, stem diameter, age, presence and type of graft. On the fruits was measured: the total weight (g), the shape of the fruit (height / diameter), weight (%) and thickness (mm) of the exocarp, mesocarp and endocarp, the amount (ml) of juice,

the number of sectors, the number of seeds per fruit, TSS (° Brix) and TA (mg of citric acid / 100 mg). The weight and thickness of the fruit tissues were measured with a precision balance and a caliber. The quantity of juice was measured after extraction of juice by manual pressure. The extent of TSS and TA was performed following the same basis as like above.

### *Essential oil analysis*

#### *Plant material (leaves, fruits)*

From the same 4 species chosen for phenotypic comparison, ripe fruits and leaves at their maximum development were also taken, in March, from the periphery of 3 trees, for essential oil extraction.
By the fruits of Pompia of Sardinia used for phenotypic measurements, it was separated the rind in order to make both the essential oil extraction. Considering the low number of available fruits, fruit rinds were grouped into two groups corresponding to two geographically distinct areas: Oliena and Siniscola. The availability of the leaves being greater, the sample was taken from 6 individuals divided into 4 areas (0150 and 08= Oliena; T5 and ME2 = Siniscola; B = Bitti; M3 = Milis) (see Table 1).

**Table 1:** List of accessions and material adopted in this analysis

| Species | Variety | Origin | Accession No | Material adopted |
|---|---|---|---|---|
| *C. limon* | Santa Teresa lemon | CRB Citrus[1] | ICVN010626 | leaves, fruits |
| *C. medica* | Poncire commun citron | CRB Citrus | ICVN010701 | leaves, fruits |
| *C.* x *aurantium* | Florida sour orange | CRB Citrus | ICVN010852 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | CRB Citrus | ICVN0110244 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | Oliena, Sardinia | O150 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | Oliena, Sardinia | O8 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | Siniscola, Sardinia | T5 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | Siniscola, Sardinia | ME2 | leaves, fruits |
| *C.aurantium* x *C. medica* | Pompia | Bitti | B | leaves |
| *C.aurantium* x *C. medica* | Pompia | Milis | M3 | leaves |

[1]CRB Citrus INRA-CIRAD San Giuliano Corsica, France

#### *Extraction method*

The essential oil (EO) of leaves and rind of fruit have been extracted by hydrodistillation with the help of a Clevenger type apparatus. They were placed 200 g of the fruit peel, or 300 g of leaves, in a 2 l glass bowl. The material was then covered with distilled water. The set has been brought and kept in ebullition for 3 hours (Lota, 1999). The water vapor product, containing the essential oil volatile compounds, was recovered after condensation of the vapors, following contact with a refrigerant. The essential oil is recovered and separated from the hydrolat by density difference.

### *Analysis method*

Gas chromatography (GC): The analysis of gas chromatography was carried out with the help of a chromatograph PerkinElmer Clarus 500. This consists of a split injector, two capillary columns (50 mx 0.22 mm ID; film 0.25 μM): polar (BP-20, polyethylene glycol) and apolar (BP-1, polymethylsilosano) and a flame ionization detector. The temperature scale is from 60 to 220°C with increase of 2°C / min; 220°C for 20 min., the column head pressure is 20 psi. The injector and the detector were thermostated at 250 ° C. The sample was injected in the split mode (1/60) using helium as carrier gas (1ml / min); the injection volume was 0.2 μl. Retention times of each compound are converted into separate retention indices for the polar and apolar columns, through the Kovats application. These indices, calculated by linear interpolation, allow to identify the constituents of each mixture on the corresponding chromatograms using a linear series of n-alkanes (C8-C22). The quantification is made without correction factor.

Gas chromatography coupled with mass spectrometry (GC / MS): The analyses were carried out through the use of PerkinElmer Autosystem XL chromatograph, equipped with an automatic injector and a non-polar column (Rtx-1, polydimethylsiloxane, 50 x 0.22 mm ID, film 0.25 μm) coupled to a mass detector PerkinElmer Turbo mass. The molecules are bombarded by an electric beam of 70 eV in an ionization source 150 ° C; the detection is performed by a tetrapole analyzer consists of an assembly of four parallel electrodes of cylindrical section. The carrier gas is helium and exercises a pressure of 43 psi to the head of the column. The program used consists of a temperature increase from 60 ° C to 230 ° C at 2 ° C / min, 230 ° C for 30 minutes. The split ratio is 1/80.

### *Data analysis*

Data were analyzed with the help of R software, using the basic packages for calculating means, standard deviations, the error and Pearson correlations between variables. The Agricolae package for analysis of variance and the LSD (least significant difference) test Fisher to α risk 0.05, and the base package by Pearson, correlations PCA realized with the Ade4 package to the PCA (Principal Component Analysis). For PCA, the values of each variable have been centered and reduced for obtaining variations of the same size among variables. For analysis of essential oil data and determine the relationships between varieties and the components which contribute to this diversity, heat maps were constructed with the help of R software using theg plots package.

## Results and discussion

### *Pompia compared to its parents and lemon*

#### *Through phenotypic traits*

The phenotype of the fruit of Pompia in relation to the other 3 species is summarized in Table 2. The fruits of Pompia are intermediate in size and weight to those of the citron, which are the largest and heavier fruit and the sour orange. Lemon fruits are very similar for these characters to Pompia. However, the shape of Pompia fruit is very similar to that of sour orange, flattened at the poles, while the overall shape of lemon, according to the height / diameter ratio, is similar to that of citron, which is a fruit of elongated shape (Figure 1a). However, in detail, there are differences between lemon and citron: the apex of lemon fruit presents a more or less pointed nipple, which is absent in citron (Figure 1a). The citron has, however, very marked aureole apex of the fruit, present in Pompia although less deep and absent in lemon (Figure 2). For skin color, sour orange stands out clearly from the other three species for its orange color due to the presence of carotenoids xanthophylls, while others are yellow, slightly more intense in Pompia (b* index is higher). The brilliance of the rind, indicated by L *, differentiated by lower values the sour orange from the other three varieties.

The thickness of the pericarp is relatively low in Pompia when compared to that of citron, but higher than that of the lemon and sour orange. The biggest differences are observed in the axis of the fruit that is empty in Pompia and sour orange, full in lemon and citron (Figure 1b).

**Table 2:** Description of fruit phenotype of Pompia, Poncire citron, Florida sour orange, Santa Teresa lemon. Alphabetic letter indicate the significant averages tested with Fisher's LSD (Least Significant Difference)

| | | Weight (g) | Height (mm) | Ø (mm) | Shape Index H/Ø | Peel colour | | | | Pericarp thickness (mm) | Endocarp thickness (mm) | Columel Ø (mm) | Axis width (mm) | Juice % | Segment number | TSS (°Brix) | TA (% citric acid) | Seed / fruit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | L* | a* | b* | CCI | | | | | | | | | |
| **Pompia** | **Mean** | 269 b | 77 b | 96 a | 0.80 a | 74 a | -1 b | 70 a | 0.2 b | 13 b | 34 a | 0 c | 27 a | 21 c | 12.3 a | 7.3 bc | 5.7 a | 26 b |
| | ***SD*** | *51.9* | *8.0* | *5.1* | *0.06* | *1.8* | *4.3* | *3.9* | *1* | *1.6* | *3.1* | *0.0* | *4.2* | *2.8* | *1.3* | *0.2* | *0.2* | *4.9* |
| | ***SE*** | *16.4* | *2.5* | *1.6* | *0.02* | *0.6* | *1.4* | *1.2* | *0.3* | *0.5* | *1* | *0.0* | *1.3* | *0.9* | *0.4* | *0.1* | *0.1* | *1.6* |
| **Citron Poncire** | **Mean** | 716 a | 135 a | 101 a | 1.34 b | 74 a | -2b | 62 bc | 0.5 b | 21 a | 28 b | 17 a | 0 c | 5 d | 11.6 a | 7.1 c | 5.8 a | 37 a |
| | ***SD*** | *153.5* | *9.2* | *7.8* | *0.11* | *1.7* | *3.4* | *6.3* | *0.8* | *3.3* | *5* | *8.2* | *0.0* | *1.1* | *0.7* | *0.2* | *0.2* | *13* |
| | ***SE*** | *48.6* | *2.9* | *2.5* | *0.04* | *0.6* | *1.1* | *2.0* | *0.2* | *1.1* | *1.6* | *2.6* | *0.0* | *0.4* | *0.2* | *0.1* | *0.1* | *4.1* |
| **Sour orange Florida** | **Mean** | 151 c | 61 c | 75 b | 0.82 a | 65 b | 31 a | 65 b | 7.2 a | 7 c | 30 b | 0 a | 11 b | 28 a | 9.0 b | 9.2 a | 4.0 b | 30 ab |
| | ***SD*** | *11.5* | *3.2* | *3.2* | *0.04* | *1.7* | *2.2* | *1.8* | *0.7* | *1* | *1.3* | *0.0* | *2.5* | *2.7* | *0.8* | *0.1* | *0.3* | *5.2* |
| | ***SE*** | *3.6* | *1* | *1* | *0.01* | *0.5* | *0.7* | *0.6* | *0.2* | *0.3* | *0.4* | *0.0* | *0.8* | *0.8* | *0.3* | *0* | *0.1* | *1.6* |
| **Lemon Santa Teresa** | **Mean** | 171 c | 88 c | 66 c | 1.32 b | 75 a | -3b | 59 c | 0.7 b | 8 c | 25 c | 6 b | 0 c | 25 b | 9.5 b | 7.5 b | 5.6 a | 15 c |
| | ***SD*** | *31.2* | *6.8* | *4.9* | *0.07* | *2.9* | *4.3* | *4.7* | *1* | *1.4* | *1.4* | *1.1* | *0.0* | *1.3* | *1.1* | *0.4* | *0.5* | *6.5* |
| | ***SE*** | *9.9* | *2.2* | *1.6* | *0.02* | *0.9* | *1.4* | *1.5* | *0.3* | *0.5* | *0.4* | *0.4* | *0.0* | *0.4* | *0.3* | *0.1* | *0.1* | *2.1* |
| | ***LSD*** | *75.0* | *6.5* | *5.0* | *0.06* | *1.9* | *3.3* | *4.0* | *0.79* | *1.9* | *2.8* | *3.7* | *2.2* | *1.9* | *0.9* | *0.2* | *0.3* | *7.3* |

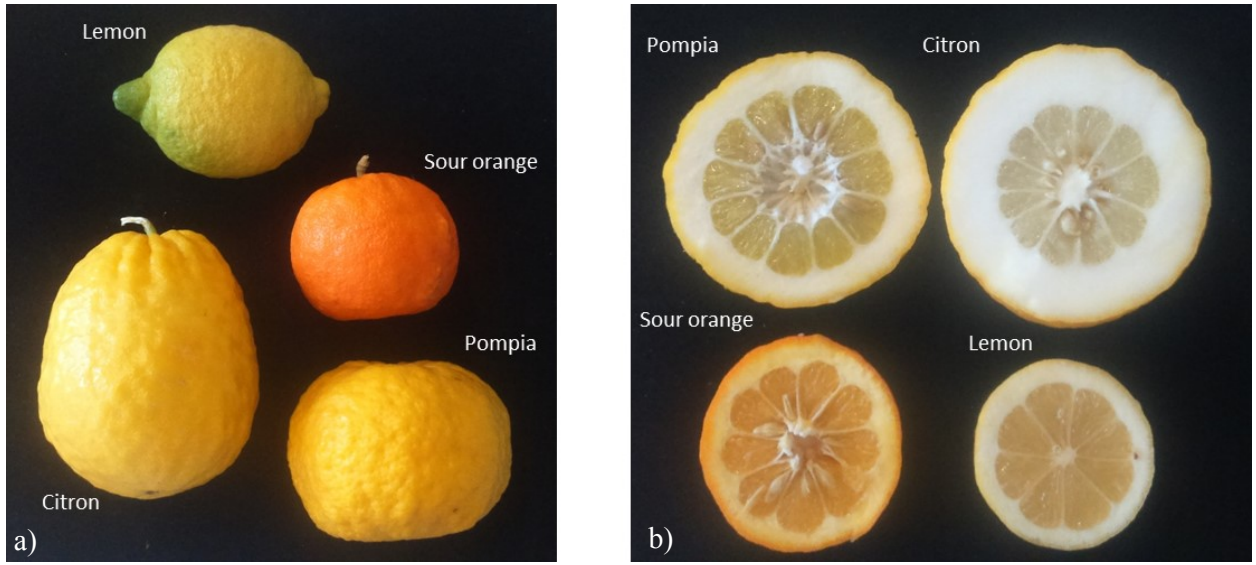



**Figure 1:** Fruit of Pompia, citron, sour orange and lemon a) fruit side, b) inside view of the fruit cut on the equatorial plane


**Figure 2:** Apex of Pompia and citron fruit with aureole

This axis is of different size and may contain, when it is full, a columel of variable diameter, as in the case of the citron and lemon. Color of the pulp changes in the 4 species, but not measurable because the unit used measure the radiation reflected from the matter and pulp adsorbs most of the radiation emitted by the apparatus.

The percentage of juice is very low in the citron and higher into sour orange, while the Pompia, for this character comes close to lemon. The number of the segments of the fruit is the only character where Pompia presents the highest values respect to the other three species. Sugar content and acidity of Pompia are similar to those of lemon and citron. The number of seeds per fruit is high, but less than that in sour orange and citron. However, the fruits come from trees that are in collection, which contains many other nearby species and, consequently, the pollen pressure during flowering is very different, and so the number of seeds per fruit. For example, the fertility of the pollen is normally low and lemon fruits derived from commercial orchards have, in general, many fewer seeds than those of our study (less than 10 seeds).

The description of the measured characters in the seeds and leaves is summarized in Table 3. By examining the seeds, those of Pompia are intermediate for weight and form, compared to those of its parent, and close to those of lemon. The presence of a curved beak at the end of the seed, formed by the integuments of chalaza, is very marked in the citron seeds and is almost always present, although less markedly, in Pompia seeds (Figure 3). Note also that the sour orange seeds present on the surface streaks or longitudinal wrinkles (Figure 3). Barely perceptible wrinkles are also present on the surface of Pompia seeds. The number of embryos for seed is very variable: in citron, seeds are monoembryonic, as in all varieties belonging to this species (Ollitrault et al., 2003); the seeds inPompia are polyembryonic with a high rate, > 2, higher than in the lemon, and in sour orange that was shighly polyembryonic (3.1 in average). The leaves of Pompia are of equivalent dimensions to those of sour orange, but similar in shape to those of the lemon.

**Table 3:** Description of seed and leaf phenotype of Corsican Pompia specimen, Poncire citron, Florida sour orange, Santa Teresa lemon. Alphabetic letter indicate the significant averages tested with Fisher's LSD (Least Significant Difference)

| | | Seed | | | | Leaf | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight (mg) | Embryo /seed | Length (mm) | Width (mm) | Area ($cm^2$) | Lenght (cm) | Width (cm) | Shape index |
| **Pompia** | **Mean** | **167 b** | **2.4 b** | **11.6 bc** | **6.4 b** | **49 b** | **10.8 c** | **5.6 bc** | **1.9 b** |
| | *SD* | *43.8* | *0.9* | *1.4* | *0.7* | *11.5* | *1.3* | *0.7* | *0.2* |
| | *SE* | *11.3* | *0.2* | *0.4* | *0.2* | *3.6* | *0.4* | *0.2* | *0.1* |
| **Citron** | **Mean** | **129 c** | **1.0 d** | **10.8 c** | **6.3 b** | **73 a** | **14.7 b** | **6.2 b** | **2.4 a** |
| | *SD* | *12.0* | *0* | *0.9* | *0.4* | *13.1* | *1.0* | *0.6* | *0.2* |
| | *SE* | *3.1* | *0* | *0.2* | *0.1* | *4.1* | *0.3* | *0.2* | *0.1* |
| **Sour orange** | **Mean** | **206 a** | **3.1 a** | **14.2 a** | **6.9 a** | **44 b** | **12.2 b** | **5.0 c** | **2.5 a** |
| | *SD* | *33.8* | *1.0* | *2.1* | *0.9* | *6.4* | *1.3* | *0.7* | *0.4* |
| | *SE* | *8.7* | *0.3* | *0.5* | *0.2* | *2* | *0.4* | *0.2* | *0.1* |
| **Lemon** | **Mean** | **174 b** | **1.6 c** | **12.0 b** | **6.5 ab** | **69 a** | **13.2 a** | **7.3 a** | **1.8 b** |
| | *SD* | *41.5* | *0.5* | *1.8* | *0.6* | *10.8* | *1.1* | *0.7* | *0.1* |
| | *SE* | *10.7* | *0.1* | *0.5* | *0.2* | *3.4* | *0.3* | *0.2* | *0.0* |
| | *LSD* | *25.7* | *0.5* | *1.2* | *0.5* | *9.7* | *1.1* | *0.6* | *0.2* |

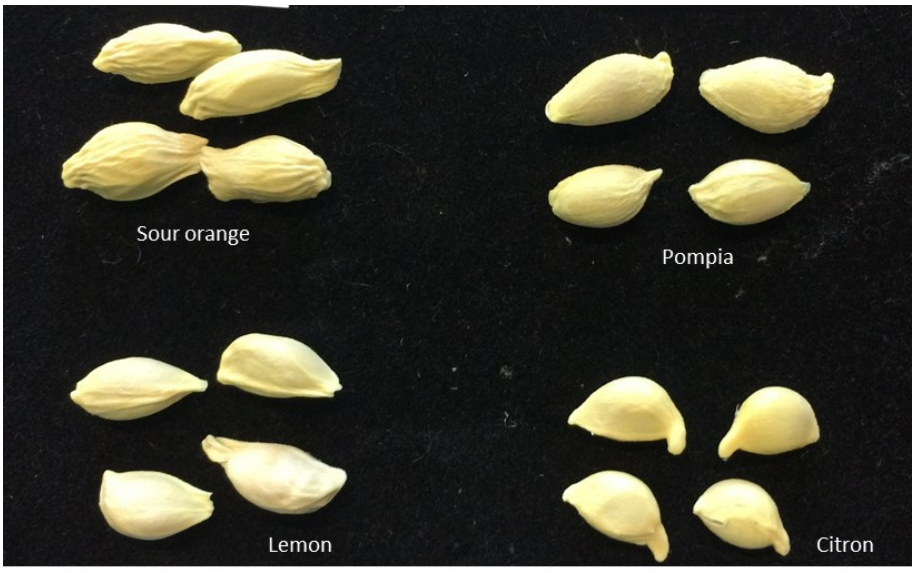



**Figure 3:** Seed morphology of Pompia, lemon, sour orange and citron

A phenotypic characterization of Pompia was carried out by D'Aquino et al. (2005) based on objective and subjective descriptors of the tree, leaves, fruit and seeds. The observed trees were grown in culture specialized in Siniscola, Sardinia. This description provides a basis for comparisons of Pompia phenotype observed in other cultural and environmental conditions. In their study, it was taken account of the evolution of some characters, renewing observations every month over a period of six months (October to March). Comparing data observed in January, it can be noted that, for most of the characters of Pompia grown in Corsica are very close to that Pompia grown in Sardinia. The trees grown in Sardinia, observed by D'Aquino et al. (2005), are located in commercial orchards, while in Corsica the trees are located in the collection with other citrus species and varieties. The same rootstock, sour orange, was used in both cases. However some differences can be noticed: in Corsica the fruits are smaller, a little more juicy and sweet, and a little less acidic. This may be due to changes in growth conditions, cultural technology or the environment. In collection, the main objective is the preservation and not the productivity of trees. Consequently, the annual pruning and fertilization would not meet the same requirements of purposes: nitrogen fertilization in fact tends to increase the size of the fruit. Among the Sardinian phenotype of D'Aquino et al. (2005) and the Corsican phenotype of the present study, the greatest variability was observed for the number of seeds per fruit, with an average of 10 in Sardinia against 20 in Corsica. This difference can be explained by the variation of the pollen pressure during flowering between the two geographical sites.

The average phenotype of Pompia described by Mignani et al. (2004) on trees cultivated in Sardinia, it is closer to that of Pompia in Corsica than that described by D'Aquino et al. (2005). The average weight of the fruit, for example, is 287 g with a difference of only 17 g more of the observed average value in Corsica.

It can therefore be concluded that these observed changes in different Pompia phenotypic aspects are negligible and although the environment is very different between Corsica and Sardinia, it has only a very low influence on the expression of the main qualitative characteristics of the fruit.

PCA have not taken into account all of the measured variables due to their strong correlation. Examples of this are the weight and the diameter of the fruit (Table 4). The variables independence is a condition without which you cannot have a representation of the real diversity that is, not based on over-representation of a character or characters in a very close relationship to another.

For example, the weight, the height and the diameter of fruit are very much related to each other. Using these 3 variables should arbitrarily increase the role of the weight and size of the fruit in the

overall variability. Similarly, we have maintained the thickness of the various portions of the fruit and not their weights.

Furthermore, there are correlations that do not have a biological sense, and this is due to the fact that sampling covered a few species. For example, the content of juice is negatively correlated to the weight of the fruit, this is given by the fact that the citron is a large fruit, with a thick skin and its pulp is not very juicy. The same happens between the colors indices of the pulp and the TSS and TA. The correlation is simply due to the fact that for these parameters the variability is made only from sour orange, while the other 3 species are not different. The biosynthetic pathway of sugars and organic acids has no relation to that of carotenoids.

The variables taken into consideration were: fruit weight, fruit shape, color of the peel (the three indices L *, a * and b *), the percentage of the pericarp thickness in relation to the diameter of the fruit, the size of the recess axis, the diameter of the columel, the number of segments of the fruit, the number of seeds per fruit, the percent in juice, its acidity and its sugar content, the shape and the surface of the leaf, the weight, the shape of the seed and the number of embryos for seed. The other characters were discarded by PCA.

PCA in the first two axes (Figure 4) represents 62% of total phenotypic diversity of the sample; this suggests that the diversity structure is representative of the real diversity. The dispersion of 4 varieties around their barycentre is very low; this is due to the fact that there is a stronger inter-species variability that intra-species. In the second PCA (Figure 4) corresponding to the axes 1 and 3, the proportion of represented variability is almost identical (60%) to the first, but the dispersion of the species is different.

**Table 4:** Correlation between Pompia specimen fruit variables in Corsica. Values corresponding to high correlations between pair of variables (> 0.75) are bold typed

| | | V1 | V2 | V3 | V4 | V5 | V6 | V7 | V8 | V9 | V10 | V11 | V12 | V13 | V14 | V15 | V16 | V17 | V18 | V19 | V20 | V21 | V22 | V23 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| V1 | Weight | 1.00 | | | | | | | | | | | | | | | | | | | | | | |
| V2 | Height | **0.90** | 1.00 | | | | | | | | | | | | | | | | | | | | | |
| V3 | fruit diam | **0.79** | 0.57 | 1.00 | | | | | | | | | | | | | | | | | | | | |
| V4 | Shape index | -0.46 | **-0.75** | 0.07 | 1.00 | | | | | | | | | | | | | | | | | | | |
| V5 | L. color index | 0.34 | 0.52 | 0.24 | -0.50 | 1.00 | | | | | | | | | | | | | | | | | | |
| V6 | a. color index | -0.41 | -0.59 | -0.34 | 0.55 | **-0.86** | 1.00 | | | | | | | | | | | | | | | | | |
| V7 | b. color index | -0.17 | -0.32 | 0.27 | 0.61 | -0.14 | 0.26 | 1.00 | | | | | | | | | | | | | | | | |
| V8 | CCI | -0.41 | -0.59 | -0.34 | 0.55 | **-0.87** | **1.00** | 0.25 | 1.00 | | | | | | | | | | | | | | | |
| V9 | Pericarpthickness | **0.94** | **0.86** | **0.85** | -0.37 | 0.40 | -0.50 | -0.02 | -0.50 | 1.00 | | | | | | | | | | | | | | |
| V10 | Endocarpthickness | 0.13 | -0.17 | 0.57 | 0.60 | -0.15 | 0.07 | 0.48 | 0.06 | 0.11 | 1.00 | | | | | | | | | | | | | |
| V11 | Columel diam | **0.83** | **0.84** | 0.44 | -0.67 | 0.39 | -0.40 | -0.26 | -0.40 | 0.68 | -0.06 | 1.00 | | | | | | | | | | | | |
| V12 | Axis width | -0.31 | -0.52 | 0.32 | **0.84** | -0.16 | 0.12 | 0.62 | 0.12 | -0.15 | 0.72 | -0.60 | 1.00 | | | | | | | | | | | |
| V13 | Endocarpwidth | -0.45 | -0.48 | -0.49 | 0.20 | -0.51 | 0.49 | -0.18 | 0.49 | -0.58 | 0.14 | -0.37 | -0.07 | 1.00 | | | | | | | | | | |
| V14 | Pericarpweight | **1.00** | **0.90** | **0.77** | -0.47 | 0.34 | -0.41 | -0.18 | -0.41 | **0.93** | 0.10 | **0.83** | -0.33 | -0.44 | 1.00 | | | | | | | | | |
| V15 | Proportion pericarp | **0.90** | **0.86** | **0.79** | -0.42 | 0.43 | -0.55 | -0.10 | -0.54 | **0.93** | 0.07 | 0.69 | -0.19 | -0.60 | **0.90** | 1.00 | | | | | | | | |
| V16 | Endocarpweight | 0.60 | 0.51 | 0.68 | -0.10 | 0.28 | -0.32 | 0.14 | -0.32 | 0.58 | 0.49 | 0.49 | 0.13 | -0.28 | 0.56 | 0.41 | 1.00 | | | | | | | |
| V17 | Juice weight | -0.13 | -0.24 | 0.31 | 0.45 | -0.01 | -0.12 | 0.31 | -0.12 | -0.09 | **0.77** | -0.28 | 0.72 | 0.12 | -0.17 | -0.11 | 0.45 | 1.00 | | | | | | |
| V18 | Juice percent | **-0.93** | **-0.92** | -0.74 | 0.52 | -0.46 | 0.52 | 0.12 | 0.52 | **-0.94** | 0.04 | **-0.78** | 0.33 | 0.59 | **-0.93** | **-0.94** | -0.49 | 0.28 | 1.00 | | | | | |
| V19 | Segment number | 0.48 | 0.37 | 0.73 | 0.12 | 0.45 | -0.49 | 0.35 | -0.49 | 0.57 | 0.44 | 0.24 | 0.40 | -0.56 | 0.46 | 0.55 | 0.55 | 0.36 | -0.50 | 1.00 | | | | |
| V20 | TSS | -0.52 | -0.66 | -0.49 | 0.49 | **-0.82** | **0.93** | 0.10 | **0.93** | -0.63 | 0.01 | -0.47 | 0.08 | 0.61 | -0.52 | -0.68 | -0.36 | -0.12 | 0.63 | -0.59 | 1.00 | | | |
| V21 | TA | 0.45 | 0.59 | 0.41 | -0.48 | **0.87** | **-0.88** | -0.05 | **-0.89** | 0.55 | -0.02 | 0.40 | -0.06 | -0.55 | 0.45 | 0.57 | 0.33 | 0.07 | -0.57 | 0.53 | **-0.84** | 1.00 | | |
| V22 | n. seed/ fruit | 0.54 | 0.29 | 0.58 | 0.10 | -0.18 | 0.20 | 0.14 | 0.19 | 0.47 | 0.34 | 0.39 | 0.00 | -0.05 | 0.55 | 0.43 | 0.25 | -0.15 | -0.48 | 0.25 | 0.08 | -0.06 | 1.00 | |
| V23 | n.seed/ segment | 0.32 | 0.09 | 0.31 | 0.15 | -0.43 | 0.46 | 0.08 | 0.46 | 0.22 | 0.23 | 0.22 | -0.07 | 0.17 | 0.33 | 0.18 | 0.04 | -0.25 | -0.25 | -0.10 | 0.37 | -0.32 | **0.93** | 1.00 |

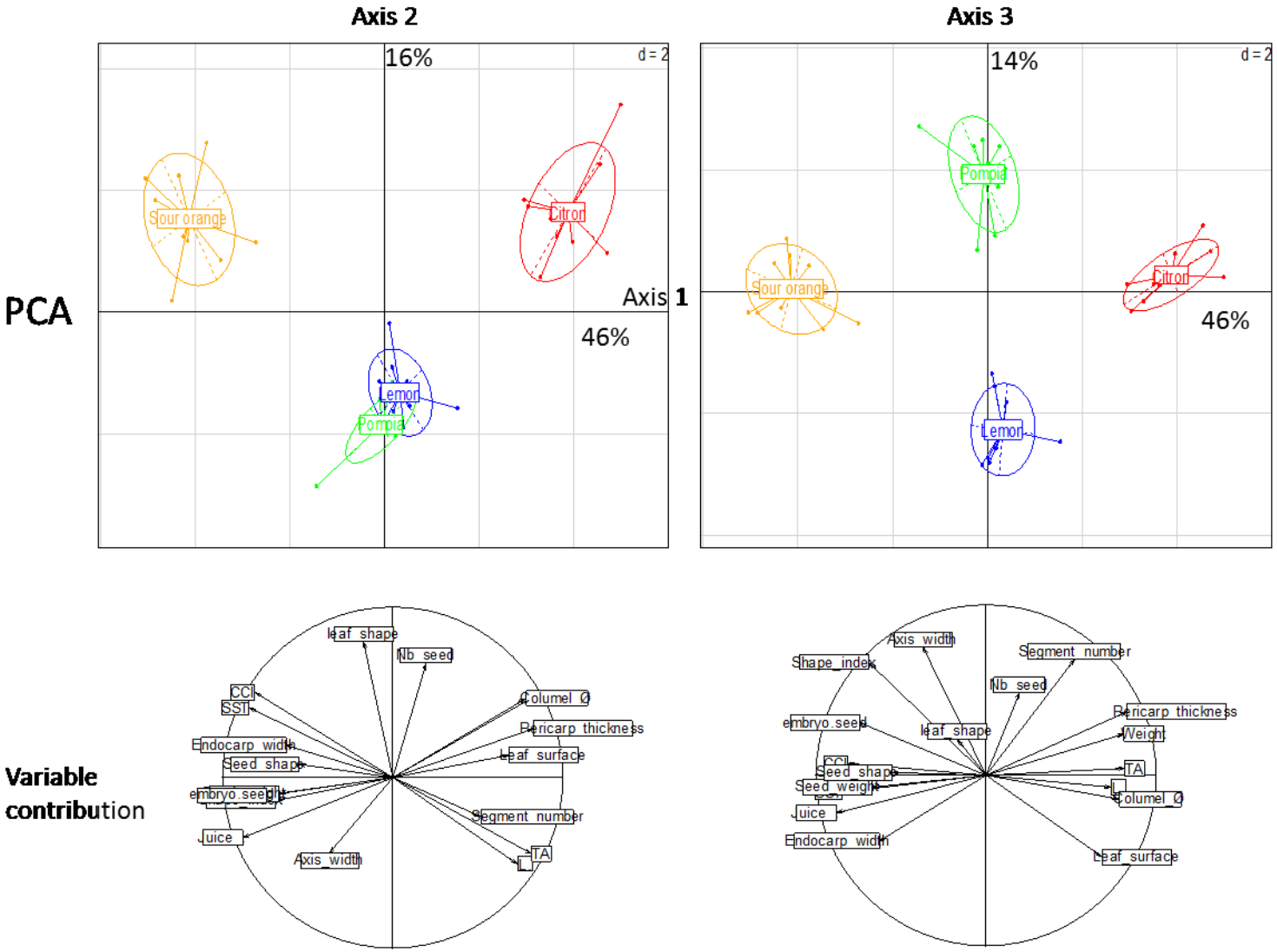



**Figure 4:** Principal Component Analysis of the diversity revealed by 18 phenotypic characters from fruit, leaf and seed, among the samples of Pompia, Poncire citron, Florida sour orange and Santa Teresa lemon and the contribution of the characters to the dispersion on the axis 1, 2 and 3

The sour orange and citron systematically have an opposite phenotype (axis 1), lemon and Pompia positions are different in the two graphs. In the first, they are mixed, while in the second, and are clearly separated by axis 3. The contribution of variable to varieties dispersion gives information about the distinguishing features between lemon and Pompia: the central axis diameter and the presence of the columel, the weight and shape of the fruit. In the two graphics that maximize the diversity, lemon and Pompia always positioned equidistant from the citron and sour orange. It can be concluded that the phenotype of the two hybrids is, in large part, intermediate to the two parents, although this depends on the number of varieties and from measured variables.

***Chemotypes of Pompia its parents and lemon.***

Given the fact that have not been realized repetitions of extraction and analysis of essential oils, for the comparison of the profiles of 4 citrus, we will focus exclusively on the majority compounds: >3% essential oil of the leaves and > 1% on essential oil of the rinds.

The percentages of the compounds present in the essential oil of analyzed citrus leaves are listed in Table 5. As can be seen, there are numerous differences between the 4 citrus, quantitative and also qualitative (presence / absence). If we only refers to the 15 major compounds (in bold in Table 5), the EO profile of Pompia leaves is close to that of the citron; only the percentage of neryl acetate in Pompia is closer to that of sour orange. It appears a profile with about 30% of limonene, 20% of geranial, 15% of neral (major compounds) for Pompia and citron, while the lemon has a profile, which differs especially for the high percentage of β-pinene (25% of limonene, 15% of geranial, 15% of β-pinene and 10% of neral). Sour orange chemotype is completely different: 35% of linalyl acetate; 25% linalool, 10% α-terpineol. In Pompia EO only limonene and (E)-β-ocimene have higher rates than those of the other three citrus. This closeness in composition between Pompia and citron is illustrated in the representation of Heat map (Figure 5), where the main contributors are the 15 compounds with a proportion over 1%, for all citrus analyzed. More than 80% of the overall variability is represented by only 6 components.

**Table 5:** Proportions (%) of all the compounds detected in the leaf essential oil of Pompia, lemon, citron and sour orange

| Compounds | RI[a] | RI[p] | Pompia | Lemon Santa Teresa | Citron Poncire | Sour orange Florida |
|---|---|---|---|---|---|---|
| α-Thujene | 921 | 1013 | tr | 0.1 | tr | tr |
| α-Pinene | 929 | 1013 | 0.2 | 1.0 | 0.3 | 0.3 |
| Amphene | 941 | ND | 0.0 | 0.1 | 0.0 | tr |
| 6-methylhept-5-en-2-one | 959 | 1337 | 0.8 | 0.7 | 0.7 | 0.0 |
| **Sabinene** | 963 | 1121 | 0.4 | **3.1** | 1.0 | 0.5 |
| **β-Pinene** | 969 | 1110 | 0.7 | **16.7** | 1.0 | **4.2** |
| Octanal | 977 | ND | 0.0 | 0 | 0.1 | 0 |
| **Myrcene** | 979 | 1159 | 1.0 | 0.9 | 0.9 | 2.8 |
| a-Phellandrene | 995 | ND | 0.0 | tr | 0.0 | 0.0 |
| δ-3-Carene | 1004 | 1147 | 0.6 | 0.4 | tr | tr |
| a-Terpinene | 1010 | ND | 0.0 | 0.1 | tr | 0.1 |
| p-Cymene | 1011 | 1270 | 0.2 | 0.1 | 0.1 | tr |
| **Limonene*** | 1020 | 1200 | **36.0** | **23.8** | **27.4** | **3.9** |
| β-Phellandrene* | 1020 | 1209 | 0.4 | 0.6 | 0.1 | 0.1 |
| (Z)-β-Ocimene | 1024 | 1231 | 0.5 | 0.4 | 0.6 | 0.9 |
| **(E)-β-Ocimene** | 1035 | 1248 | **5.4** | 1.8 | 1.0 | 2.7 |
| γ-Terpinene | 1047 | 1243 | 0.0 | 0.4 | 0.3 | 0.2 |
| *trans*-Sabinene hydrate | 1051 | ND | 0.0 | 0.1 | 0.0 | 0.0 |
| *cis*-LinalooloxideTHF | 1056 | ND | 0.0 | 0.0 | tr | tr |
| Terpinolene | 1077 | 1281 | 0.0 | 0.5 | 0.1 | 0.5 |
| Nonanal | 1080 | 1392 | 0.1 | 0.0 | 0.0 | 0.0 |
| **Linalool** | 1082 | 1544 | 1.1 | 1.4 | **3.0** | **26.7** |
| *cis*-Limonene oxyde -1,2 | 1115 | ND | 0.0 | tr | 0.1 | 0.0 |
| *trans*-Limonene oxyde -1,2 | 1119 | ND | 0.0 | 0.0 | 0.5 | 0.0 |
| **Citronellal** | 1129 | 1477 | 2.1 | 1.4 | **3.3** | tr |
| Isoneral | 1146 | ND | 0.5 | 0.6 | 0.8 | 0.0 |
| Isogeranial | 1156 | ND | 0.7 | 0.9 | 1.2 | 0.0 |
| Terpinen-4-ol | 1160 | 1598 | 0.1 | 0.5 | 0.2 | 0.3 |
| **α-Terpineol** | 1170 | 1691 | 0.2 | 0.5 | 1.4 | **9.5** |
| Decanal | 1182 | ND | 0.0 | 0.1 | 0.2 | 0.0 |
| **Nerol*** | 1208 | 1796 | 0.3 | 3.8 | 0.7 | 1.9 |
| Citronellol* | 1208 | 1762 | 0.2 | 0.0 | 0.0 | 0.0 |
| **Neral** | 1213 | 1680 | **15.6** | **13.1** | **16.6** | 0.0 |
| **Geraniol** | 1232 | 1843 | 0.4 | 0.5 | 1.4 | **5.1** |
| **Geranial** | 1241 | 1730 | **20.4** | **16.5** | **22.0** | 0.0 |
| **Linalylacetate** | 1244 | ND | **0.0** | 0.0 | 0.0 | **32.4** |
| Undecanal | 1284 | ND | **0.0** | 0.2 | 0.2 | 0.0 |
| methylGeraniate | 1300 | 1680 | 0.2 | 0.0 | 0.0 | 0.0 |
| Citronellylacetate | 1332 | 1658 | 0.4 | 0.1 | 0.4 | 0.0 |
| **Nerylacetate** | 1340 | 1723 | 1.6 | **6.8** | **4.9** | 2.8 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Geranyleacetate** | 1358 | 1753 | **5.0** | 1.9 | **7.4** | **5.2** |
| β-Elemene | 1385 | 1585 | 0.2 | 0.0 | 0.0 | 0.0 |
| (E)-β-Caryophyllene | 1415 | 1591 | 0.6 | 0.4 | 0.6 | 0.3 |
| (Z)-β-Farnesene | 1433 | 1638 | 0.1 | 0 | 0.0 | 0 |
| (E)-β-Farnesene | 1445 | 1661 | tr | 0 | 0.0 | 0 |
| α-Humulene | 1447 | 1662 | tr | 0 | 0.0 | 0 |
| Germacrene D | 1473 | 1702 | 1.0 | 0 | 0.0 | 0 |
| bicyclogermacrene | 1488 | 1726 | 0.2 | 0.1 | 0.1 | 0.1 |
| *trans*-β-Bergamotene | 1480 | 1495 | 0.1 | 0 | 0.0 | 0 |
| β-Bisabolene | 1498 | 1720 | 0.0 | 0.1 | 0.1 | 0.0 |
| δ-Cadinene | 1512 | 1749 | tr | 0 | 0.0 | 0 |
| trans-Sesquisabinene hydrate | 1528 | 1985 | tr | 0 | 0.0 | 0 |
| (E)-Nerolidol | 1546 | 2035 | 0.9 | 0 | 0.0 | 0 |
| Spathulenol | 1560 | 2115 | 0.2 | 0 | 0.0 | 0 |
| Caryophyllene oxyde | 1566 | 1974 | 0.2 | 0.2 | 0.0 | 0.0 |
| α-Cadinol | 1635 | 2222 | 0.1 | 0 | 0.0 | 0 |
| β-Sinensal | 1673 | ND | 0.0 | 0.0 | 0.0 | 0.1 |
| a-Sinensal | 1725 | ND | 0.0 | 0.0 | 0.0 | tr |
| **TOTAL** | | | **98.6** | **99.7** | **98.6** | **99.3** |

Order of elution and percentages are given on an apolar column (BP1) except for compounds with an asterisk (percentage in polar column, BP20). $RI^a$ and $RI^p$: retention indices measured respectively on apolar and polar columnsTr = traces < 0.05%; ND: Not Determined

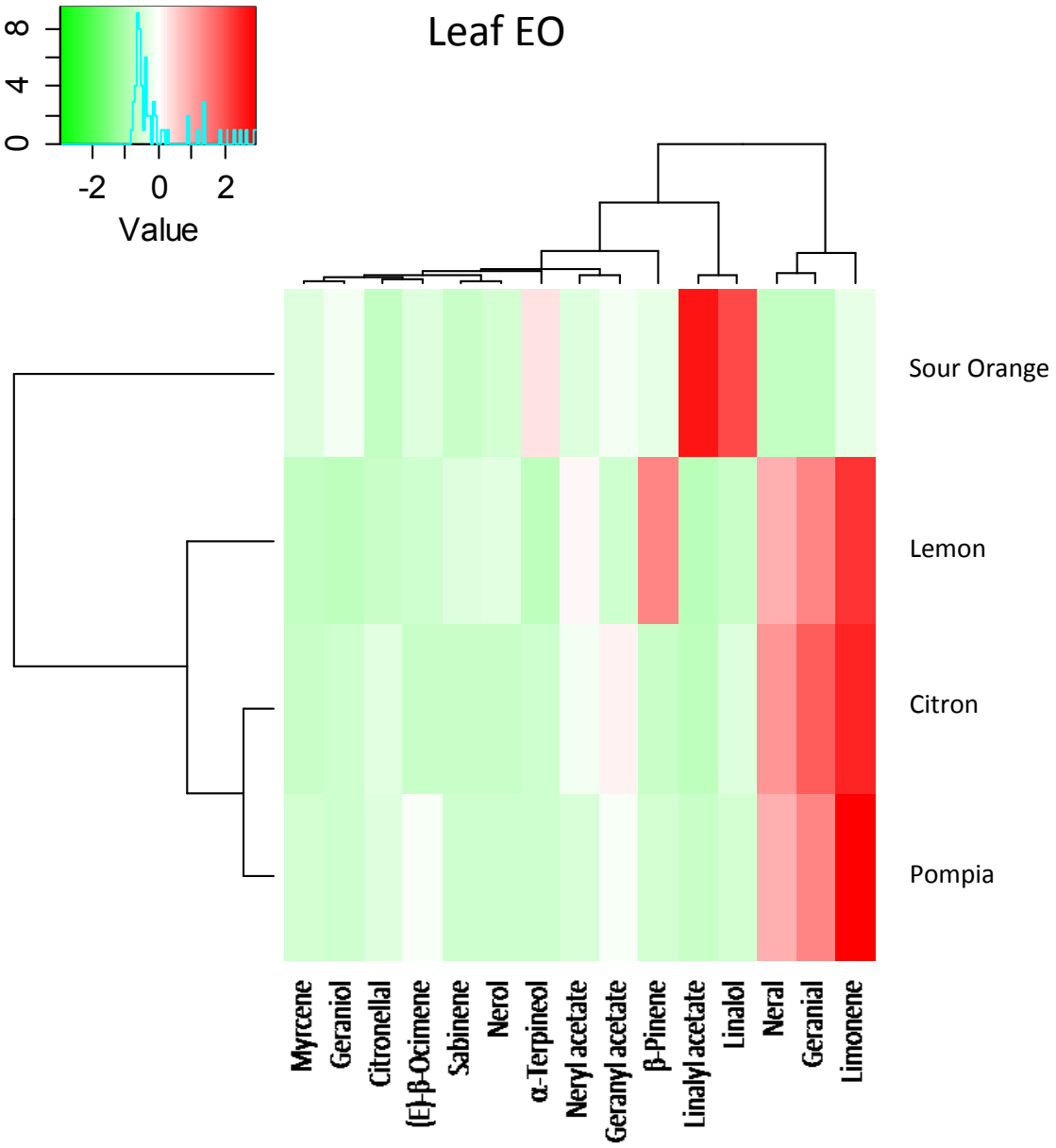



**Figure 5:** Heat map of relationships between citrus genotypes related to the contribution of the major variable compounds of Leaf EO (color range represents the variance level of the quantitative character with respect to average)

The major constituent of rind EO (Table 6) is limonene, from 50 to 93.5%, as a consequence the other compounds are contained in much lesser extent than the essential oil of the leaves. The number of compounds detected is lower compared to the oil of the leaves (respectively 39 versus 58). Based on the 12 major compounds (in bold on Table 6) it shows that EO of Pompia is very rich in limonene, > 90%, as sour orange, while limonene is in a lower percentage, around 50% in the citron and lemon. Excepted the limonene, the presence and

proportion of geraniol and nerol are close to those of citron, while the two compounds are absent in sour orange and lemon. The EO of lemon rind stands out from other 3 mainly for its elevate content in β-pinene (12%) and in sabinene. The representation of these reports on 16 components is represented in the Heat map in Figure 6.

**Table 6:** Proportions (%) of all the compounds detected in the zest essential oil of Pompia, lemon, citron and sour orange

| Compounds | **RI[a]** | **RI[p]** | Pompia | Lemon Santa Teresa | Citron Poncire | Sour orange Florida |
|---|---|---|---|---|---|---|
| α-Thujene | *921* | *1013* | 0 | 0 | 0.3 | 0 |
| **α-Pinene** | *929* | *1013* | 0.4 | 0.3 | 0.9 | 0.4 |
| Camphene | *941* | *ND* | 0.0 | 0.1 | 0.0 | 0.0 |
| **Sabinene** | *963* | *1121* | 0.1 | **1.6** | 0.2 | 0.2 |
| **β-Pinene** | *969* | *1110* | 0.2 | **12.4** | **1.3** | 0.6 |
| **Myrcene** | *979* | *1159* | **1.5** | **1.4** | **1.1** | **1.7** |
| a-Phellandrene | *995* | *ND* | 0.0 | tr | 0.0 | tr |
| a-Terpinene | *1010* | *ND* | 0.0 | 0.2 | 0.1 | 0.0 |
| **p-Cymene** | *1010* | *1270* | tr | **1.2** | **4.3** | 0.0 |
| **Limonene*** | *1020* | *1200* | **90.6** | **64.3** | **50.0** | **93.5** |
| β-Phellandrene* | *1020* | *1209* | 0.2 | 0.4 | 0.2 | 0.3 |
| (Z)-β-Ocimene | *1024* | *1231* | tr | 0.1 | 0.7 | tr |
| **(E)-β-Ocimene** | *1035* | *1248* | 0.3 | 0.1 | 1.0 | 0.2 |
| **γ-Terpinene** | *1047* | *1243* | 0.1 | **8.1** | **9.7** | tr |
| *Cis*-Linalool oxyde THF | *1056* | *ND* | 0.0 | 0.0 | 0.0 | 0.2 |
| Terpinolene | *1077* | *1281* | tr | 0.4 | 0.5 | tr |
| Nonanal | *1080* | *1392* | 0.3 | 0.2 | 0.2 | 0.0 |
| **Linalool** | *1082* | *1544* | 0.5 | 0.4 | 0.7 | 1.0 |
| *trans*-p-mentha-2,8-dien-1-ol | *1102* | *1625* | 0.2 | 0.0 | 0.0 | 0.0 |
| *cis*-p-mentha-2,8-dien-1-ol | *1114* | *1667* | 0.2 | 0.0 | 0.1 | 0.0 |
| *trans*-Limonene oxyde-1,2 | *1119* | *ND* | 0.0 | tr | 0.2 | 0.0 |
| Citronellal | *1129* | *1477* | 0.1 | tr | 0.4 | 0.0 |
| iso-neral | *1139* | *ND* | 0.0 | 0.0 | 0.1 | 0.0 |
| iso-geranial | *1156* | *ND* | 0.0 | 0.0 | 0.3 | 0.0 |
| Terpinen-4-ol | *1160* | *1598* | 0.1 | 0.5 | 0.5 | 0.1 |
| **α-Terpineol** | *1170* | *1691* | 0.3 | 0.9 | 1.1 | 0.4 |
| Decanal | *1182* | *ND* | 0.0 | 0.0 | 0.0 | 0.1 |
| *trans*-Carveol | *1195* | *1837* | 0.1 | 0.0 | 0.0 | 0.0 |
| **Nerol*** | *1208* | *1796* | **1.1** | 0.0 | **2.5** | 0.0 |
| Citronellol* | *1208* | *1762* | 0.5 | 0.0 | 0.0 | 0.0 |
| **Neral** | *1213* | *1680* | 0.5 | **1.4** | **5.8** | 0.1 |
| **Geraniol** | *1232* | *1843* | **1.7** | 0.0 | **2.4** | 0.0 |
| **Geranial** | *1241* | *1730* | 0.6 | **1.9** | **7.4** | 0.1 |
| Citronellylacetate | *1332* | *1658* | tr | 0.0 | 0.0 | 0.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Nerylacetate** | *1340* | *1723* | tr | 0.6 | **1.8** | 0.1 |
| **Geranylacetate** | *1358* | *1753* | 0.1 | 0.5 | **1.3** | 0.2 |
| (E)-β-Caryophyllene | *1415* | *1591* | 0.1 | 0.0 | 0.3 | 0.0 |
| *trans*-α-Bergamotene | *1430* | *1579* | 0.1 | 0.3 | 0.3 | 0.0 |
| β-Bisabolene | *1495* | *ND* | 0.0 | 0.5 | 0.5 | 0.0 |
| **TOTAL** | | | **99.5** | **97.7** | **95.84** | **99.14** |

Order of elution and percentages are given on an apolar column (BP1) except for compounds with an asterisk (percentage in polar column, BP20). $RI^a$ and $RI^p$: retention indices measured respectively on apolar and polar columnsTr = traces < 0.05%; ND: Not Determined

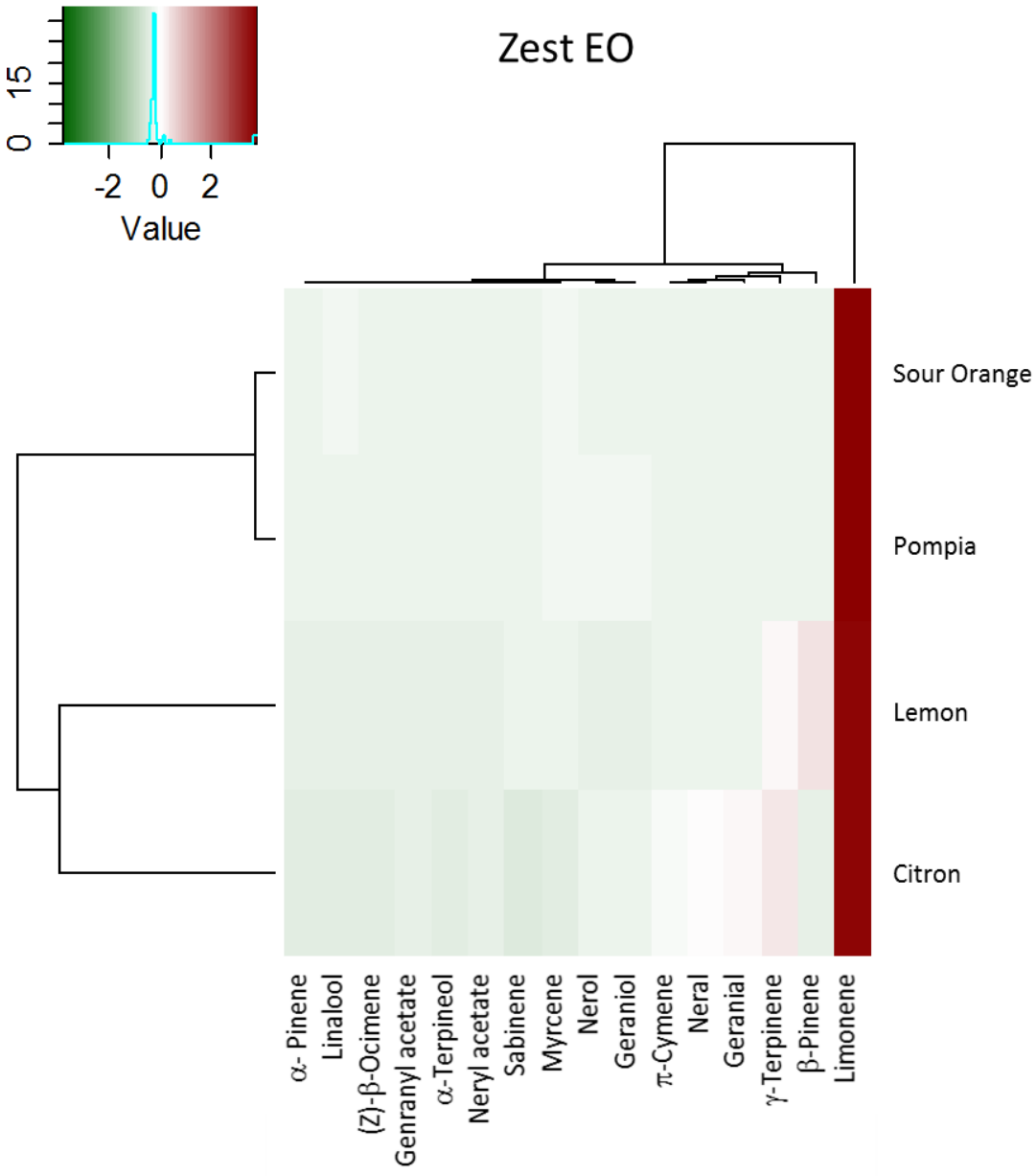



**Figure 6:** Heat map of relationships between citrus genotypes related to the contribution of the major variable compounds of zest EO (color range represents the variance level of the quantitative character with respect to average)

***Phenotypic Diversity of Pompia specimens of Sardinia***

*Leaf description and pomology*

The common characters observed in all sampled plants of Pompia were: the assurgent posture, features already observed by Chessa et al. (1994). Characters as the vigor, the average height of the plant and stem diameter vary greatly and are more closely associated with growing conditions and plant health. Most of the sampled plants are located in private gardens and trees are not found in optimal health and growing conditions, probably due to the fact that Pompia is not a commercial product on a large scale, but known and appreciated only by a market niche. The presence of the rootstock, always represented by *C. aurantium*, seems related to age of the plant. In general, the older individuals are concentrated in the town of Oliena and are not grafted.

The measurements on the fruits are summarized in Table 7. The comparison among Pompia samples of Sardinia, divided into the various pick zones, showed differences in phenotypic characters also very important as fruit weight, sugar content and acidity.

The Milis samples have a lower percentage of mesocarp, an important characteristic that depreciates the product intended for processing. The fruits of Milis, moreover, have a greater number of seeds and higher acidity that is also found in the fruits picked in Oliena site. This variability of phenotypic traits intra and inter individual there is already evidence from Mignani et al., (2004). The fruits analyzed by D'Aquino et al. (2005) are bigger and less sweet and more acidic than fruits analyzed in our study, when compared with the fruits collected in the same month, in January. By comparing the data of D'Aquino et al. (2005) acquired in Siniscola, they equates with our averages values with the exception of the number of seeds per fruit (10 seeds against 20) and acidity (6.45 against 5.30). Our data is also in line with those reported by Mignani et al., (2004). Comparing the averages for Siniscola site, common in both studies, however, we can see the differences for TSS (7.93 versus 7:50 in our study) and TA (5.6 against 5.3).

Overall the differences found across the 3 studies compared (Mignani et al., 2004; D'Aquino et al., 2005 and our) could be due to diversity of the sampling areas, the cultivation methods and crop management, the sampling period and the sample size, affecting more characters that show higher variability. The fruits were collected and analyzed over a period not specified in the first work; from October to January in the second and January in the third. In addition, the

first two studies are close together in terms of year, 2004 and 2005, and the third after a long time, about 10 years.

The size of the sampling, not specified in the work of Mignani et al. (2004), was 36 fruits per sample for a total of 7 samples in the work of D'Aquino et al. (2005), and 66 total fruits divided into the 4 with drawal areas in the present study. Finally, it is noteworthy that the three studies have sampled individuals Pompia in different areas of the island. The first in five areas (Limpiddu, Orosei, Posada, Siniscola, Torpè), the second in a single site (Siniscola), while our study involved four areas (Bitti, Milis, Oliena and Siniscola). The only common area to the 3 studies is Siniscola.

**Table 7:** Description of Pompia fruit phenotype in Bitti, Oliena, Siniscola e Milis. The value of each parameter is the sum of the averages of 3 fruits per each sample grouped by area

| AREA | Weight (g) | H (mm) | Ø (mm) | Shape Index (H/Ø) | Peel thickness (mm) | % peel | Mesocarp thickness (mm) | % mesocarp | Endocarp thickness (mm) | Juice (ml) | Segment number | Seeds / fruit | TSS (°Brix) | TA (% citric acid) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bitti n=1 | 330.70 | 80 | 110 | 0.73 | 1.70 | 18.80 | 14.00 | 36.70 | 21.00 | 39.00 | 11.70 | 17.00 | 7.1 | 5.09 |
| Oliena n=7 | 205.30 | 73 | 90 | 0.81 | 2.00 | 20.50 | 10.00 | 32.70 | 20.00 | 27.10 | 12.30 | 17.50 | 8.41 | 5.55 |
| Siniscola n=12 | 304.90 | 75 | 99 | 0.76 | 2.20 | 19.50 | 10.00 | 38.00 | 22.00 | 39.90 | 12.70 | 19.80 | 7.11 | 5.14 |
| Milis n=2 | 252.17 | 66.5 | 91.5 | 0.73 | 1.83 | 14.78 | 6.83 | 25.8 | 23.5 | 44.17 | 12.08 | 25.08 | 7.37 | 5.40 |
| **Means** | **273.27** | **73.6** | **97.5** | **0.76** | **1.93** | **18.35** | **10.21** | **33.30** | **21.63** | **37.54** | **12.20** | **19.85** | **7.50** | **5.30** |

n=number of samples esaminated

### *Variability of Essential oil*

51 compounds were detected in the essential oils of Pompia in the samples collected in Sardinia (Table 8), 26 of these were present in both fruits and leaves. The number of compounds in the essential oil of the leaves is higher than those of the fruits rinds (41 versus 36), but some of them were detected only in the rinds as trans-p-mentha-2, 8-dien-1-ol, cis-p-mentha-2,8-dien-1-ol, trans-α and β-bergamotene-bisabolene. Concerning the variability among samples, the profiles of the rind are qualitative and quantitative different, although very close. Limonene, geraniol and nerol have different percentages between Oliena and Siniscola and, if we consider the trace compounds, γ-terpinene, trans-carveol, neryl acetate, geranyl acetate, bicyclogermacrene and (E)–neridol were detected exclusively in Siniscola

group. Although the percentages of these molecules are low (0.1 to 0.2%) the accuracy of the analysis is that (<0.005%) it can say that these differences are real. Oliena and Siniscola are two municipalities within the province of Nuoro. Siniscola is near the sea, whereas Oliena is located inland. To be able to say with certainty that the observed differences can be explained by these geographical positions the analysis should carried out with a larger sampling.

For the essential oil of the leaves have three distinct profiles: O150, M3 differ from other samples that have a similar profile. The changes are most in quantity and concern primarily 9 molecules: limonene, (E)-β-ocimene, citronellal, neral, geranial, neryl acetate, geranyl acetate, (E) -β-caryophyllen and (E) -nerolidol.

**Table 8:** Chemical composition of fruit and leaf essential oil samples from Pompia grown in different localities of Sardinia

| Compounds | RI[a] | RI[p] | Leaf | | | | | | Zest | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | O150 | T5 | ME2 | O8 | B | M3 | Oliena | Siniscola |
| α-Thujene | 921 | 1013 | 0 | 0 | tr | tr | tr | 0 | 0 | 0 |
| α-Pinene | 929 | 1013 | 0.2 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 |
| 6-Methylhept-5-en-2-one | 959 | 1337 | 0.4 | 1.1 | 1.2 | 0.5 | 0.5 | 1.2 | 0 | 0 |
| Sabinene | 963 | 1121 | 0.4 | 0.4 | 0.5 | 0.3 | 0.3 | 0.5 | 0.1 | 0.1 |
| β-Pinene | 969 | 1110 | 0.9 | 0.5 | 1 | 0.6 | 0.5 | 0.7 | 0.1 | 0.2 |
| Myrcene | 979 | 1159 | 0.7 | 1 | 1.2 | 0.9 | 0.8 | 1.3 | 1.6 | 1.4 |
| d-3-Carene | 1004 | 1147 | 0.4 | 0.8 | 0.8 | 0.4 | 0.4 | 0.6 | 0 | 0 |
| p-Cymene | 1010 | 1270 | 0.4 | 0.2 | 0.1 | 0.2 | 0.3 | 0.1 | 0 | tr |
| Limonene* | 1020 | 1200 | 21.3 | 44.4 | 41.1 | 31.1 | 31.1 | 46.8 | 92.7 | 88.4 |
| β-Phellandrene* | 1020 | 1209 | 0.2 | 0.2 | 0.5 | 0.2 | 0.2 | 0.8 | 0.2 | 0.2 |
| *(Z)*-β-Ocimene | 1024 | 1231 | 0.2 | 0.4 | 0.4 | 0.4 | 0.3 | 1.1 | 0 | tr |
| *(E)*-β-Ocimene | 1035 | 1248 | 2.5 | 4.6 | 4.9 | 4.9 | 3.4 | 11.8 | 0.2 | 0.3 |
| γ-Terpinene | 1047 | 1243 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 |
| Terpinolene | 1077 | 1281 | 0 | 0 | 0 | 0 | 0 | 0 | tr | tr |
| Nonanal | 1080 | 1392 | 0.1 | tr | 0 | 0 | tr | 0.1 | tr | 0.3 |
| Linalool | 1082 | 1544 | 1 | 1.1 | 1 | 1 | 1.1 | 1.5 | 0.5 | 0.5 |
| *trans-p*-mentha-2,8-dien-1-ol | 1102 | 1625 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.2 |
| *cis-p*-mentha-2,8-dien-1-ol | 1114 | 1667 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.2 |
| Citronellal | 1129 | 1477 | 2.8 | 2.1 | 1.5 | 3.4 | 2.2 | 0.4 | 0.1 | 0.1 |
| Isoneral | 1139 | ND | 0.3 | 0.4 | 0.6 | 0.7 | 0.5 | 0.5 | 0 | 0 |
| Isogeranial | 1156 | ND | 0.4 | 0.5 | 0.9 | 1 | 0.8 | 0.8 | tr | 0 |
| Terpinen-4-ol | 1160 | 1598 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| α-Terpineol | 1170 | 1691 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.6 | 0.3 | 0.3 |
| *trans*-Carveol | 1195 | 1837 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| Citronellol* | 1208 | 1762 | 0.3 | 0.5 | 0.1 | 0.1 | 0.1 | tr | 0.3 | 0.6 |
| Nerol* | 1208 | 1796 | 0.3 | 0.2 | 0.3 | 0.2 | 0.2 | 0.3 | 0.6 | 1.6 |
| Neral | 1213 | 1680 | 15.7 | 13.5 | 15.5 | 17.4 | 19.5 | 11.7 | 0.5 | 0.5 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Geraniol | 1232 | 1843 | 0.5 | 0.3 | 0.4 | 0.4 | 0.5 | 0.3 | 0.9 | 2.4 |
| Geranial | 1241 | 1730 | 21.1 | 17.5 | 19.9 | 23 | 26 | 14.9 | 0.6 | 0.5 |
| Methylgeraniate | 1300 | 1680 | 0.6 | 0.2 | 0.1 | 0.2 | 0.2 | 0 | 0 | 0 |
| Citronellylacetate | 1332 | 1658 | 0.7 | 0.3 | 0.2 | 0.4 | 0.5 | tr | 0 | tr |
| Nerylacetate | 1340 | 1723 | 2.1 | 1.5 | 1.6 | 2 | 1.8 | 0.3 | 0 | 0.1 |
| Geranylacetate | 1358 | 1753 | 5 | 5 | 4.6 | 7.9 | 6.2 | 1.2 | 0 | 0.2 |
| β-Elemene | 1385 | 1585 | 0.9 | 0.2 | tr | 0 | 0 | 0 | 0 | 0 |
| *(E)*-β-caryophyllene | 1415 | 1591 | 1.6 | 0.3 | 0.3 | 0.6 | 0.3 | 0.6 | 0.1 | 0.1 |
| *trans*-α-Bergamotene | 1430 | 1579 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.1 |
| *(Z)*-β-farnesene | 1433 | 1638 | 0.5 | 0 | 0 | tr | 0 | 0 | 0 | 0 |
| *(E)*-β-farnesene | 1445 | 1661 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | tr |
| α-Humulene | 1447 | 1662 | 0.1 | tr | tr | 0.1 | tr | 0.1 | 0 | 0 |
| Germacrene D | 1473 | 1702 | 6 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Bicyclogermacrene | 1488 | 1726 | 0.5 | 0 | 0.1 | 0.2 | 0 | 0.2 | 0 | 0.1 |
| *trans*-β-bergamotene | 1498 | ND | 0.2 | 0.1 | 0 | 0.1 | 0.1 | 0.1 | 0 | 0 |
| β-Bisabolene | 1498 | 1720 | 0 | 0 | 0 | 0 | 0 | 0 | 0.2 | 0.3 |
| δ-Cadinene | 1512 | 1749 | 0.3 | tr | tr | tr | tr | tr | 0 | 0 |
| trans-Sesquisabinene hydrate | 1528 | 1985 | 0.2 | 0 | 0 | 0.3 | 0 | 0 | 0 | 0 |
| *(E)*-Nerolidol | 1546 | 2035 | 3.8 | 0.4 | 0.2 | 0.2 | 0.3 | 0.4 | 0 | 0.1 |
| Spathulenol | 1560 | 2115 | 0.7 | 0 | 0.2 | 0.3 | 0.2 | 0 | 0 | 0 |
| Caryophyllene oxyde | 1566 | 1974 | 0.5 | 0.2 | 0.2 | 0 | 0.3 | 0.1 | 0 | 0 |
| T-Muurolol | 1623 | 2177 | 0.3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| α-Cadinol | 1635 | 2222 | 0.5 | 0 | 0.1 | 0.1 | 0 | 0 | 0 | tr |
| α-Bisabolol | 1666 | 2209 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | tr |
| **TOTAL** | | | **95.1** | **98.7** | **100.1** | **99.5** | **99** | **99.3** | **99.5** | **99.2** |

Order of elution and percentages are given on an apolar column (BP1) except for compounds with an asterisk (percentage in polar column, BP 20). Tr = traces < 0.05%; ND: Not Determined

Three molecules have qualitative variations: germacren-D was present in relatively high proportion (6%) in O150, in most low percentages in T5 and absent in the others; (Z) -β-farnesene and T-muurolol were present at 0.5% and 0.3% only in O150.

Differences are observed in samples of the same area. Two samples of Oliena (O150 and O8) differ mainly in the content of germacrene-D and (E)-nerolidol and, to a lesser level (E)-β-ocimene and limonene. On the other hand, the samples of Siniscola (T5 and ME2) are closer to each other, while the sample of Bitti (B) is closer to those of Oliena. The sample of Milis, municipality located in the province of Oristano, in the western part of Sardinia, has a profile that differs from othersfor the lower levels in citronellal, neral and geranial and a higher content of limonene and (E) -β-ocimene. As for the variability in essential oil in the rinds, the effect of the environment or the geographical position can only be pointed out through a

larger sampling. It can therefore be assumed that within a region the variability can be potentially higher than among samples collected in different regions.

Is it an effect of the environment, tree sanitary status or genetic modifications at the origin of these changes in the essential oil composition?

At present this study does not allow to choose any of these possibilities. The effect of the environment on the composition of essential oil has already been observed many times during pathogen attack or under abiotic stress where some volatile compounds are synthesized by the plant as a response (Blée, 2002; Loreto and Schnitzler, 2010; Niinemets et al., 2013; Vieira et al., 2016). It is also true that the trees from which we took the samples were in precarious plant health and physiological conditions.

In order to confirm these results, the analysis should be reproduced for each location and at different fruit ripening periods, since the essential oil composition of Pompia evolves with the development of the fruit (Fenu et al., 2010). Through sensorial analysis it can be verified also the repercussions that these observed changes in the essential oil of the rind have on the taste and aroma of the fruit.

**Conclusions**

Depending on the examined characteristics, Pompia has greater proximity with lemon and sour orange or citron. For example, regarding the shape of the seeds or the acidity and the sugar content, it is closer to the citron. Phenotypic analysis carried out from quantitative data or from discrete variables do not reject the parental relation of Pompia with sour orange and citron, but instead confirms it, as well as for lemon. In general, Pompia phenotype is no longer close to one of its parental, contrary to visual impression that occurs when fruits of the 3 citrus are observed: the yellow color of the skin and the shape of the fruit make it appear Pompia closer to citron.

The phenotype of a hybrid is not always intermediate to his parents, can also be distinctly different, or closer to one parent, this depends on the genetics of the observed phenotypic traits.

The main components of the essential oils of the fruit rind and of the leaves of Pompia, of the Poncire citron, Santa Teresa lemon and Florida sour orange, are similar to those observed in 1997-1998 (Lota, 1999) in a study on the same varieties in collection at the INRA-CIRAD of

San Giuliano. This suggests a certain stability over time regardless of the age of the trees. Moreover, on two studies on the essential oil composition of Pompia fruit rind in Sardinia reported different results: limonene is 77.5% according to Fenu et al., (2010) and 93.3% in the work of Camarda et al., (2013).

Petretto et al. (2015) by a different process of extraction from the bark of the birds (saturation in the head space), also obtained a rich Pompia profile on limonene, 94.1%, close to that of the sour orange. Nevertheless, this sour orange profile differs from the Pompia profile, by a relatively high concentration in linalool, about 5% against 0.1%. If the lemon profile is very close to what it observed in the presence, the citron resulted very different. These differences are mainly due to limonene, more present in the analisys of Petretto et al. (2015) (71% versus 50%) and a lower concentration in nerol, neral, geraniol, geranial, neryl acetate and geranyl acetate which do not exceed 1%. Moreover, the percentages of the same components are between 1.3 and 7.4% in the essential oil of citron fruit rind in the present study. The cause of these variations may be due to a cultivar effect: Diamante versus Poncire. The variability of the composition of the essential oils between these two cultivars has already been highlighted (Luro et al., 2012). Also the different extraction methods could be a source of variability in the EO composition.

How is it that the oil essential profile resulted close to leaves of one parental and oil of the fruit rind to the other parent?

This difference shall be the result of a different regulation of the synthesis of volatile compounds between the flavedo and the leaf. It must also consider the predominance of limonene in the essential oil of the rind that constitutes over 90% of the analyzed oil in Pompia and in sour orange. In a relation of proportionality it is evident that, in this case, the other molecules are, in proportion, limited and their quantitative variations can be under or over estimated. In the essential oil of the leaves limonene is at most 1/3 of the totality of the compounds, leaving more visibility to the variation of the minor components. In the study of Petretto et al. (2015), the Pompia profile was close without distinction to that of orange, grapefruit, sour orange and chinotto, because they all had an high percentage of limonene, from 91.5% to 96.7%. Although this feature is shared by Pompia and sour orange, its maternal parent, the PCA was notenoughto determine who among the grapefruit, orange and sour orange was the parental Pompia.

The foliar EO compositions of Pompia, and a lesser degree of lemon, are very close to those of citron. Is it enough to consider a dominant inheritance of the volatile and the aromatic components of citron?

There is a known citrus that is also a hybrid of sour orange x citron, which is the Marrakech limonette (*C. limetta*), also known under the name of Tunis bergamot (Curk et al., 2016). Except for the essential oil composition of the rind (Delort and Naef, 2011) we did not find studies with the composition of its leaf oil. The bergamot, which is a hybrid sour orange x lemon (Curk et al., 2016) has an EO profile closer to that of sour orange leaves than to lemon with 39.7% linalool, 19.9% of acetate linalyl and 12.1% of a-terpineol (Lota, 1999).

The Rangpur lime (*C. limonia*), Khatta (*C. karna*) and the lemons Volkamer (*C. limonia*) and Rough (*C. jambhiri*) are hybrids resulting from spontaneous crosses of mandarin x citron (Curk et al., 2016). According to Lota et al. (1999) the essential oil compositions of Rangpur, Khatta and Rough leaves would resemble each other for the main compounds, 28-47% of β-pinene, 11 to 27% of limonene and 9-15% citronellal. This profile does not match that of the citron, nor to that of *C. reticulata*. Lemon Volkamer (or Volkameriana) has a profile of main compounds, 30% sabinene, 26% limonene, citronellal 14% and 10% linalool (Lota et al, 1999), which no longer corresponds to the EO profile of the citrons.
It can be concluded that the inheritance of chemical profiles of the essential oils of the leaf is not of dominant type and also no parental type, and that there are a great deal of possible chemical profiles in citron descendants.

The predominance of the limonene in essential oil Pompia and sour orange is a handicap for the analysis of the variation of the other compounds. A quantitative analysis that highlights any type of compound, may give further information about the inheritance of EO components of the rind of the fruit. Otherwise, olfactory sensory analysis will also give us information on inheritance of aroma in Pompia.

## References


Blée E., 2002. Impact of phyto-oxylipins in plant defense. Trends Plant Sci. 7 (7): 315-322

Camarda I., Mazzola P., Brunu A., Fenu G., Lombardo G., Palla F., 2013. Un agrume nellastoriadellaSardegna: Citrus limón var. PompiaCamardavar. nova. Quad. Bot. Amb. Appl. 24: 109-118

Chessa I., Mulas M. and Pala M. 1994. Gliagrumi p339-360. In: Patrimonio genetico di speciearboree da frutto. Le vecchievarietadellasaedegna. Carlo Delfino Editore, Sassari, Italy.

Curk F., Ollitrault F., Garcia-Lor A., Luro F., Navarro L. and Ollitrault P. 2016. Phylogenetic origin of limes and lemons revealed by cytoplasmic and nuclear markers Annals of Botany Doi:10.1093/aob/mcw005.

D'Aquino S., Fronteddu F., Usai M., Palma A., 2005. Qualitative and physiological properties of 'Pompia', a citron-like fruit.Plant Genetic Resources Newsletter 143: 40-45

Delort E. and Naef R. 2011 Volatile Components in less common Citrus species. In: Citrus oils: composition, advanced analytical techniques, contaminants and biological activityEdited by Dugo G. and Mondello L. CRC Press (N.W. USA)

Fancello F., Petretto G.L., Zara S., Sanna M.L., Addis R., Maldini M., Foddai M., Rourke J.P., Chessa M., Pintore G., 2016. Chemical characterization, antioxidant capacity and antimicrobial activity against food related microorganisms of Citrus limon var. Pompia leaf essential oil. LWT-Food Science and Technology, 69: 579-585

Fanciullino A.L.; Tomi F.; Luro F.; Desjobert J.M.; Casanova J., 2006. Chemical variability of peel and leaf oils of mandarins. Flavour and FraganceJournal , 21(2) : 359

Fenu G., Carai A., Foddai M., Azara E., Careddu S., Usai M., 2010. Composition and seasonal variation of Citrus mostruosa essential oil from Sardinia. Int. J. Essent. Oil Therap, 4: 23-25

Güney M., Oz A.T., Kafkas E.,2015Comparison of lipids, fatty acids and volatile compounds of various kumquat species using HS/GC/MS/FID techniques. Journal of the Science of Food and Agriculture, 95; 1268–1273

Jiménez Cuesta M.J., Cuquerella J., Martínez Jávega J.M., 1981. Determination of a color index for citrusdegreening . In Proc. of the International Society of Citriculture, Vol. 2, 750-753

Loreto F., Schnitzler J.P., 2010. Abiotic stresses and induced BVOCs. Trends Plant Sci. 15 (3): 154–166

Lota M.L. 1999. Les huiles essentielles d'agrumes : caractérisation par RMN du carbone-13 CPG-IK et CPG/SM. Thèse de Doctorat de l'Université de Corse Pascal Paoli, Discipline : Chimie Organique et Analytique. pp 293.

Lota M.L., de Rocca Serra D., Tomi F., Bessiere J.M., Casanova J., 1999. Chemical composition of peel and leaf essential oils of Citrus medica L. and C. limonimedica Lush. Flavour and fragrance journal, 14(3): 161-166

Luro F., Venturini N., Costantino G., Paolini J., Ollitrault P., Costa J., 2012. Genetic and chemical diversity of citron (Citrus medica L.) based on nuclear and cytoplasmic markers and leaf essential oil composition. Phytochemistry, 77: 186-196

Mignani I., Mulas M., Mantegazza R., Lovigu N., Spada A., Nicolosi E., Bassi D., 2004. Caratterizzazione morfologica, biochimica e molecolare di accessioni di "Pompia", agrume della Sardegna. In: Atti delle VII giornate scientifiche SOI, Castel dell'Ovo, Napoli

Niinemets Ü., Kännaste A., Copolovici L., 2013. Quantitative patterns between plant volatile emissions induced by biotic stresses and the degree of damage. Front. Plant Sci. 4: 262

Ollitrault P., Jacquemond C., Dubois C., Luro F., 2003. Citrus. In : Hamon, P. ; Seguin, M. ; Perrier X.; Glaszmann X., Genetic diversity of cultivated plants. CIRAD, Montpellier, 193-197

Paoli M., de Rocca Serra D., Tomi F., Luro F., Bighelli A., 2016. Chemical composition of the leaf essential oil of grapefruits (*Citrusparadisi*Macf.) in relation with the genetic origin. Journal of Essential Oil Research. DOI:10.1080/10412905.2016.1140090

Petretto G.L., Sarais G., Maldini M.T., Foddai M., Tirillini B., Rourke J.P., Chessa M., Pintore G., 2015. Citrus monstruosa discrimination among several Citrus species by multivariate analysis of volatiles: a metabolomic approach. Journal of Food Processing and Preservation http://dx.doi.org/10.1111/jfpp.12674

Sutour S., Luro F., Bradesi P., Casanova J., Tomi F., 2016. Chemical composition of the fruit oils of five Fortunella species grown in the same pedoclimatic conditions in Corsica (France) NPC 11 (2): 259-262

Van Den Dool H., Kratz P.D.J., 1963. Chromatography, 11: 463-471

Vieira D.D.S.S., Emiliani G., Michelozzi M., Centritto M., Luro F., Morillon R., Loreto F., Gesteira A., Maserti B**.,** 2016. Polyploidization alters constitutive emission of volatile organic compounds (VOC) and improves membrane stability under water deficit in Volkamer lemon (Citrus limoniaOsb.) leaves. Environmental and Experimental Botany 126: 1-9

# GENERAL CONCLUSIONS

*Citrus monstruosa* "Pompia" (*Rutaceae*) is an endemic *Citrus* of Sardinia, widespread in the area of Baronia (eastern Sardinia) and with less intensity, spread almost all the island. Pompia trees can be found mainly in urban and peri-urban gardens and whose presence is greatly tied to tradition. Only in Siniscola area, specialized crops of Pompia were present, today for the most part to the state of neglect.

First phenotypic and molecular characterizations have pointed a high degree of similarity between Pompia, lemon (*C. limon*) and citron (*C. medica*), making hypothesis on a putative genetic origin of Pompia as an hybrid of citron and lemon. Nevertheless, the used molecular markers were dominant and so not fully adapted to phylogenic studies.

By using codominant molecular markers, (SSR and InDels) of nuclear and cytoplasmic genomes, widespread through the genome, Pompia and 15 citrus representing the different species of the genus *Citrus* were genotyped. The results suggested that Pompia is a product of a cross between a sour orange (*C. aurantium*) and a citron, where the citron was the pollinator. This kind of cross, was also at the origin of lemon. The genotypes of lemon and Pompia were very close. This close relationship of Pompia with lemon and citron was supported by the analysis of essential oil composition but pondered by the global phenotype analysis (fruit and leaf morphology and composition) where Pompia appeared distinct as much with the citron and lemon as with sour orange. The fruit size, pulp acidity and peel colour were the characters that have suggested to consider in the past Pompia as a kind of citron.

The DNA barcoding, even if gave some indication about the genetic relationship of Pompia and closer citrus species, was not enough informative for distinguish the parent species from closer related species. On the contrary, molecular analysis with SSRs and Indels was the best choice to clarify genetic inheritance and to indicate the cross sour orange x citron at the origin of Pompia. For that, the proposed taxonomy of Pompia should be: *Citrus aurantium* x *Citrus medica* var *pompia*.

Even if we discovered the parents of Pompia, it will not really be possible to recreate it by crossing because the sour orange, maternal parent of Pompia, is very heterozygous due to its

status as mandarin-pummelo hybrid. Each hybrid of the offspring of this cross will be a unique genotype and therefore a unique phenotype, as is Pompia.

The rarity of this genotype confers some fragility to his existence if nothing is done to preserve and multiply it. It would, also, be important to deepen the analysis with a larger sample targeted for a greater knowledge of the variability intra species. Taking into account the fact that it is currently extremely difficult to find the material useful to perform morphological and chemical analysis because of the rarity of plants and neglect into which is the Sardinian heritage of Pompia.