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Identification of Chestnut Hybrids Using SSR Markers and Bioinformatic Tools

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DECLARATION

I, Toufiq Soale Yussif, a student in the Instituto Politécinco de Bragança - Escola Superior Agrária, declare that the work submitted to the department is a product of my effort and has not been submitted for any degree. It contains no material previously published by another person or materials accepted for the award of any other degree of the University except where acknowledgment has been made in the text.

Traditional foods, as vessels of heritage and identity, deserve vigilant preservation to enrich future generations.

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ABSTRACT

Chestnut trees serve as a vital resource for food and timber production, yet invasive pests and diseases increasingly threaten their survival and productivity. In Europe, hybridization programs have been launched to develop resilient rootstocks, such as the ink disease-resistant CA90 hybrid (*Castanea sativa* × *Castanea crenata*), to combat pathogens like *Phytophthora cinnamomi*. However, distinguishing these hybrids from conventional varieties remains challenging, as current methods rely heavily on field observations and morphological traits. To address this gap, this study identified molecular markers using Simple Sequence Repeats (SSR) microsatellite markers and bioinformatics to differentiate CA90 hybrids from other chestnut varieties.

We analyzed 35 chestnut samples, including three CA90 controls, hybrids, and non-hybrid plants from Portugal, to establish genetic profiles based on SSR band patterns and motif variations. From 43 existing SSR markers, nine primers with null allelic features and low observed heterozygosity (Ho) were selected and modified for this study. Polymerase Chain Reaction (PCR) amplification and agarose gel electrophoresis were employed to visualize Deoxyribonucleic Acid. (DNA) bands, followed by Sanger sequencing of 27 amplified products to confirm genetic variations. This approach identified 31 SSRs across 22 sequences, with trinucleotide repeats dominating (67.74%), followed by dinucleotide (22.58%), mononucleotide (6.45%), and hexanucleotide (3.23%) motifs. Across the nine loci, 18 alleles were detected, ranging from one to three alleles per locus among the samples. Crucially, the CP4 locus emerged as a novel, hybrid-specific marker, exclusively present in CA90 samples, and should be combined with primer loci CP2, CP6, CP9, and CP10 during identification on gel for cost-effectiveness.

These SSR-based markers offer a reliable, cost-efficient solution to identify disease-resistant CA90 chestnut hybrids, surpassing traditional morphological methods. By enabling precise selection of resilient rootstocks, they enhance sustainable cultivation and pathogen management. These tools can optimize breeding programs, boost orchard productivity, and protect genetic diversity, proving essential for mitigating pest and disease threats while ensuring the long-term health of chestnut ecosystems.

Keywords: Chestnut, *Castanea sativa,* Microsatellite Markers (SSR), hybrids, CA90

RESUMO

Os castanheiros são um recurso vital para a produção alimentar e madeireira, mas a sua sobrevivência e produtividade são ameaçadas por pragas e doenças invasoras. Na Europa, foram inicializados programas de hibridização para desenvolver porta-enxertos resilientes, como o híbrido CA90 (*Castanea sativa* × *Castanea crenata*), resistente à doença da tinta (*Phytophthora cinnamomi*). Contudo, a distinção destes híbridos de variedades convencionais permanece um desafio, pois os métodos atuais dependem de observações de campo e características morfológicas. Para combater estas dificuldades, este estudo identificou marcadores moleculares usando marcadores microssatélites de repetições de sequência simples (SSR) e bioinformática para diferenciar o híbrido CA90 de outras variedades de castanheiro.

Foram analisadas 35 amostras, incluindo três controlos CA90, híbridos e plantas não híbridas de Portugal, para estabelecer perfis genéticos baseados em padrões de bandas SSR e variações de motivos. De 43 marcadores SSR existentes, foram selecionados e modificados nove primers com alelos nulos e baixa heterozigosidade observada (Ho). Utilizou-se amplificação por PCR e eletroforese em gel de agarose para visualização das bandas de DNA, seguida de sequenciação de Sanger de 27 produtos amplificados para confirmar variações genéticas. Esta abordagem identificou 31 SSR em 22 sequências, com repetições trinucleotídicas a dominar (67,74%), seguidas de motivos dinucleotídicos (22,58%), mononucleotídicos (6,45%) e hexanucleotídicos (3,23%). Nos nove loci, detetaram-se 18 alelos, variando entre um a três alelos por locus. O locus CP4 destacou-se como um marcador exclusivo do CA90, devendo ser combinado com os loci CP2, CP6, CP9 e CP10 em análises de gel para maior custo-efetividade.

Estes marcadores SSR oferecem uma solução fiável e económica para identificar híbridos CA90 resistentes a doenças, superando métodos morfológicos tradicionais. Ao permitir a seleção precisa de porta-enxertos resilientes, estes marcadores promovem o cultivo sustentável e o maneio de patógenos. Estas ferramentas podem otimizar programas de melhoramento, aumentar a produtividade de pomares e proteger a diversidade genética, sendo essenciais para mitigar ameaças de pragas e doenças, garantindo a saúde a longo prazo dos ecossistemas de castanheiro.

Palavras-chave: Castanha, *Castanea sativa*, Marcadores microssatélites (SSR), Híbridos, CA90

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LIST OF ABBREVIATIONS

EST-SSRs:	Expressed Sequence Tag-derived Simple Sequence Repeats
AFLPs:	Amplified Fragment Length Polymorphism
EST:	Expressed Sequence Tags
SSRs:	Simple Sequence Repeats
STRs:	Short Tandem Repeats
FAO:	Food And Agriculture Organization
QTLs:	Quantitative Trait Loci
BACs:	Bacterial Artificial Chromosomes
CHV1:	*Cryphonectria hypovirus* 1
GPS:	Global Positioning System
INIAV:	National Institute for Agricultural and Veterinary Research
ddH_20:	Double-Distilled Water

1.0 Introduction

The economic and wildlife significance of chestnut trees (*Castanea*: *Fagaceae*) has long been recognized in Europe, Asia, and North America. Chestnut is significant due to its multifaceted contributions to these continents, and produces nuts around 2.353 Tg worldwide. China (1.965 Tg), Bolivia (84.01 Gg), Turkey (63.58 Gg), the Republic of Korea (53.384 Gg), and Italy (53.28 Gg) are the countries with the largest chestnut production (FAO, 2020). They provide valuable timber and nuts and contribute to the overall biodiversity of forests. Nutritionally, they are a healthy food option with low fat and cholesterol, rich in carbohydrates, fiber, vitamins, and minerals (Borges *et al*., 2008; Borges *et al*., 2007; Meleti *et al*., 2024). Culturally, they are staple food sources integral to traditional practices and festivals, and were promoted to fight hunger (Chapman *et al*., 2022). Economically, they offer livelihood opportunities. Ecologically, chestnut trees enhance the growth of mushrooms and aromatic herbs, contributing to the maintenance of forest biodiversity (Baptista *et al*., 2010).

Understanding and preserving the value of chestnuts ensures their continued significance in various aspects of human life and the environment. However, chestnut trees (*Castanea* spp.) are susceptible to invasive pests and diseases that can seriously jeopardize their existence and productivity. Chestnut ecosystems are currently threatened by different stress factors (natural or anthropogenic), such as climate change, abandonment of traditional orchards, wildfire, and an increased incidence of pests and diseases (Freitas *et al*., 2021). Towards the end of the nineteenth century, chestnut populations significantly declined due to diseases such as chestnut canker caused by the fungus *C. parasitica* and ink disease caused by the Oomycete *Phytophthora cinnamomi* (Petri) and *Phytophthora cambivora* (Rands). These diseases drastically threatened European and American chestnuts, killing an estimated 4 billion trees at that time (Fernandes *et al*., 2022). Also, high humidity in grove areas has hindered the establishment of new groves and the preservation of existing ones due to the disease's advancement (Serrazina *et al*., 2015).

In the pursuit of countering these destructive diseases, it became necessary to seek research-driven solutions capable of mitigating or eradicating the influence of these pathogens. The scientific community applied numerous research techniques to determine the cellular, molecular, and genetic relationships that underlie all biotic and abiotic stresses in the chestnut tree, identifying a promising approach: establishing hybridized plants (Pereira-Lorenzo *et al*., 2016). More common control approaches are correctly managing nurseries/orchards, using resistant rootstocks for propagation, or planting resistant hybrids for production (Hardham, 2005). Interspecific hybrid chestnut plants derived from crosses of Asian and European (‘*Castanea crenata* X *Castanea sativa’* or Vice Versa) sources have proven notably resistant to ink diseases (Santos *et al*., 2017). In the last decades, the significant decline in chestnut populations led to breeding programs in Portugal, France, and Spain to obtain hybrids tolerant to ink disease while maintaining fruit production and quality traits to satisfy commercial demands (Serrazina *et al*., 2015). These programs promoted the need for farmers to plant hybridized plants to protect their farms from future biotic and abiotic challenges. However, there is a lack of straightforward strategies for identifying and maintaining these established hybrid plants, leading to farmers unintentionally cultivating non-hybrid plants, ultimately resulting in pathogen attacks and the death of their trees in subsequent years. Selecting the right cultivars is essential in establishing and sustaining productive orchards. Supporting nurseries and growers in maintaining accurate genetic records can provide valuable insights into the performance of specific cultivars.

In Portugal, the cultivation of chestnut trees holds great importance, particularly in the cold region of Trás-os-Montes, which accounts for more than 80% of production (34,000 to 38,000 tons) (Ribeiro *et al*., 2020). The price of a single *C. sativa* plant in Portugal varies based on the height, with costs typically falling within 2 to 4 euros. In contrast, hybrid plants such as “Ferosacre” (CA90) and other hybrids command a higher price range, typically 8 to 12 euros per plant. However, in certain instances, rootstock vendors may offer *C. sativa* plants while asserting that they are hybrid cultivars. To date, some farmers still depend on traditional methods of hybrid identification, such as morphological characteristics and field observations. However, these methods have limitations in distinguishing closely related individuals and genetically similar plants (Marinoni *et al*., 2003). Chestnut varietal characterization has been carried out in recent decades using different approaches, and more recently, the development of DNA markers has provided a direct study of genotypes that enable the identification of chestnut hybrids (Martin *et al*., 2009). Molecular techniques, particularly microsatellite markers, have revolutionized the field of hybrid identification by providing precise means of differentiating individuals based on their genetic profiles.

The development of molecular markers facilitated the evaluation of chestnut materials (Gobbin *et al*., 2007; Pereira-Lorenzo *et al*., 2010, 2011) and expressed sequence tags SSRs (EST-SSRs) (Martín *et al*., 2010; Pereira-Lorenzo *et al*., 2017). Hybrid identification plays a crucial role in plant breeding, ensuring genetic purity and enhancing crop improvement programs. In Portugal, chestnut genetic diversity was initially assessed through a study employing simple sequence repeat (SSR) markers, which successfully identified traditional Portuguese chestnut varieties (Costa *et al*., 2005). The findings indicated a relatively low level of genetic variability among these varieties. Braga *et al*. (2023) reported that *Castanea crenata* and *C. mollissima* have served as the genetic foundation for Portuguese chestnut breeding programs. More recently, Costa *et al*. (2011) initiated a breeding program focused on the development of *C. sativa* × *C. crenata* and *C. sativa* × *C. mollissima* hybrids. From this effort, four F1 hybrids were selected for large-scale propagation based on their *in vitro* rooting ability, resistance to ink disease, and favorable performance under field conditions (Fernandes *et al*., 2020; Marinoni *et al*., 2003). Additionally, a clonal collection of Japanese chestnut hybrids was crossed with European chestnut (*C. sativa*) to generate improved genotypes aimed at nut, timber, and rootstock production for local cultivars(Pereira-Lorenzo *et al*., 2016). Furthermore, a group of hybrids exhibiting strong resistance to *Phytophthora cinnamomi* is currently being propagated and released as enhanced genetic material for new rootstocks (Santos *et al*., 2017).

However, it is evident from European research articles that chestnut research focuses on the genetic diversity and germplasm of chestnut populations, excluding tools that can specifically identify chestnut hybrids to enable farmers to buy and plant resistant chestnut plants from commercial rootstock vendors. Therefore, the fight against plant diseases and the development of molecular tools to differentiate chestnut hybrids and non-hybrid plants is necessary. In this study, I aim to develop and validate a genetic identification tool based on microsatellite (SSR) markers to accurately distinguish ink disease-resistant chestnut hybrids, particularly CA90 (a *Castanea sativa* × *Castanea crenata* hybrid), from other varieties. The goal is to support the selection and deployment of resilient rootstocks for sustainable chestnut production and disease management. This research is especially relevant given the current absence of cultivar certification and validation systems within the chestnut industry. The application of genetic marker technology will contribute to preserving genetic integrity and enhancing the traceability and reliability of chestnut breeding programs.

1.1 Objectives of Study

The spread of ink disease, caused by *Phytophthora cinnamomi*, poses a significant threat to chestnut cultivation, with serious implications for productivity, sustainability, and forest health. The primary objective of this dissertation is to utilize microsatellite marker analysis in identifying and characterizing chestnut hybrids and non-hybrids through Simple Sequence Repeat (SSR) markers.

The specific objectives include:

- Selecting the most effective SSR primer combinations to differentiate the CA90 (*Castanea sativa* × *Castanea crenata*) hybrid from other chestnut varieties.
- Validating the diagnostic tool's accuracy and reproducibility across different chestnut populations.
- Establishing a standardized protocol for the molecular identification of CA90 hybrids to support certification and validation programs within the chestnut industry.

2.0 Literature Review

2.1 Chestnut Species, Cultivars, and Rootstocks.

Chestnuts originated from the genus *Castanea*, in the *Fagaceae* family, alongside other important tree species, such as beeches and oaks (*Quercus sp*.) (Clark *et al*., 2023). The genus *Castanea* comprises three sections: *Eucastanon* (chestnuts), *Hypocastanon* (Henry chestnut), and *Balanocastanon* (chinquapins). Although there are thirteen species of chestnut in the world, the *Eucastanon* species holds greater economic significance and includes the European chestnut (*Castanea sativa* Mill), American chestnuts (*Castanea dentata* (Marshall) Borkh), Chinese chestnuts (*Castanea Mollissima* Blume), and Japanese chestnuts (*Castanea crenata* Sieb. and Zucc) (Fernandes *et al*., 2022; Mellano *et al*., 2012).

Chestnut is cultivated using vegetative propagation (rooted cuttings) or seedlings (Song *et al*., 2021). The most successful propagation is by grafting or budding onto a seedling rootstock, preferably of the same variety, to minimize graft incompatibility. *Castanea* trees typically grow 12-15 meters high, possess slender yellowish sapwood, tannin-rich brown heartwood, and have a robust root system. They are moderately heat-loving, well-suited for ecosystems, and flourish best in temperate zones between 300 and 500 latitudes, with a yearly rainfall of approximately 800 mm (Massantini *et al*., 2021). Soils rich in calcium, low pH, and poor drainage are unfavorable for these trees (Semenyutina *et al*., 2022). The *C. mollissima* can withstand temperatures as low as -28.8 ◦C and resist chestnut blight. The Japanese chestnut, *C. crenata*, characterized by narrow leaves with epithelial scales on the underside, possesses low-quality nuts and is vulnerable to low temperatures. The European chestnut (*C. sativa*) produces dark brown chestnuts with white stripes (**Figure 1**) and has long, hairy leaves with coarse-toothed margins **(**Pereira-Lorenzo *et al*., 2019**)**. The American chestnut tree, *C. dentata*, has lanceolate leaves with a bluntly acuminated apex and hairless surfaces on both sides, with high-caliber nuts (Massantini *et al*., 2021).

Figure 1. Chestnut species - adapted from Massantini *et al*. (2021).

The Japanese (*Castanea crenata* Siebold and Zucc.) and Chinese (*Castanea mollissima* Blume) chestnut species resist diseases. Still, their nuts have little commercial value and are not suitable as rootstocks due to grafting incompatibility with *C. sativa* . These species have proven to resist chestnut blight, with *C. crenata* also showing resistance to ink disease. While the nuts of these species are edible, they are generally considered inferior in flavor compared to other chestnut varieties. Additionally, the incompatibility with *C. sativa* limits their use as rootstocks. Therefore, despite their disease resistance, their nuts' commercial value and incompatibility with *C. sativa* hinder their widespread utilization (Fernandes *et al*., 2020). The only native species to spread in Europe is *C. sativa* , a multipurpose species for its high-quality nuts, timber, and flour production. *C. sativa* has been widely cultivated throughout Europe since ancient times (Clark *et al*., 2023). In Europe, the coastal regions of Spain, France, and Portugal hold significant importance as the origin of the indigenous species of *C. sativa* (Krebs *et al*., 2004).

According to (STATS FAO, 2022), *C. sativa* was chastised in Europe, leading to a decline in their nut production by 251,549 tons from 1961 to 2015. The decline was attributed to the forest area production replacement by other crops in many regions, the abandonment of ancient chestnut groves, natural dieback, pests, and diseases (Freitas *et al*., 2021). However, since 2015, chestnut production in Europe has increased due to various factors. One significant factor is the influence of climate change, which has positively impacted chestnut production in recent decades (Freitas *et* *al*., 2021). Research and breeding efforts have also contributed to this increase, indicating a positive impact on chestnut production since 2015 (Fernandes *et al*., 2022).

2.1.1 Establishment of Chestnut Hybrids and Rootstocks.

The repercussions of biotic and abiotic stress factors, predictions on implications of climate change on pathogens' shift distribution, coupled with the consequent manifestation of distinct disease phenotypes (Burgess *et al*., 2017) led to the development of novel chestnut hybrids that are better suited to prevailing climatic conditions and biotic stress. Chestnut clones may be referred to as genetically identical chestnut trees reproduced through somatic embryogenesis. This technique involves creating thousands of new embryos from a specific tree, which can then propagate trees with desired traits, such as resistance to chestnut ink diseases (McGuigan *et al*., 2020). Chestnut seedlings can be used as rootstock for grafting scions of the same cultivar, as this can help prevent graft failure and ensure successful growth (Huang *et al*., 1994).

There are hundreds of chestnut cultivars around the world. In Portugal, the most popular have included ‘Longal,’ ‘Judia,’ ‘Cota,’ ‘Amarelal,’ ‘Lamela,’ ‘Aveleira,’ ‘Boaventura,’ ‘Trigueira,’ ‘Martaínha’ and ‘Negral.’ In Italy, marron-type cultivars are 'Luserna,' 'Chiusa Pesio,' 'Castel del Rio,' 'Marradi,’ and ‘Fiorentino.’ Also, some *C. sativa* traditional French cultivars include 'Verdale,' 'Arizinca,' 'Bouche Rouge,' 'Toumive,' 'Savoye,' 'Belle Epine,' ' Châtaigne de Laguepie,' 'Sardonne,' and 'Dorée de Lyon' (Mellano *et al*., 2012) and in Australia: De Coppi Marone, Buffalo Queen, Red Spanish, Purton’s Pride are the known varieties. Bouche de Betizac is a clone promoted in Europe as a *Phytophthora*-resistant rootstock, but in Australia, Menzies is the currently favored rootstock due to its reported resistance to *Phytophthora.* Growers have observed that some rootstocks have better resistance than others (Horticulture Innovation Australia, 2014).

Breeding initiatives in the 20th century produced the genotypes currently accessible on the European market (Pereira-Lorenzo & Fernandez-Lopez, 1997). In the early centuries, the hybrid clone CHR-151 (formerly denominated HS) was used as a rootstock to introduce resistance to the ink disease. Pereira-Lorenzo and Fernandez-Lopez (1997) also reported that the Chinese cultivar “Vanuxem" is one of the sources of immune genes in *C. mollissima* for the large breeding program. Numerous chestnut hybrids have been documented in Europe through morphological assessments since the early this century (Freitas *et al*., 2021). In Portugal, the initial crossbreeding efforts involving different species began in 1947 under the guidance of Bernardino Barros Gomes, using the *C. crenata* cultivar 'Tamba' as the male parent (Pereira-Lorenzo *et al*., 2017).

The INIAV's Chestnut Breeding Program, inaugurated in 2006 in Europe {France, Spain, and Portugal} (Santos *et al*., 2017) whose primary aim was to develop and select interspecific hybrids through open or controlled breeding, specifically for use as resistant rootstocks, singled out hybrids exhibiting strong resistance to *P. cinnamomi* and production of high-quality fruit nuts suitable for rootstocks. see (**Table 1**).

Table 1. The most used chestnut hybrids.

Hybrids	**Reference**
(i) Marsol (CA07) (ii) Maraval (CA74) (iii) Ferosacre (CA90) (iv) Marigoule (CA15) (v) Marlhac (CA118)	(Larue & Petit, 2023)

These hybrids originate from crosses between *C. crenata* and *C. sativa* or the reverse. The interspecific hybridization in Portugal artificially induced crossings between (*C. sativa* x *C. crenata* and *C. sativa* x *C. mollissima*) produced two full-sib progenies used to introduce resistance genes for *P. cinnamomi* (ink disease) and *C. parasitica* (chestnut blight) from Asian species into the susceptible European chestnut species (Costa *et al*., 2011). This project focused on developing and distributing improved chestnut rootstocks resistant to ink disease. It uses pedigree and molecular characterization to select and multiply these new generations of rootstock to enhance national and international productivity in the chestnut sector by disseminating these new clones. More recently, Photoautotrophic micropropagation has been used to multiply and root valuable ink-resistant hybrids for use as rootstocks, showing a success rate of up to 71% for some genotypes (Aldrey *et al*., 2018).

Assessing genetic diversity holds significant importance in formulating conservation strategies and breeding initiatives aimed at producing cultivars that exhibit resistance to pathogens like the Chinese wasp *Dryocosmus kuriphilus,* the *P. cinnamomi*, and the *C. parasitica* while also ensuring high nut quality. The first studies were: i) the interspecific hybrids Bouche de Bétizac (*C. sativa* "Bouche Rouge" × *C. crenata* 'CA04') from France (Dini *et al*., 2012; Sartor *et al*., 2015),ii) the 'Pugnenga' cultivar of *C. sativa* Mill, an Italian cultivar native to the Cuneo Province (Piedmont), iii) the 'Savoye,' a cultivar of *C. sativa* Mill native to France (Sartor *et al*., 2015). Several chestnut hybrids confer resistance to pathogen stress (**Table 2**), indicating the level of resistance and the use of chestnut clones as rootstocks for the market.

Table 2. Chestnut species and hybrids and their level of disease susceptibility.

Species / Hybrid clones	**Rootstock**	**Level of susceptibility to *P. cinnamomi***	**Reference**
C. dentata	No	Susceptible to both ink disease and chestnut blight	(Clark *et al*., 2023)
C. sativa	No	Highly susceptible to ink disease, chestnut blight, and the impact of the Chinese gall wasp	(Fernandes *et al*., 2021)
C. crenata	No	Carries resistance to *P. cinnamomi*,	(Pavese *et al*., 2021)
C. mollissima	No	It resists two significant invasive pathogens: *C. parasitica* (Murr.) Barr and *P. cinnamomi* Rands	(Westbrook *et al*., 2019)
Ferosacre (CA90)	Yes	Resistant to *P. cinnamomi*	(Vannini *et al*., 2001)
Bouche de Bétizac (A125)	Yes	Exhibits complete resistance to the gall wasp	(Sartor *et al*., 2009)
Precoce Migoule (CA48)	Yes	It exhibits resistance to chestnut- to Asian gall wasp insects and chestnut anthracnose.	(Anagnostakis, 2012.)
'Marsol' (CA07)	Yes	Exhibits resistance against *Phytophthora* root rot and some resistance to chestnut blight	(Vahdati *et al*., 2021)
'Maraval' (CA74)	Yes	Resistant to *P. cinnamomi*	(Vannini *et al*., 2001)
'Marigoule' (CA15)	Yes	Resistant to *P. cinnamomi*	(Larue & Petit, 2023)
'Marlhac' (CA118)	Yes	Resistant to *P. cinnamomi*	(Vannini *et al*., 2001)

2.2 Molecular Identification of Chestnut.

In Mediterranean countries, identifying chestnut cultivars and evaluating genetic resources are significant, as in Spain (Ramos-Cabrer & Pereira-Lorenzo, 2005), Portugal (Costa *et al.*, 2005; Goulao *et al.*, 2001) and Italy (Boccacci *et al.*, 2004). Several methods have been used to characterize distinct chestnut varieties. In the past few decades, traditional chestnut varieties have been characterized according to their morphology, ripening period, and geographical origin (Gobbin *et al.*, 2007). More recently, the development of DNA markers has made it possible to directly analyze genotypes, allowing for the identification of different cultivars of chestnuts (Martin *et al.*, 2009). **Table 3** outlines in descending order the molecular methods of the identifications that were used to characterize chestnut diversity until the development of the more recent microsatellite markers (SSRs).

Table 3. Various molecular methods are used in the identification of chestnut genetic variability.****---** Represents the best markers in the study.

Method of Identification	**Markers**	**References**
The first markers used to describe the genetic diversity of *C. sativa* were isozymes.	Dia (diaphorase, E.C. 1.6.4.3), Est (esterase, E.C.3.1.1.3), Idh (isocitric dehydrogenase, E.C.1.1.1.42), Lap (leucine aminopeptidase, E.C.3.4.11.1), **Pgi (phosphoglucose isomerase E.C.5.3.19)**,**	(Fineschi *et al.*, 1994)
	Isocitrate dehydrogenase (Idh; EC 1.1.1.42), phosphoglucoisomerase (Pgi; EC 5.3.1.9), phosphoglucomutase **(Pgm; EC 2.7.5.1.)**,** malate dehydrogenase (Mdh; EC 1.1.1.37), and shikimate dehydrogenase (Skdh; EC 1.1.1.25).	(Ramos-Cabrer & Pereira-Lorenzo, 2005)

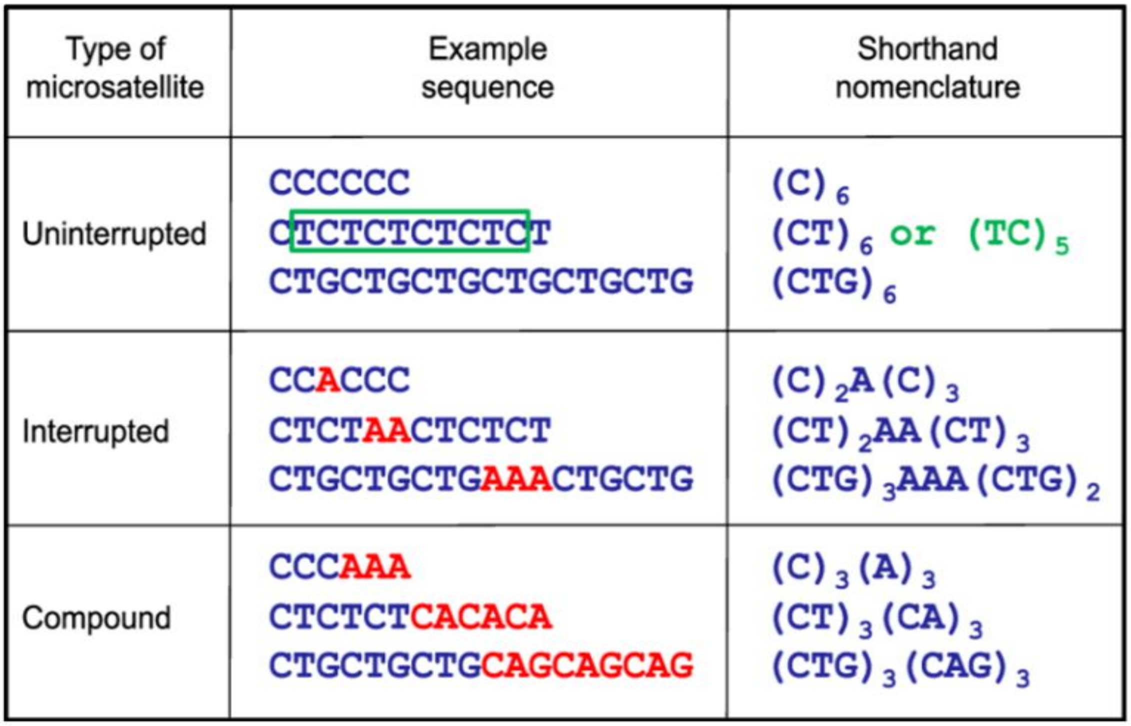
Table 3. Continued

Method of Identification	**Markers**	**References**
The second was random amplified polymorphic DNAs (RAPDs)	**Ul****, U2, **U3**, ** U4, U5, U6, U7, U8, U9, U10, U11, U12, U13, U14, U15, U16, U17, U18**, U19**** OPA01, OPA02, OPA03, OPA05, OPA07, OPA09, **OPA10**, OPA13****, OPA15, OPE01, OPE03, OPE04, **OPE06**, OPE07****, OPE08, OPE14, OPE15, OPE16, OPE19, OPE20. OPA (02, 04, 07, 10, and 15), OPB (08), OPD (20), OPE (**01**,** 04, 16, and 19), and OPX (17)	(Galderisi *et al*., 1998) (Carvalho *et al*., 2004) (Casasoli *et al*., 2001)
ISSR	HVH(CA)7, HVH(TG)7, VHV(GT)7, DBD(AC)7, (CA)8R, (GA)8YG, (AG)8YT, Failed-((AT)8YC, (AG)8YC, (GA)8YC, (GT)8YC (AGC)4YR and (TCC)5RY) UBC 810, **834**,** 836, **841****, and 890	(Goulao *et al*., 2001) (Abdelhamid *et al*., 2014)
Single Nucleotide Polymorphism (SNP)	A3079, A5096, A7075, A8045, A9081, B0033, B0042, B0081, B1077, B3127, C0114, C1115, C3057, C5126, D3098, D3104, E2081, F0116, F1081, F3045, G1038, G3081, G3111, G4120, G5075, G5135, H2081, H2132, H4081, I0081, J1105, J1108, K0044, K4081, L1081, L1117, L4081, L4081, and **(A4081, A8095, A8121, B2081, B2082, B5060, C1083, C2122, C4088, C4092, C7034,C7035, C7040, C9098, E0104, E1081, E1091, E2115, F0090, F0115, F3081, G0115, G4023, G5083, H0106, I2081, J0109, J2081, K0049, K0081, K1126, L0115, L2056)****	(Nunziata *et al*., 2020)
Expressed sequence tags (EST)	FIR015, FIR030, GOT014, POR026, POR042, WAG004, WAG005, WAG011, WAG017.	(Martín *et al*., 2010 and 2017)

The methodologies employed in **Table 3** failed to yield precise outcomes in discerning the genetic diversity among chestnut specimens. However, in the last twenty years, microsatellite markers (SSRs) have been primarily used to estimate population genetic diversity accurately (Buck *et al*., 2003; Marinoni *et al*., 2003; Pereira-Lorenzo *et al*., 2010). Nonetheless, the primary and dependable tool is microsatellite markers, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), a widely embraced method for evaluating genetic diversity in numerous fruit species (Bini *et al*., 2023).

2.2.1 Microsatellite Markers (SSR) as Genetic Barcodes

Microsatellites are sequences with one or a few bases tandemly repeated for varying numbers of times (Maia *et al*., 2008). Microsatellites arose about 25 years ago, (Beier *et al*., 2017) and remain a commonly used genetic marker system in plant genetics and breeding (Hu *et al*., 2020) and forensics (Butler, 2005). They are essential for plant varietal identification, population genetics, phylogenetic studies, marker-assisted selection, segregation analysis, population structure study, and evaluation of genetic links between individuals (Gao *et al*., 2013). SSRs can be found in different regions of genes: coding sequences, untranslated sequences (5' -UTR and 3' -UTR), and introns, where the expansions and contractions can lead to gene gain or loss of function (Maia *et al*., 2008). These molecular techniques allow for exploring a plant species' genetic makeup by detecting distinct genetic patterns associated with each genotype and analyzing specific DNA segments called molecular markers. Identifying differences (polymorphisms) within the sequence of DNA nucleotides establishes these molecular markers (Jiang, 2013). They detect polymorphisms within repeated DNA sequences by employing specialized primers that match the regions adjacent to the microsatellites. These primers are especially valuable as they unveil differences in these repetitive DNA sequences through their binding to the sequences surrounding the microsatellites (Amiteye, 2021). These repetitive sequences can range from 1 to 6 bases, resulting in mono-nucleotide, di-nucleotide, tri-nucleotide, and tetra-nucleotide microsatellites (Silvanini *et al*., 2011). Microsatellites consist of uninterrupted, interrupted, and compound repeats of nucleotides (**Figure 2)** (Brazda *et al*., 2020).



Type of microsatellite	Example sequence	Shorthand nomenclature
Uninterrupted	CCCCCC CTCTCTCTCTCT CTGCTGCTGCTGCTGCTG	$(C)_6$ $(CT)_6$ or $(TC)_5$ $(CTG)_6$
Interrupted	CCACCC CTCTAACTCTCT CTGCTGCTGAAACTGCTG	$(C)_2A(C)_3$ $(CT)_2AA(CT)_3$ $(CTG)_3AAA(CTG)_2$
Compound	CCCAAA CTCTCTCACACA CTGCTGCTGCAGCAGCAG	$(C)_3(A)_3$ $(CT)_3(CA)_3$ $(CTG)_3(CAG)_3$

Figure 2. Nomenclature: Variations Of Microsatellite Repeat. Adapted from Brazda *et al*. (2020)

Adjusting the sequence frame in (**Figure 2**) brings attention to redundancy within each repeat, encompassing various sequences. The green box specifically highlights (TC) repeats within (CT) repeats. While classic examples of microsatellites involve uninterrupted repeats of the same sequence, the term 'interrupted' (or 'imperfect') is applied when one or more bases disrupt the repeat array, as indicated in red bases. Genomes often contain neighboring repeats of two types, called 'compound' microsatellites. (Brazda *et al*., 2020).

In the last 20 years, microsatellite markers (SSRs) have been suitable for genetic variability detection (Pereira-Lorenzo *et al*., 2016; Rasoarahona *et al*., 2023). Hybrid identification is crucial in breeding, ensuring genetic purity and enhancing crop improvement programs. SSR markers provide an efficient molecular approach for distinguishing parental lines from hybrids, facilitating chestnut breeding programs and genetic conservation strategies (Bai *et al*., 2023)**.** Alessandri *et al*. (2022) monitored gene flow between wild and cultivated chestnut trees, revealing the significant role of hybridization in shaping genetic diversity through SSR markers. (Tumpa *et al*., 2022) also confirmed the high effectiveness of both genomic SSR (gSSR) and expressed sequence tag SSR (EST-SSR) markers in describing the genetic diversity of sweet chestnut, highlighting their utility in hybrid identification**.** A recent study (Kara *et al*., 2023) in Turkey used drought-associated SSR and EST-SSR markers to evaluate tolerance and analyze the genetic relationships among economically important chestnut cultivars. The study revealed 100% polymorphism among the chestnut cultivars, demonstrating the high level of genetic variation captured by microsatellite markers. Another comprehensive analysis involving 216 chestnut accessions, from Japanese, Chinese, European, and American species and Japanese–Chinese hybrids, utilized SSR markers to assess genetic diversity and relationships. The study identified 21 synonym groups and 189 distinct genotypes, demonstrating the effectiveness of SSR markers in distinguishing between chestnut species and their hybrids (Nishio *et al*., 2011). More recently, SSRs have been used in maize genomic and evolutionary breeding (Zhao *et al*., 2023), the validation of candidate genes in sugarcane (Divakar *et al*., 2023), olive gene validation (Arbeiter *et al*., 2017; Yadav *et al*., 2021), and *Quercus* genetic diversity (Thunder *et al*., 2022). Microsatellites within genes of plants have diverse functions (**Figure 3)**, including regulation of gene expression, involvement in plant metabolism, and implications for genetic stability and disease (Li *et al*., 2004).

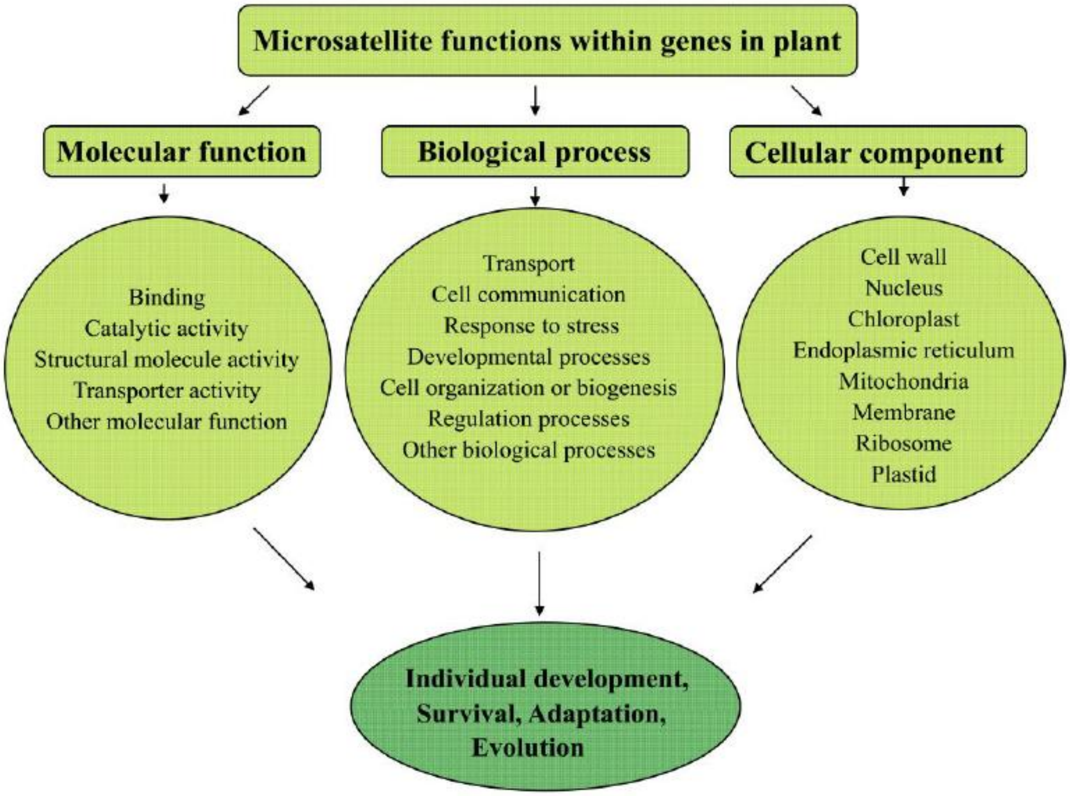


Figure 3. Functions of Microsatellites within the Plant Genome, adopted from Gao *et al*. (2013).

2.2.2 The commonly utilized SSR markers in *Castanea*

The aims of this research underscore the need to outline frequently utilized microsatellites within the genome of the chestnut and the *Fagaceae* family. This investigation encompasses the following sets: i) CsCAT, consisting of di-nucleotide repeats (Botta, 2001; Marinoni *et al*., 2003), ii) EMCs, characterized by tri-nucleotide repetitions (Buck *et al*., 2003); iii) OCI, OAL, RIC, and CIO, were sourced from the *Castanea* species (Gobbin *et al*., 2007), and iv) SSRs acquired from the *Quercus* gene, such as QpZAG and QrZAG (Barreneche *et al*., 2004).

2.3 The Characterization of the European Chestnut Population Using SSRs

Apart from the European breeding programs, there have been fruitful research endeavors of numerous European scholars dedicating their efforts to investigating chestnut diseases, enhancing production methods, and advancing breeding techniques. I provide an overview of some notable European research initiatives. As seen in (**Table 4**)

Table 4. Various SSR markers currently utilized in the chestnut tree population and cultivar identification. ****---** Represents the best marker in the study

Materials	**SSR Markers Used**	**Results Obtained**	**Reference**
A total of 787 accessions from Spain, Portugal, and France.	CsCAT3, CsCAT14, CsCAT16, CsCAT41, EMCs2, EMCs14, EMCs15, QpZAG36, QpZAG110, QrZAG96.	Their research unveiled two main origins of genetic variation within chestnut tree populations in the Iberian Peninsula: one in the northern region and the other in the central area.	(Pereira-Lorenzo *et al*., 2010).
593 samples from Spain and Portugal.	**CsCAT3**,** CsCAT14, CsCAT16, CsCAT41, EMCs2, EMCs14, EMCs15, QpZAG36, QpZAG110, QrZAG96.	They identified ten primary cultivar groups rooted in specific regions, shedding light on the regional genetic connections.	(Pereira-Lorenzo *et al*., 2011).
Analyzed 100 grafted chestnut trees corresponding to 34 traditional names in Spain	CsCAT1, CsCAT2, CsCAT3, CsCAT16, CsCAT6, **EMCs25**, EMCs38****	Found 61 alleles across seven microsatellites, showing regional variations in genetic diversity, with Huelva and Malaga having distinct genetic patterns.	(Martin *et al*., 2009).
Sixteen chestnut populations covering the distribution range of the species in Spain	CsCAT1, CsCAT2, CsCAT3, CsCAT6, CsCAT16**, EMCs25**,** EMCs38	They identified three genetic clusters in Spanish chestnut accessions representing different geographical regions.	(Martín *et al*., 2012).
A similar study compared chestnut and Wild chestnut populations from Spain, Greece, Turkey, and Italy	CsCAT1, CsCAT3, CsCAT6, CsCAT16, EMCs25 , EMCs38.	Demonstrated genetic divergence between the western (Italian and Spanish) and the eastern (Greek and Turkish) populations.	(Mattioni *et al*., 2013).
Analyzed 16 chestnut populations from the forest of Spain.	CsCAT1, CsCAT2, **CsCAT3**,** CsCAT6, CsCAT16, EMCs25, EMCs38.	They identified the El Bierzo region as a hot spot with high genetic variability, entirely separated from other European populations.	(Martín *et al*., 2012) Confirmed by (Quintana *et al*., 2015)

Table 4. Continued

Materials	**SSR Markers Used**	**Results Obtained**	**Reference**
Characterized 1,608 wild chestnut trees in Europe.	CsCAT1, CsCAT2, CsCAT3, CsCAT6, CsCAT14, CsCAT16, EMCs25 and EMCs38. And (**FIR030**, GOT014**,** PIE227, PIE228, PIE233, PIE260, POR009, POR026, and WAG004) from EST expressed *Quercus robur* and *Quercus petraea.*	Identifying three main gene pools and a structural barrier separating eastern from central and western European populations.	(Martín *et al*., 2017)
They used 327 sweet chestnut trees from Central Europe and the western part of the Balkan Peninsula.	CsCAT01**, CsCAT02**, CsCAT03**,** CsCAT04, CsCAT06, CsCAT14, CsCAT16, CsCAT17, EMCs15 , OAL	They hypothesized that genetic distribution was mainly due to natural diffusion. Thus, two groups could have originated from glacial refugia in Central Europe and the southern parts of the Balkan Peninsula. In contrast, the group's south could have originated via migrations from Asia Minor.	(Poljak *et al*., 2017)
They analyzed 271 accessions from Spain, Italy, and France.	CsCAT14, CsCAT2, CsCAT41B, QrZAG4, CIO EMCs25	They identified two significant clusters (Spanish and Italian) and an additional genetic substructure with highly diversified Italian clusters. These results confirm findings from previous research by Mattioni *et al*. (2017), which showed a division between cultivars from northwest Spain and those from central and southern Spain and southern Italy.	(Pereira-Lorenzo *et al*., 2017)
Selected 102 giant trees: 84 from Spain, 16 from Italy, and 2 from Portugal.	A set of 24 SSR markers, developed for *C. sativa* (Buck *et al*., 2003; Gobbin *et al*., 2007; Marinoni *et al*., 2003a) for *Quercus petraea* and *Quercus robur*.	They conducted genetic analysis on ancient giant chestnuts in Italy and the Iberian Peninsula, finding no distinct genetic structure between wild and cultivated trees.	(Pereira-Lorenzo *et al*., 2019).

Table 4. Continued

Materials	**SSR Markers Used**	**Results Obtained**	**Reference**
Over 600 *C. sativa* trees and coppice stools in Britain and Ireland.	(CsCAT-1, **-2**, -3**, -6**,** -14, -16; and EMCs-25, **-38**)**	Analyzed sweet chestnut trees in England and Wales and concluded genetic connections to France, Spain, Portugal, Italy, and Romania, essential refugia during the Last Glacial Maximum.	(Jarman *et al*., 2019).
Finally, in a recent study, 693 trees were genotyped at 24 SSRs and 1401 trees at 13 SSRs.	CsCAT41, EMCs15, **EMCs2**,** QpZAG36, QrZAG4, QrZAG96, EMCs14, CsCAT14, CsCAT6**, CsCAT1, CsCAT17, CIO, OCI, OCI, OCI, and OAL	They identified two main genetic clusters in Switzerland: one related to the European chestnut genetic dataset and another representing a specific genetic group in Switzerland, possibly originating from medieval times.	(Bouffartigue *et al*., 2020).

3.0 Materials and Methods

3.1 Laboratory of Study

All chestnut leaf samples were collected across Portugal, clearly labeled, and transported in sealed envelopes to the Molecular Biology Laboratory at the Polytechnic Institute of Bragança. There, I performed PCR amplification, gel electrophoresis, and DNA band visualization. The amplified products were then sent to the University of Salamanca for sequencing.

3.2 Plant Materials

A total of 35 samples were collected in summer 2023, comprising hybrids and traditional Portuguese chestnut varieties, within the municipality of Bragança, Portugal. These included 12 samples from St^{a} Comba, 10 from Vila-Boa de Serapicos, and 13 from Deifil Green Biotechnology, a research institute focused on *in vitro* propagation of plant species with limited natural reproductive capacity. **Table 5**. Provides a detailed description of each accession. Three samples, identified as CA90 hybrids (*C. sativa* × *C. crenata*), served as positive controls and exhibited distinct genetic profiles within the Sta Comba group. Following collection, the leaves were cleaned with 70% ethanol and stored at -20°C until DNA extraction.

Table 5. Details of the 35 chestnut samples, including laboratory-designated codes. M = mark; P = place.

Lab code	Samples	GPS / Field Tag	Sampling location
A	CA90 positive control	M111111,2 P222973,9	St^{a} Comba
B	CA90 positive control	M110916,0 P2225509	St^{a} Comba
C	CA90 positive control	M110932,6 P222509,7	St^{a} Comba
D	*C. sativa*	TD	Vila-Boa de Serapicos
1	CA90	T1	Vila-Boa de Serapicos
2	CA90	T2	Vila-Boa de Serapicos
3	CA90	T3	Vila-Boa de Serapicos
4	CA90	T4	Vila-Boa de Serapicos
5	CA90	T5	Vila-Boa de Serapicos
6	CA90	T6	Vila-Boa de Serapicos
7	CA90	T7	Vila-Boa de Serapicos
8	CA90	T8	Vila-Boa de Serapicos
9	CA90	T9	Vila-Boa de Serapicos

Table 5 continued

Lab code	**Samples**	**GPS / Field Tag**	**Sampling location**
10	*C. mollissima* 60907	T10	Deifil Green Biotechnology
11	*C. mollissima* E2604	T11	Deifil Green Biotechnology
12	*C. mollissima* Y0204	T12	Deifil Green Biotechnology
13	*C. mollissima* Z1408	T13	Deifil Green Biotechnology
14	CA90	T14	Deifil Green Biotechnology
15	Martaínha 2	T15	Deifil Green Biotechnology
16	Bouche de Betizac 1	T16	Deifil Green Biotechnology
17	Bouche de Betizac 2	T17	Deifil Green Biotechnology
18	Martaínha 1	T18	Deifil Green Biotechnology
19	Cota	T19	Deifil Green Biotechnology
20	Judia	T20	Deifil Green Biotechnology
21	Marsol	T21	Deifil Green Biotechnology
22	Précoce Migoule	T22	Deifil Green Biotechnology
23	Putative CA90	M111478,8 P223081,2	Stª Comba
24	Putative CA90	M11609,9 P222967,4	Stª Comba
25	Putative CA90	M111649,4 P222888,7	Stª Comba
26	Putative CA90	M111494,7 P222815,0	Stª Comba
27	Putative CA90	M111497,6 P223077,5	Stª Comba
28	Putative CA90	M111191,6 P222934,5	Stª Comba
29	Putative CA90	M111512,1 P222808,6	Stª Comba
30	Putative CA90	M111486,4 P222934,5	Stª Comba
31	Putative CA90	M111477,6 P222821,3	Stª Comba

The plants' ages ranged from 7 months to 7 years. The 12 Freguesia de Stª Comba samples were GPS-mapped, while the remaining 19 samples were manually collected, and trees labeled as T1 to T22 for future identification and morphological investigations.

3.3 DNA Extraction.

The research utilized silica-based membrane technology (Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit, Catalog number K0792) for total genomic extraction following the manufacturer's protocol. The kit was particularly important as it reduced the necessity for costly resins, toxic phenol-chloroform extraction processes, and labor-intensive alcohol precipitation procedures.

3.4 Molecular Marker Analysis

3.4.1 SSR Primer Selection and Amplification.

In order to define a set of microsatellite sequences useful for the fingerprinting and mapping of the chestnut plants, nine (9) SSR primer pairs were selected and modified from the studies of (Santos *et al*., 2015), an already developed 43 simple sequence repeat (SSR) markers from expressed sequence tags (ESTs). The selection criterion was based on null allelic nature and low observed heterozygosity (Ho) values (0.000 to 0.552) of primer pairs, aiming to target specific genotypes in disease-resistant hybrids and improve selective breeding outcomes. Out of the nine(9) primer pairs selected and modified from that study, loci CcPT 0002 and CcPT 0003 were maintained but renamed CP2 and CP3 according to this study. The modifications affected CP4 to CP11, including adding one or two nucleotides to the 3’ end of the forward and reverse primers to enable their binding specificity. As seen in (**Table 6**).

Table 6. Primer sequences used for SSR analysis and their characteristics.

No	Primer code	Forward (5'-3')	Reverse (5'-3')	Annealing Tm (℃)
1.	CP2	AGTTCTCCACGAGGCTCAAA	TCCAAGCTGGAGAATCATCA	55.3
2.	CP3	GGTGCCCAGATTTACGAGAA	ATCGCTTGGAGTCACAGCTT	57.3
3.	CP4	GCTGCTTCACAACCTTCCTC	GCAAGAGATTCCCTTTGCTG	57.3
4.	CP5	ACACATGGGGGTGTGAACTT	TTATGGGAAACGGCATCTTC	55.3
5.	CP6	CCTGTGAGGCTAAGAGAGCG	ACCACGTCGGTGCTTCTAGT	59.4
6.	CP8	TCGTCCCCTTCTTCATCATC	ATATGGCCAAAAACCCATCA	53.2
7.	CP9	TTCCACCCAATTGTTACCAC	GATGAAGAAGGGGACGA	55.3
8.	CP10	ATCCATGAGTGAAAGCCACC	TGGAACAAGAAGCCTCGATT	55.6
9.	CP11	TCATCCAAGAAGCCCTCAAC	TTCTGCCTCTTTTGTTGCCT	55.3

3.4.2 Primer Dilution

All the primers were obtained from Isogen Life Sciences, and following the provider's instructions, we added ddH_20 to obtain (100 pmol/µL) to each stock primer. A working solution was then prepared from the concentrated primers by diluting the primer to a ddH20 (5:95) ratio.

3.4.3 Simple Sequence Repeat Amplification

Target genes were amplified for the study using a BIO-RAD T100TM thermocycler, and PCR amplification reactions were set up using BIORON GmbH Reactives in concentrations of 0.2 mM dNTPs, 1X reaction buffer, 0.8X KCl, 1.5 mM $MgCl_2$, 2.5 units DFS-DNA Taq polymerase, 2.5 µM each of Forward and Reverse primers, 4 µLgenomic DNA (20 ng/µL), 0.6 mg/mL BSA, and 29.25 µL deionized water were used in a 50µL PCR mix for each sample. DNA amplification reactions were performed using thermocycling conditions of initial denaturation, final denaturation, annealing, and extension, respectively: 94 ◦C-2 min; 94 ◦C-10 s, 57 ◦C-20 s, and 72 ◦C-30 s for 36 cycles and 72 ◦C-5 min. The thermocycler's internal temperature was adjusted to 4°C to maintain the amplified DNA in an optimal state, thereby preventing any denaturation of the DNA strands. A summary of the primers used (**Table 6**) with little program modifications from (Botta, 2001).

3.4.3.1 Determining PCR Conditions

The above-described (**3.4.3**) reaction conditions were standardized in final concentrations and volumes shown in **Table 7** using the relationship. $CiVi = CfVf$ But; $Vi = \frac{CfVf}{Ci}$ where *Ci* = initial concentration of the re-actives, *Vi* = initial volume of reaction, *Cf* = final concentration, and *Vf* = final volume of reaction = 50µL. All the initial volumes of Re-actives were summed and deducted from the final volume (*Vf*), and the results represented the quantity of ddH_2O.

Table 7. Calculations leading to reactive initial volumes.

Re-actives	**Initial concentration** ***(Ci)***	**Final concentration** ***(Cf)***	**Initial volume** $Vi = \frac{CfVf}{Ci}$ **(µL)**
dNTPs,	10 mM	0.2 mM	1
Reaction buffer	10 X	1 X	5
Kcl	10 X	0.8 X	4
$Mgcl_2$	100 mM	1.5 mM	0.75
DFS-DNA polymerase	5 units/ µL	2.5 units	0.5
Forward primer	10 µM	2.5 µM	1.25
Reverse primer	10 µM	2.5 µM	1.25

Table 7. continued

Genomic DNA	4 µg/µL	0.32 µg/ul	4
BSA	10 mg/mL	0.6 mg/ml	3
ddH_2O	-	-	29.25

3.5 Agarose Gel Electrophoresis and Band Visualization.

A laboratory technique used for separating and analyzing macromolecules, such as DNA, RNA, and proteins, based on their size and charge. It is a fundamental tool in molecular biology and biochemistry and is commonly employed to separate DNA fragments and analyze RNA and Proteins. Agarose gel electrophoresis relies on the electric field-induced separation of negatively charged nucleic acids. This separation occurs within the agarose gel matrix, where smaller molecules move swiftly and migrate a greater distance than the more prominent bands. Agarose gel enables estimating the molecular weight of DNA fragments post-digestion with restriction enzymes and DNA analysis following PCR amplification (Lee *et al.*, 2012).

3.5.1 Agarose gel Preparation (2%)

All the plates used for the electrophoresis were cleaned with warm water, rinsed with ddH_2O, and dried with 100% ethanol wipes. The quantity of agarose needed was based on the volume of the gel to be prepared.

Fr of gel.$Agarose(grams) = \frac{2}{100} * Total\ volume\ of\ gel\ (in\ millilitres)\ tray$— Eqn (1)

Afterward, 1 g of agarose was added to 100 mL of TBE buffer in a conical flask. The resulting solution was mixed and swirled gently to ensure the agarose powder was well dispersed in the TBE buffer. Subsequently, the agarose-TBE mixture was heated in a microwave until the agarose completely dissolved. The resulting mixture was stained with 0.02 µL ethidium bromide and poured into an already-leveled gel-casting tray with a comb inserted at one end to create wells for sample loading. The agarose gel was allowed to solidify for approximately 10-15 minutes at room temperature, and the combs were gently removed.

3.5.2 Gel Loading and Band Visualization

The gel was placed into the gel electrophoresis chamber and covered with 0.5X TBE buffer, ensuring the gel wells were placed at the negative pole to successfully run the DNA toward the positive pole. After amplification, 6 μL of each PCR product, consisting of 5 μL DNA and 1 μL loading dye, was loaded onto the already prepared 1.5% agarose gel, incubated for 55 min in 0.5 × TBE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), and run at voltages between 60 and 80 depending on the size of the gel casting tray. PCR products were labeled with ethidium bromide and visualized using the Bio-Rad gel DocTM Ez Imager (Image Lab application 5.2.1). Proper safety precautions were followed when treating ethidium bromide during and after visualization, as it is hazardous. According to the institution's guidelines, agarose gels and buffers were disposed of appropriately.

3.6. Sequencing of PCR Products

Besides visualizing the primer pairs' gel electrophoresis results, 27 DNA amplicons were randomly chosen from combinations of nine primers and chestnut samples. For each primer, a minimum of three distinct bands from different tree samples were selected to undergo sequencing and identify SSR motifs, ensuring reliability and reproducibility. The selected bands were purified using the DNA Clean & Concentrator kit (ZYMO Research, Irvine, CA, USA) to eliminate potential inhibitors and contaminants that could interfere with the sequencing process.

Since sample preparation before sequencing is a crucial step in the genomics workflow, several precautions were taken during sample preparation, including using appropriate methods to ensure the integrity of the biological material (Roy *et al*., 2018). To obtain high-quality DNA for sequencing, we performed a sample quality assessment using a nanodrop to measure DNA concentration and purity, measuring nucleic acid absorbance at 260 nm (A260), protein contamination absorbance at 280 nm (A280), and organic compounds like phenol or chaotropic salts at A280.

Afterward, the concentrations were calculated using the NanoDrop provided direct concentration readings based on the absorbance at 260 nm using the Beer-Lambert Law.

$$DNA\ concentration\left(\frac{\mu\text{g}}{\text{mL}}\right) = \text{A260} * \text{Dilution Factor} * 50\text{—Eqn (2)}$$

This helped identify potential issues early, ensuring that only high-quality samples were sequenced to obtain consistent PCR results. The purity assessment was computed using the A260/A280 ratio, considering that the ideal ratio for pure DNA is between 1.8 and 2.0. Lower values (e.g., 1.5) may indicate protein contamination and A260/A230 Ratios, where the ideal ratio is typically between 2.0 and 2.2. Lower values can indicate contamination by organic solvents or salts (Desjardins *et al*., 2010).

The samples were then sent to the University of Salamanca for Sanger sequencing. To help understand the genotypes of the chestnut varieties and their potential traits, including mutational analysis, gene expression, genetic mapping, functional genomics, comparative genomics, molecular breeding, and pathogen detection.

3.7. Bioinformatic Analysis

The raw DNA sequence reads generated by Sanger sequencing were first subjected to quality control and trimming using BioEdit version 7.2.5 to remove low-quality regions and retain high-confidence sequences. Subsequently, the processed sequences were aligned using ClustalW version 2.1 to identify genetic variations and ensure accurate comparative analysis. Homology searches were performed using BLASTn (NCBI) against the GenBank database to identify similar sequences and confirm species identity. Simple sequence repeats (SSRs) were identified using MISA-web, applying parameters that targeted motif lengths ranging from 1 to 6 base pairs, with a minimum of five repeat units and the detection of compound microsatellites. To infer phylogenetic relationships, a tree was constructed in MEGA11 (Molecular Evolutionary Genetics Analysis software, version 11) using the Maximum Likelihood (ML) method based on the Tamura-Nei model. Clade support was evaluated using bootstrap analysis with 100 replicates. Clades with bootstrap values greater than 70% were considered strongly supported, those between 50% and 70% were moderately supported, and values below 50% indicated weak support.

4.0 Results

4.1 Effects on Primer Locus Modification

The primer modifications influenced the outcome of this study, as the altered primers consistently amplified different genomic regions, potentially representing new variants or loci. These were designated as CP2, CP3, CP4, CP5, CP6, CP8, CP9, CP10, and CP11 when compared to the study (Santos *et al*., 2015) from which the original primers were selected and modified, variations were observed in SSR motifs, allele numbers, and allele sizes, except for CP2, which remained unmodified. The markers CP2 and CP11 had the lowest number of alleles (one), while CP3 and CP6 shared the highest number (three). Regarding motif types, CP3, CP6, CP8, and CP10 exhibited compound motifs, with CP3 showing the highest. See. (**Table 8**).

Table 8. Comparison of results for the modified SSR primer loci: number of alleles (Na), *-No motifs found

LOCI	Sample	Na	Motif	Reference	Loci	Na	Motif	Reference
CP2	Marsol	1	(GTG)$_9$	This study	CcPT 0002	1	(GTG)$_9$	(Santos *et al*., 2015).
CP2	CA90		*	This study				
CP2	*C. sativa*		*	This study				
CP3	Marsol	3	(ATC)$_6$	This study	CcPT 0003	7	(ATC)$_8$	(Santos *et al*., 2015).
CP3	*C. sativa*		(ATC)$_6$	This study				
CP3	*C. mollissima* . Z1408		(ATC)$_6$	This study				
CP3	CA90		(TGA)$_5$tcatgatgaccacaaggattgaagttagtcacagcatctcggccaccaacgcgttgggccgcgatgtcgtacgcttttgca(TCT)5	This study				
CP3	Martainha		(TGA)$_8$tcatcagtaccacccggcgggttgaagttcacaacatcttctccaccacaaaagcgggggggccatgatgtcatattttgttg(CTT)5	This study				
CP4	CA90	2	(GA)$_{10}$	This study	CcPT 0004	6	(CT)$_{10}$	(Santos *et al*., 2015).
CP4	CA90		(GA)$_{11}$	This study				

Table 8. continued

LOCI	**Sample**	**Na**	**Motif**	**Reference**	**Loci**	**Na**	**Motif**	**Reference**	Sample
CP5	CA90	3	$(CT)_{8}$	This study	CcPT 0005	7	$(CT)_{1}$	(Santos *et al*., 2015).	
CP5	*C. sativa*		$(CT)_{12}$	This study					
CP5	COTA		$(AG)_{7}$	This study					
CP6	Marsol	2	$(GAA)_{5}$	This study	CcPT 0006	4	(TTC)	(Santos *et al*., 2015).	
CP6	CA90		$(GAA)_{5}$ gaggaagaagaac$(A)_{12}$	This study					
CP6	CA90		*	This study					
CP6	*C. sativa*		*	This study					
CP8	*C. sativa*	2	$(CTCAGA)_{5}$ gtacaacaaccgacagca $(AAG)_{11}$	This study	CcPT 0008	5	$(TCT)_{1}$	(Santos *et al*., 2015).	
CP8	CA90		$(A)_{12}$ $(TCT)_{8}$	This study					
CP9	CA90	2	$(AG)_{10}$	This study	CcPT 0009	8	(TC)	(Santos *et al*., 2015)	
CP9	*C. sativa*		$(TC)_{8}$	This study					
CP9	CA90		*	This study					
CP10	CA90	2	$(CAC)_{6}$ $(AAG)_{5}$	This study	CcPT 0010	5	(GGT)	(Santos *et al*., 2015).	
CP10	*C. sativa*		$(CAC)_{6}$ $(AAG)_{5}$	This study					
CP10	CA90		$(GGT)_{5}$ gggggagccttc $(TCT)_{6}$	This study					
CP10	CA90		$(GGT)_{5}$ gggggagccttc $(TCT)_{6}$	This study					
CP11	CA90	1	$(GGT)_{7}$	This study	CcPT 0011	5	(CAC)	(Santos *et al*., 2015).	

4.2 Locus Amplifications and Band Patterns

To achieve the research objectives, we employed agarose gel electrophoresis to visualize PCR products and evaluate the effectiveness of various primer pairs, particularly in detecting null alleles. This technique allowed the identification of primers capable of producing distinguishable banding patterns between hybrid and non-hybrid chestnut plants. In this study, all primer sets gave good amplification across all loci in CA90 samples, but not in other varieties due to their null allelic nature.

Although the gel images showed monomorphic bands, sequencing revealed high levels of polymorphism, except for primer CP8, which displayed polymorphic bands even on the agarose gel. However, accurate detection of SSR primer polymorphism typically requires high-resolution gels or capillary electrophoresis. Due to equipment limitations, this study relied solely on agarose gel electrophoresis, representing a constraint. Therefore, we recommend further validation using high-resolution electrophoretic techniques.

A heatmap (**Figure 4**) was generated from the visual banding patterns observed from all the gel images (**See Appendix Figures S2–S3**). This heatmap served as a profile for selecting the most informative primer pairs capable of clearly distinguishing hybrids from non-hybrid varieties.

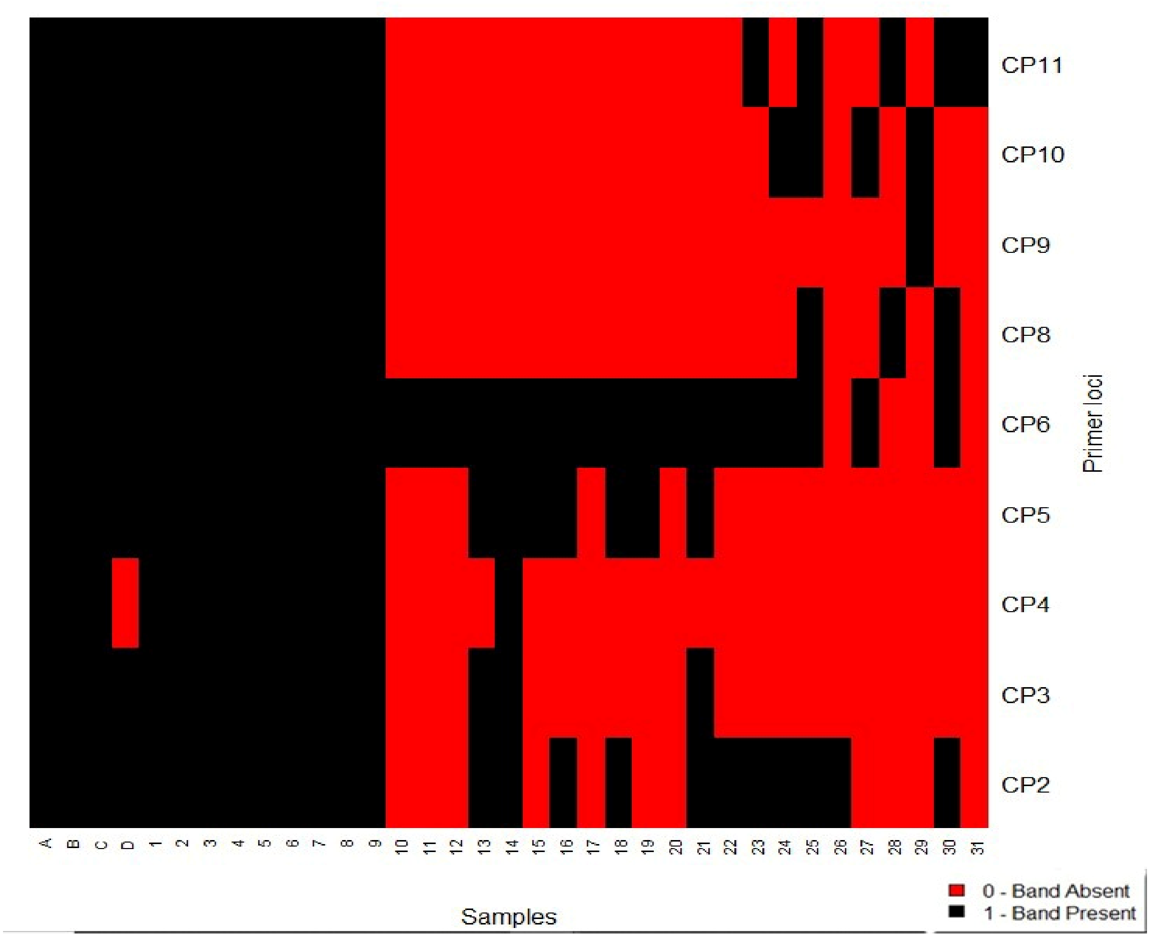


Figure 4. A Heatmap of band patterns from gel images. (ABC)—CA90 controls, (D)—*C. sativa* ,(1–9)—CA90, (10)—*C. mollissima* 60907, (11)—*C. mollissima* E2604, (12)—*C. mollissima* Y0204, (13)—*C. mollissima* Z1408, (14)—CA90-deifil, (15)—Martainha 2, (16)—Bouche de Betizac 1, (17)—Bouche de Betizac 2, (18)—Martainha 1, (19)—Cota, (20)—Judia, (21)—Marsol, (22)—Precoce migoule, (23–31)—Putative CA90

4.3 Gel Image Of The Novel Locus CP4

The primer locus CP4 demonstrated strong monomorphism on gel, consistently showing bands in all three CA90 controls and all samples from Vila-Boa de Serapicos and the CA90 sample from Deifil Green Biotechnology. Notably, this primer did not show bands in any other hybrid variety or species, including *C. sativa*. This specificity suggests that CP4 (**Figure 5**) may be a reliable marker for distinguishing the CA90 genotype, making it a valuable tool for genetic identification and differentiation in these samples.

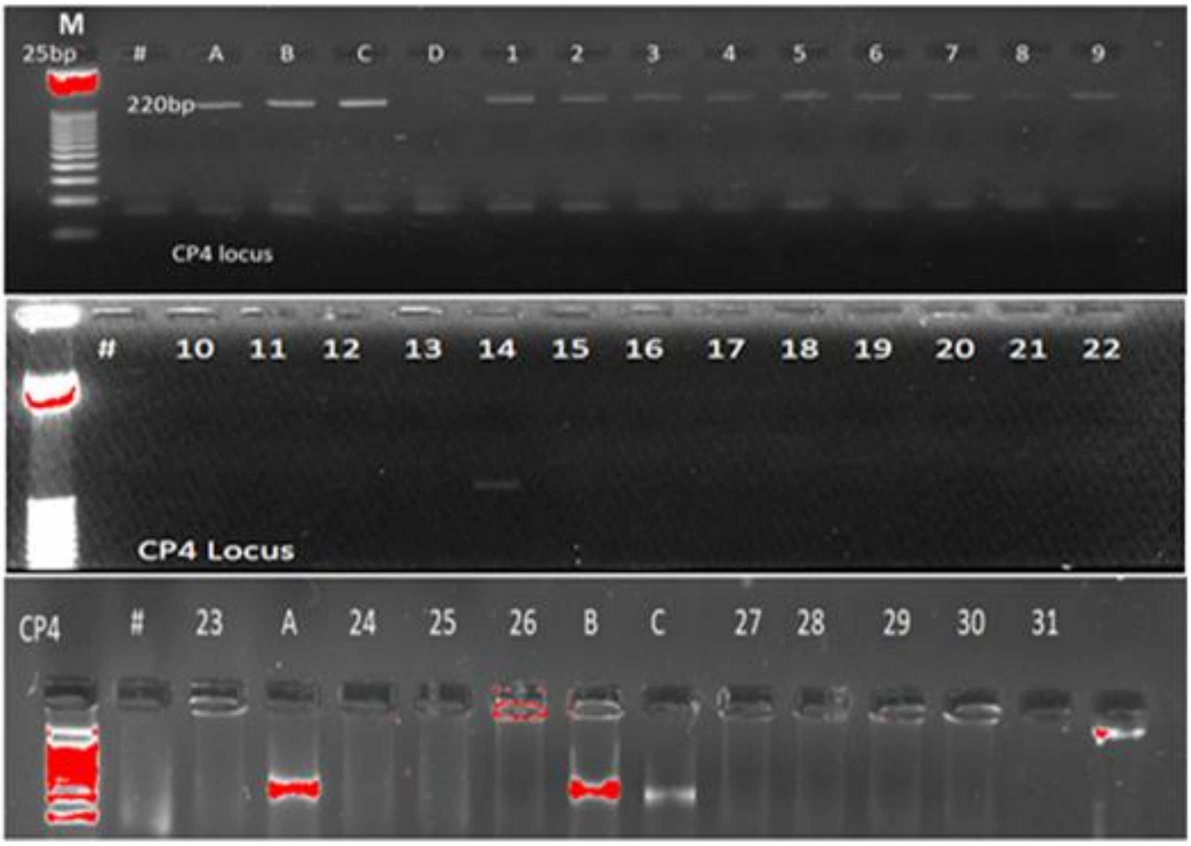


Figure 5. Gel Image of primer locus CP4: (M)-25bp ladder, (#)-H2O Control, (ABC)—CA90 controls, (D)—C. sativa,(1–9)—CA90, (10)—C. mollissima 60907, (11)—C. mollissima E2604, (12)—C. mollissima Y0204, (13)—C. mollissima Z1408, (14)—CA90-deifil, (15)—Martainha 2, (16)—Bouche de Betizac 1, (17)—Bouche de Betizac 2, (18)—Martainha 1, (19)—Cota, (20)—Judia, (21)—Marsol, (22)—Precoce migoule, (23–31)—Putative CA90 .

4.4 Sequence analysis

To validate the gel electrophoresis results, the raw DNA sequence reads were first assessed for quality using BioEdit (v7.2.5), then aligned with ClustalW, and finally analyzed using MISA-web following the protocol by Beier *et al*. (2017), for microsatellite detection (**Table 8**). The 27 sequences, comprising 8,899 base pairs, were examined.

From these, 31 SSRs were identified across 22 sequences, nine containing multiple SSRs, and six were involved in compound formations. SSR search parameters were set to include motif lengths of 1–6 base pairs, a minimum of 5 repeats, and no spacing for compound SSR detection. It was realized that Trinucleotide repeats were the most prevalent (67.74%), followed by dinucleotide (22.58%), mononucleotide (6.45%), and hexanucleotide (3.23%) types. Across the nine loci, 18 alleles were identified, with 1 to 3 alleles observed per locus among the 35 samples.

4.5 Sequences BLAST

The obtained sequences were processed manually, and the consensus sequence created through BioEdit v7.2.5 was compared with the GenBank NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/, accessed on 10 January 2024) using the BLASTn (nucleotide Basic Local Alignment Search Tool) algorithm. The sequences showing a nucleotide similarity greater than 95% were accepted and mostly belonged to *Quercus* (**Table 9**).

Table 9. BLAST Results of Sequences Showing Above 95% Similarities in two different rankings.

Primer loci	**Sample**	**Sequence (Ranking 1)**	**Accession number**	**Sequence (Ranking 2)**	**Accession number**
CP2	CA90 - C	PREDICTED: *Quercus lobata* UPF0496 protein 4-like mRNA (97.63%)	XM_031110133	*Castanea crenata* mRNA, microsatellite: PEB30, complete sequence (99.37%)	AB610059
CP2	*C. sativa* - D	PREDICTED: *Quercus lobata* UPF0496 protein 4-like mRNA (97.03%)	XM_031110133	*Castanea crenata* mRNA, microsatellite: PEB30, complete sequence (98.73%)	AB610059
CP2	Marsol-21	*Castanea crenata* mRNA, microsatellite: PEB30, complete sequence (97.91%)	AB610059	*Quercus variabilis* isolate HB chromosome 4b(96.44%)	CP129436
Cp4	CA90-C	PREDICTED: *Quercus* lobata linoleate 13S-lipoxygenase 3-1, chloroplastic-like (LOC115989795), mRNA (95.12%)	XM_031113671	PREDICTED: *Quercus robur* linoleate 13S-lipoxygenase 3-1, chloroplastic-like (LOC126724874), mRNA (93.54%)	XM_050429304

Table 9. continued

Primer loci	**Sample**	**Sequence (Ranking 1)**	**Accession number**	**Sequence (Ranking 2)**	**Accession number**
CP5	CA90-A	PREDICTED: *Quercus* lobata trihelix transcription factor GT-2-like (LOC115994903), mRNA (96.15%)	XM_0311192421	*Quercus dentata* isolate Qdleaves-2020 chromosome 6 (96.15%)	CP116449
CP5	*C. sativa* - D	*Quercus cerris* genome assembly, chromosome 10 (97.17%)	OY770015	*Quercus variabilis* isolate HB chromosome 6b (97.17%)	CP129438
CP6	*C. sativa* - D	PREDICTED: *Quercus suber* squalene monooxygenase-like (LOC111990232), Mrna(95.95%)	XM_024022019	*Quercus cerris genome assembly, chromosome 8* (95.50%)	OY770013
CP9	CA90- C	*Quercus cerris genome assembly, chromosome 2*(96.05%)	OY770007	*Quercus variabilis* isolate HB chromosome 4b(95.65%)	CP129448
CP9	*C. sativa* - D	*Quercus cerris* genome assembly, chromosome 2(96.52%)	OY770007	PREDICTED: *Quercus suber* WRKY transcription factor 22 (LOC111983699), mRNA(96.51%)	XM_024015355
CP9	CA90-2	PREDICTED: *Quercus suber* WRKY transcription factor 22 (LOC111983699), mRNA (96.76%)	XM_024015355	*Quercus cerris* genome assembly, chromosome 2 (96.76%)	OY770007
CP10	CA90-B	*Quercus variabilis* isolate HB chromosome 2a (95.96%)	CP129446	*Quercus variabilis* isolate HB chromosome 2b (95.66%)	CP129434
CP10	*C. sativa* - D	*Quercus variabilis* isolate HB chromosome 2a (95.96%)	CP129446	*Quercus variabilis* isolate HB chromosome 2b (95.66%)	CP129434

4.6 Phylogenetic Tree Analysis

To achieve the second objective of genetic divergence, it was necessary to consider the Phylogenetic evolution of the sequences to enable the study of evolutionary relationships among different species, genes, and other biological entities. An evolutionary history was inferred with a phylogenetic tree analysis (**Figure 6**) using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993) with a bootstrap consensus tree inferred from 100 and BioNJ algorithms to a matrix of estimated pairwise distances using the Tamura-Nei model. The analysis involved all 27 nucleotide sequences. There were 613 positions in the final dataset. The evolutionary analyses were conducted using the MEGA11 software (Tamura & Nei, 1993).

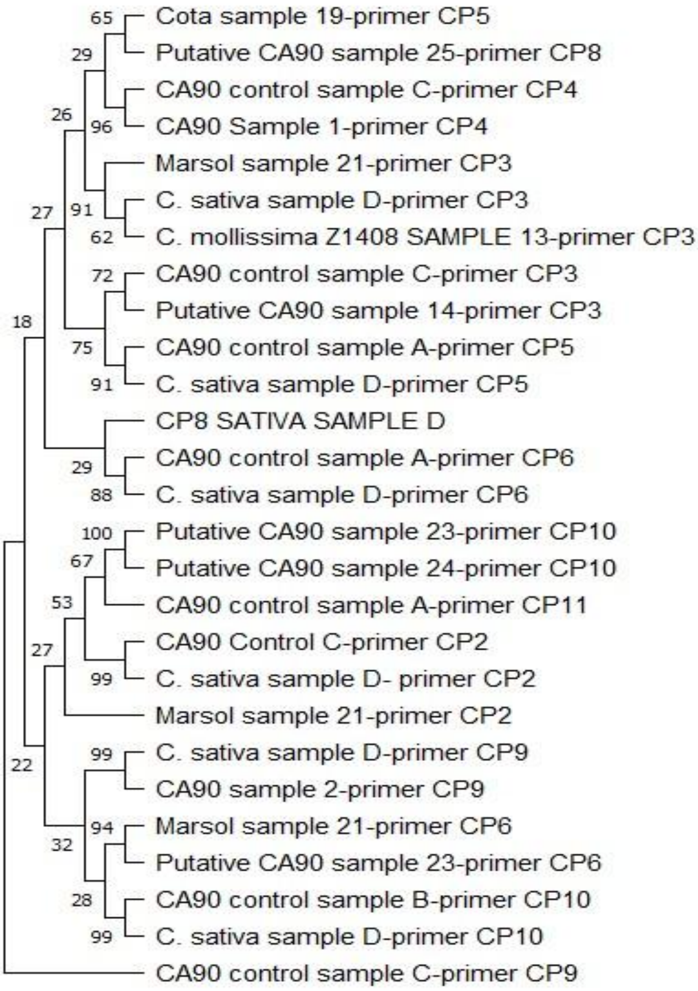


Figure 6. Bootstrap consensus phylogenetic tree showing similarities between sequences from primer loci with a Bootstrap value of 100 replicates, indicating clustering specificity according to the numbers in the tree.

5.0 Discussion

Genetic diversity is vital in enabling chestnut species to survive and adapt to challenging environmental conditions. (Freitas *et al*., 2021). The existing methods for distinguishing chestnut hybrids and varieties remain limited, as farmers still rely on morphological traits for identification. This study aimed to address this gap by developing genetic profiles for selected hybrids and Portuguese chestnut varieties based on band patterns and the existence of motifs using modified SSR markers from Santos *et al*. (2015). The analysis revealed distinct profiles for the CA90 hybrid, which showed 100% genetic identity with control samples, and *C. sativa* , which exhibited an 88.9% genetic match. A notable finding was the absence of a CP4 marker band in *C. sativa* , a locus critical for differentiating CA90 hybrids. Despite the presence of monomorphic bands in gel imaging, motif characterization across primer loci confirmed high polymorphism (**Table 8**) with limitations attributed to homoplasy, allelic variation, and genetic diversity, prompting a recommendation for future studies to employ high-resolution capillary electrophoresis for more precise polymorphism analysis.

The examination of 27 sequences identified 31 SSRs, with trinucleotide motifs dominating (67.74%), followed by dinucleotide (22.58%), mononucleotide (6.45%), and hexanucleotide (3.23%) repeats. Comparisons with (Santos *et al*., 2015), highlighted differences in motif types, allele sizes, and allele counts, with this study reporting 1–3 alleles per locus versus 1–11 in prior work (excluding unmodified CP2). These results align with (Costa *et al*., 2005). Underscoring the low genetic variability in Portuguese chestnut varieties. Markers with fewer alleles, such as those identified here, may prove valuable for population genetics research (Yang *et al*., 2010). Among the markers analyzed in this study, CP2 and CP11 exhibited the lowest allelic diversity, each presenting only a single allele. In contrast, CP3 and CP6 showed the highest allelic variation, with three alleles each. Regarding motif composition, compound motifs were observed in CP3, CP6, CP8, and CP10, with CP3 displaying the most complex motif structure. The presence of compound and complex motifs, particularly in loci such as CP3 and CP6, may enhance marker informativeness by increasing allelic variation. Such motifs are often associated with higher mutation rates, making them ideal for distinguishing closely related genotypes or detecting somatic variations within clonal lines. Intra-varietal differences were also observed, exemplified by CA90 accessions sharing identical SSR motifs but differing in repeat numbers, a pattern consistent with (Dinis *et al*., 2010), who documented similar heterogeneity in the “Judia” cultivar.

Validation of the CP4 locus using primers (5′-GCTGCTTCACAACCTTCCTC-3′ and 5′-GCAAGAGATTCCCTTTGCTG-3′) confirmed consistent SSR motifs [$(GA)_{10}$ and $(GA)_{11}$] and allele variation, underscoring the effectiveness of SSR markers in differentiating disease-resistant hybrids such as CA90. Notably, the absence of a band in *C. sativa* at the CP4 locus highlights the strong discriminatory power of this marker in distinguishing interspecific hybrids from pure species.. The BLAST analysis revealed that only the sequence from locus CP2 showed 99.37% similarities to *Castanea crenata* mRNA, a microsatellite PEB30, and a complete sequence. The same locus showed 97.63% to a PREDICTED: *Quercus* lobata UPF0496 protein 4-like mRNA in the second-ranking. Depending on the plant variety, Loci CP4, CP5, CP6, CP9, and CP10 ranged from 95% to 97.17%, mostly matching *Quercus*' genes, reflecting the scarcity of *Castanea*-specific genomic data and supporting the cross-application of *Quercus*-derived SSRs in chestnut studies (Aldrich *et al*., 2003; Barreneche *et al*., 2004). The cross-amplification of *Quercus*-derived SSR markers in *Castanea* suggests shared genomic regions among *Fagaceae* species, enabling marker transferability. However, the relatively lower similarity scores and lack of Castanea-specific sequence data highlight the urgent need to develop a dedicated SSR database for chestnut. Targeted sequencing of *C. sativa* and *C. crenata* genomes would provide a more reliable foundation for future molecular studies and reduce reliance on related genera.

Heatmap band pattern analysis revealed that samples 1–9 from Vila-Boa de Serapicos exhibited 100% genetic identity with the three control samples (ABC) across all nine primer loci, confirming their status as true CA90 hybrids. In contrast, sample 14, labeled as CA90 and sourced from Deifil Green Biotechnology, showed only 55.6% genetic similarity to the controls, casting doubt on its authenticity. Likewise, samples 23–31 from Sta. Comba did not align with the control profiles and therefore could not be classified as CA90 hybrids. Notably, sample D, identified as *Castanea sativa*, demonstrated 88.9% genetic similarity to the CA90 controls. This finding supports the hypothesis that CA90 is a natural interspecific hybrid of *C. sativa* × *C. crenata*, exhibiting genetic contributions from both parental species. The heatmap also served as a basis for selecting the most effective marker pairs for the identification of chestnut hybrids.

The phylogenetic analysis revealed distinct clustering among taxa, with bootstrap values exceeding 70% indicating robust genetic groupings. For hybrid differentiation, clusters with over 85% bootstrap confidence were prioritized to ensure specificity. The tree highlighted genetic linkages between *C. mollissima* Z1408 (a Chinese hybrid), Marsol, *C. sativa* , and CA90, suggesting shared ancestry or adaptive traits. Notably, leaf morphology differences across taxa, except for CP4, implied underlying genetic or ecological divergence. These findings collectively validate SSR markers as practical tools for hybrid identification, emphasizing the need for expanded *Castanea* genomic resources to enhance precision in future studies. The phylogenetic tree analysis validated the results obtained by gel electrophoresis and sequencing. These results reaffirmed the close genetic relatedness among samples with bands for primers CP2, CP4, CP6, CP9, and CP10 loci while highlighting considerable genetic diversity among hybrids with bootstrap values above 95%. The sequences from these loci were considered, suggesting the robustness of the inferred relationship.

Despite the utility of SSR markers in this study, the reliance on agarose gel-based detection limited resolution in identifying allelic variations, especially in loci with small size differences. To overcome this, future studies should adopt high-resolution techniques such as fluorescent capillary electrophoresis or next-generation sequencing (NGS)-based genotyping-by-sequencing (GBS) to achieve finer resolution and detect rare alleles. Expanding the number of loci and including more reference genomes from diverse *Castanea* species would also improve marker validation and hybrid identification accuracy.

These findings have significant implications for breeding programs aimed at developing disease-resistant and climate-resilient cultivars. The ability to accurately distinguish interspecific hybrids from pure *C. sativa* genotypes allows for better parent selection in hybridization efforts and ensures the authenticity of planting material in commercial nurseries. Moreover, the identification of low-diversity loci such as CP2 and CP11 may aid in monitoring inbreeding levels in endangered local populations, contributing to conservation genetics.

As global interest in sustainable agriculture and forest health grows, the genetic characterization of tree crops like chestnut is gaining momentum. This study contributes to a growing body of evidence supporting molecular tools in forestry and horticulture, facilitating the traceability, certification, and protection of high-value germplasm.

6.0 Conclusion and Recommendation

6.1 Conclusions

Genetic profiling offers a streamlined molecular approach to identify and select chestnut hybrids and varieties, which is critical for their conservation and sustainable cultivation. A key challenge lies in preventing mislabeling, such as when rootstock vendors market *C. sativa* plants as hybrid cultivars. In this study, only the **CP4 marker** proved effective for routine fraud detection using agarose gel analysis. Heatmap visualization of gel data highlighted CP4 as the most discriminatory locus, producing bands exclusively in the CA90 hybrid, while *C. sativa* consistently lacked amplification at this site. This stark contrast underscores CP4's utility in distinguishing CA90 hybrids. To optimize efficiency, primer combinations **CP2, CP4, CP6, CP9, and CP10** were identified as cost-effective tools for differentiation, as they provided clear amplification patterns and robust phylogenetic clustering without requiring sequencing or capillary electrophoresis. These primers balance accuracy with practical feasibility, making them suitable for large-scale screening.

To strengthen these findings, future efforts will focus on expanding collaborations with researchers and institutions across Portugal to increase sample diversity. Incorporating high-resolution electrophoresis techniques (e.g., capillary systems) will refine genetic profiles, enabling precise characterization of Portuguese chestnut hybrids and varieties. Such advancements will address current limitations in resolving subtle genetic variations caused by homoplasy or allelic diversity.

While molecular markers like SSRs are faster, more reliable, and cost-efficient than traditional morphological classification, both methods hold complementary value. To further enhance SSR efficacy, evaluating markers developed for closely related species within the *Fagaceae* family could broaden applicability. Integrating these tools into Portugal's chestnut industry promises multiple benefits: (1) certification of cultivars and germplasm to ensure authenticity; (2) Precise identification of species, cultivars, and rootstocks to combat mislabeling; and (3) Improved breeding programs through targeted selection of traits linked to fruit yield, size, and quality.

However, the lack of standardized certification protocols and validated marker sets for *Castanea* species remains a barrier. Expanding the SSR marker repertoire, particularly for understudied chestnut species, is essential to improve accuracy and adaptability. Additionally, investigating markers associated with pollinators could refine orchard management practices, optimizing cross-compatibility and fruit production. By bridging molecular tools with industry needs, this approach can safeguard genetic diversity while boosting agricultural productivity and economic resilience in chestnut cultivation.

6.2 Recommendations

- The limitation of chestnut genetic databases for comparisons was a challenge to this research since there are no currently standard genetic profiles of chestnut clones; therefore, establishing data repositories where researchers can deposit allelic profiles is essential to ensure long-term accessibility and comparison of data. So that genetic profiles of cultivars referencing established loci could be deposited in this database. Achieving international recognition of this technique is crucial for its commercial application and genetic certification of chestnut varieties.
- Standardizing the analysis of SSR markers across laboratories is also vital to ensure consistent results.
- Journals can implement policies requiring authors to include allelic profiles alongside their relevant publications to promote a culture of data sharing and collaboration among researchers.

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APPENDIX

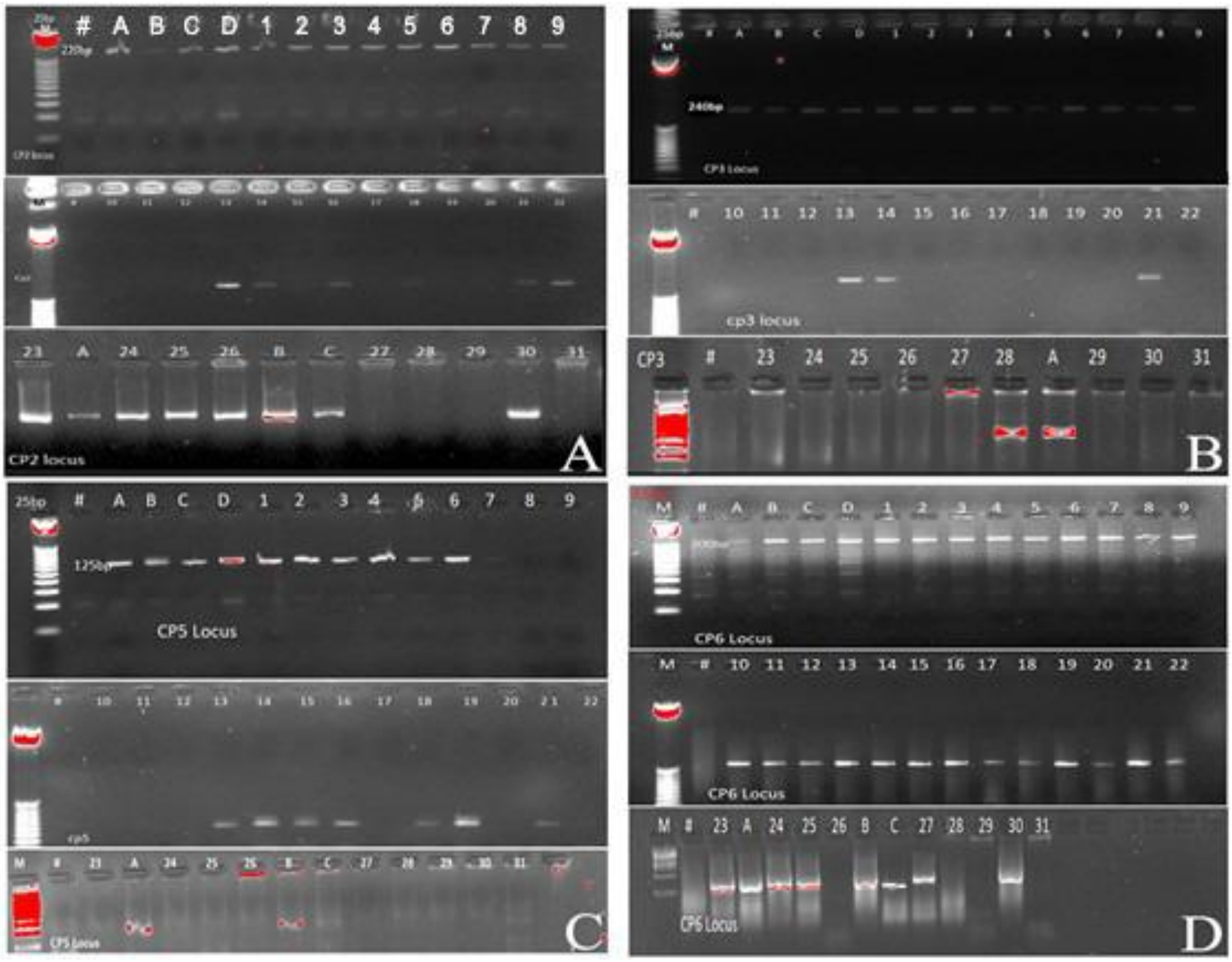

Figure S1. Gel Image of Primer Loci. A= CP2, B= CP3, C= CP5, D= CP6: (M)-25bp ladder, (#)-H2O Control, (ABC)—CA90 controls, (D)—*C. sativa* ,(1–9)—CA90, (10)—*C. mollissima* 60907, (11)—*C. mollissima* E2604, (12)—*C. mollissima* Y0204, (13)—*C. mollissima* Z1408, (14)—CA90-deifil, (15)—Martainha 2, (16)—Bouche de Betizac 1, (17)—Bouche deBetizac 2, (18)—Martainha 1, (19)—Cota, (20)—Judia, (21)—Marsol, (22)—Precoce migoule, (23–31)—Putative CA90 .

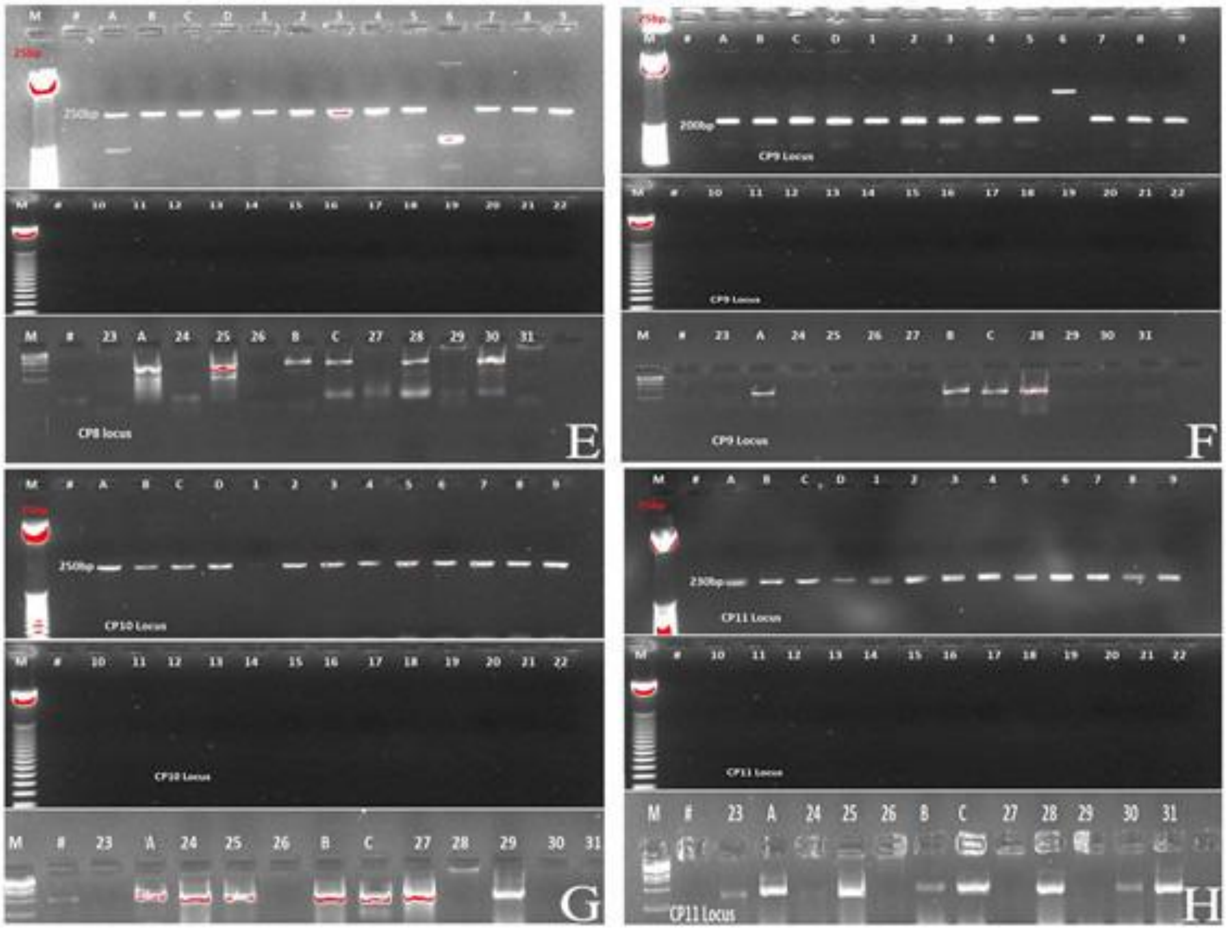

Figure S2. Gel Image of Primer Loci E= CP8, F= CP9, G= CP10, H= CP11: (M)-25bp ladder, (#)-H2O Control, (ABC)—CA90 controls, (D)—*C. sativa* ,(1–9)—CA90, (10)—*C. mollissima* 60907, (11)—*C. mollissima* E2604, (12)—*C. mollissima* Y0204, (13)—*C. mollissima* Z1408, (14)—CA90-deifil, (15)—Martainha 2, (16)—Bouche de Betizac 1, (17)—Bouche deBetizac 2, (18)—Martainha 1, (19)—Cota, (20)—Judia, (21)—Marsol, (22)—Precoce migoule, (23–31)—Putative CA90 .

Table S1. Sequences obtained from selected PCR amplified bands**.**

>CA90 Control C-primer CP2 CAAATCTGAAGCTTTATCAGCTTCATTGCAGGCTTTTCAGTCTGATGTTTCCAATATCTTGAACCAGT TGTCTTTGAATTCAAAACCCGGATCAGAAATCCTGTCCTTACCATGGATTCTACAATGTTTTGGCCTC TTACCCTCCATTCATAGAGCTTTTGCAAAGCTAGTTGTGGATATAGACTACCCCATTAACAAATGGG AAGCTGCTTCTGTRGAAGAATATCTCAACTACAGCTTGAGTTTGATGGAGCTTTTCAATTCCATGAGT TCCTCTCTTGCATATCTTGGACACGCCAGGCTATCACTAGCTCATGCTTTGAGCCTCGTGGAGAAC
>*C. sativa* sample D- primer CP2 AATCTCGAAGCTTTATCAGCTTCATTTCAGGCTTTTCAGTCTGATGTTTCCAATATCTTGAACCAGTT GTCTTTGAATTCAAAACCCGGATCAGAAATCCTGTCCTTACCATGGATTCTACAATGTTTTGGCCTCT TACCCTCCATTCATAGAGCTTTTGCAAAGCTAGTTGTGGATATAGACTACCCCATTAACAAATGGGA AGCTGCTTCTGTGGAAGAATATCTCAACTACAGCTTGAGTTTGATGGAACTTTTCAATTCCATGAGTT CCTCTCTTGCATATCTTGGACACGCCAGGCTATCACTAGCTCATGCTTTGAGCCTCGTGGAGAACT
>Marsol sample 21-primer CP2 CAGTTGGATAGCAACGAGATGAACTCATGGAATTGAAAAGCTCCATCAAACTCAAGCTGTAGTTGA GATATTCTTCTACAGAAGCAGCTTCCCATTTGCTAATGGGGTAGTCTATATCCACAACTAGCTTTGCA AAAGCTCTATGAATGGAGGGTAAGAGGCCAAAACATTGTAGAATCCATGGTAAGGACAGGATTTCT GATCCGGGTTTTGAATTCAAAGACAACTGGTTTAAGATATTGGAAACATCAGACTGAAAAGCCTGCA AGGAAGCTGATAAAGCTTCAGATTTGTGGTGGTGGTGGTGGTGGTGGTGGTGATGATGATGATTCTC CAGCTTGG
>CA90 control sample C-primer CP3 AATCTCACTCTCTCTAATAACCCATGTCCTAAACTCTCACCCTTTGATCTCCTTGTACTATCCAAACC ATAGTTACGCTTGCTTTGTTCCAACTCATCGTTGTACGTGTGTTTCCTCAACATGTCCACAATCTCAG CCTTGGAATGAGAATTCAAGAACACAGTCTCTATGTCATCCTCTTGATGATGATGATGATCATGATG ACCACAAGGATTGAAGTTAGTCACAGCATCTCGGCCACCAACGCGTTGGGCCGCGATGTCGTACGCT TTTGCATCTTCTTCTTCTTCTTCGAAAGTGCCTAACCAAACGCCTAGATGCTTCTCGTAAATCTGGGC ACCAATCTGGGCACCA
>Putative CA90 sample 14-primer CP3 TTGATAATAATACACTCTCTCTAATAACCCATGTCCTAAACTCTCACCCTTTGATCTCCTTGTACTATC CAAACCATAGTTACGCTTGCTTTGTTCCAACTCATCGTTGTACGTGTGTTTCCTCAACATGTCCACAA TCTCAGCCTTGGAATGAGAATTCAAGAACACAGTCTCTATGTCATCCTCTTGATGATGATGATGATG ATGATGATCATCAGTACCACCCGGCGGGTTGAAGTTCACAACATCTTCTCCACCACAAAAGCGGGGG GCCATGATGTCATATTTTGTTGCTTCTTCTTCTTCTTCGATGAAGGCGACTAACACAACACGATGATG CTTCTCGTACTGGTGACCACCA

>Marsol sample 21-primer CP3
AAAAATACGAAGCTGCAAAAGCTTATGACATCGCGGCCCAACGCTTTCGTGGCCGAGATGCTGTGA
CTAACTTCAAGCCCTGTGGTACTACTGATCATCATCATCATCATCACGATGACGTTGACACTGTGTTC
GTGATTTCTCATTCCCAGGCTGAGGTTGAGAACGTGTTGATGAAACGGAAATACAACTATGAGTTGG
AACTGAACCAGCGTAACTATGGTTTGGATTTGAATAGGACATCGAAAGCTGAGAGTGAAAGACTTG
GATTAGTATAAATAGAGAATTATTATTCTTTGACAGCGCGTGAACAGGTTTTTGATATTGATGTGACT
CCGACTCATAGCGATA

> *C. sativa* sample D-primer CP3
ATACGAAGCTGCAAAAGCTTATGACATCGCGGCCCAACGCTTTCGTGGCCGAGATGCTGTGACTAAC
TTCAAGCCCTGTGGTACTACTGATCATCATCATCATCATCATGATGACCTAGAGGCTGAGTTCTTGAA
TTCGCATTCGAAGGCTGATATTGTGGACGTGATGAGGAAACTGTCGAACAACCATGAGTTGGAACAT
GACTTGCGTCACTATGGTTTGGATAGTGGTTGGAGATCAACGGGGGAAAGTTTAGGAGATGGGTTAT
TACATGGAGTGAATTATTATTCGATGTATTCTCGTGAGCAGCTTTTTGAGAAAGTTGTGAATCCAACC
GATACTCCAAGCGATA

> *C. mollissima* Z1408 SAMPLE 13-primer CP3
TGCAAAAGCTTATGACATCGCGGCCCAACGCTTTCGTGGCCGAGATGCTGTGACTAACTTCAAGCCC
TGTGGTACTACTGATCATCATCATCATCATCATGATGACCTAGAGGATGAGTTCTTGACTGTGTTCTC
CAATGCTCATTTCGTGGCCGAGATTGTGGACCTGTCGTACAACCACGAGTTGGAACATGACTTGCAT
CACTATGGGTTGGATTGTGGTTGGAAATCTACGGGGGAAACTTTAGGAGATGGTTTATGACATGGAT
TGAATTATTATTCGAAGTATTCTCCTGAGCAACTTTGTGAAAAACTTTTTAATACAACTGATCCTCCA
AGCGATAA

>CA90 control sample C-primer CP4
GCTACAGAAAAAAGCTGAAAGCAAACTGTAGAAATAGAGCATTTTTATTTTTGAGAGAGAGAGAGA
GAGAGAGTCACATATAGAATACTGTGTTTTTGTAGTGAATAAAACAAGATGGCAATGGGAAAAGAA
TTCATGGGTTGTTCTATAATTGAGAGGTCTTCTTTTGTTTCATCAACAAAAGTGTTTTTGAATCATGGT
AAGAGCTTCAACAACAACAACATGTTCTTGGTTAAACCTTTGCAGAAAAGGAGGGTATTGGTGCCTT
TGAGGAAGGTTGTGAAGCAACAACGCTAATCAA

>CA90 Sample 1-primer CP4
TGCATGCTACAGAAAAAAGCTGAAAGCAAACTGTAGAAATAGAGCATTTTTATTTTTGAGAGAGAG
AGAGAGAGAGAGAGTCACATATAGAATACTGTGTTTTTGTAGTGAATAAAACAAGATGGCAATGGG
AAAAGAATTCGTGGGTTGTTCTATAATTGAGAGGTCTTCATTTGTTTCATCAACAAAAGTGTTTTTGA
ATCATGGTAAGAGCTTCAACAACAACAACATGTTCTGGGTTAAACCTTTGCAGAAAAGGAGGGTATT
GGTGCCTTTGAGGAAGGTTGTGAAGCAACA

>CA90 control sample A-primer CP5 ACTAAGCCTCCTAAATTTACTTATTCCACTCTCTCTCTCTCTCTTTTATATCAACTAATGATGAACATATACTACATAGATAGAAGATGCCGTTTCCCATAAA
> *C. sativa* sample D-primer CP5 CTAAGCCTCCTAAATTTACTTATTCCACTCTCTCTCTCTCTCTCTCTCTTTTATATCAACTAGTGATGAACATATACTACATAGATAGAAGATGCCGTTTCCCATAAA
>Cota sample 19-primer CP5 CTTTAATTGTTCTCATTAGTTGATATAAAAGAGAGAGAGAGAGTGAAATAGTGAAATTTAAGATGCTAGTGTGTAATTGTGTACTTCTTTCTTTTGATCGTTCACACCCCCATGTGTACATGTGTA
>CA90 control sample A-primer CP6 CGTACGACGTGTCTTGAGCGGCTACGTGGTAGACAACACCCCCGTGGTACTACAGCCCGACGTGGTACTATTAGACCGCCGTGTTGGAAGATGATCTACCATTTTATACCCGGTGGAGTATTTTTGGCTACTATGTGGGGTTTTGTTTTGTTGTACGGGGTCAAACCAAAAAAAACCCCAAAACCTTCAAGGGAAATAACAAAAAAGGGGTTTGTAAAAATGTTAAATAACAGTACTTGTCAGTCTGATATTACTATAAGCACCGACGTGGTATG
>*C. sativa* sample D-primer CP6 CGTACGACGTGGTCTTGTGAGCTAAGATGAGTGTGAGTACAACTCCCTCGTGGTATCTTCTTCTTCTTCATCCAATCAGTCACCAATCTTTGAAGATGATCTACGAGTATATACTCGGTGGAGTAGTTTTGGCTACTATGTTGGGTTTTGTTTTGTTGTACGGTGTCAAAGCAAAGAAGACCACAAGAGCTTCAATGGAGATAACAAGAAATGGGTTTGTAAAGATGTTGGATAACAGTACTTGTCAGTCTGAGATTACTAGAAGCACCGACGTGGT
>Marsol sample 21-primer CP6 TCTAAGGCCCCTCTGTGCTTCTCTGCCCTTCTTGTTCTCTCCATTGCCGCTCTCGTGTTGTGGTTGCTTTTGCTCCGAACCGTAAACAAAACCCAACCCCATAACCATAACTACTCCTCCGCCTATATACTCGTAGATCTACATCATCGATTGGTGACGGATAGGGAGAGGATGAAGAAGAAGAAGAATAGGAAGAAGAACAAAAAGAATAAATAACATTGAAACTTTCTTCTCTCTTGTGGTGCGGGGGCTCTCTTATCCTCACCGGACAGGACCCTACTTTTCAGCCCAGGGTGGCAATCCTAACCCCCACGGTGCTCGACCTGCTGCGCCACTTTGCCAATACCCACCAAGCTGTTCTTGGTTTGCTCCCGCGATCCTACCAAGACAACCCAAAAGGGCGAGGAAATTTAGTTTTATTCCTCTCTTACT

>Putative CA90 sample 23-primer CP6
CTCTTCATATGCTTTTCTTCCCTTTCTTGTTCTCGCCATTGCCGCTCTTGCTCTCGTGGTTGTCTTTGCT
CCGACCCCCTAAACCAAACCCAACCCCATCACCATAACTACTCCTCCTCCTCTATGCTCATACTCCTT
CATCATCGATTGGGGACGGAGAGGGTGAGGATGAAGAAGAAGAAGAAGAGGAAGAAGAACAAAA
AAAAAAAATAACATTGAAATTTTCTTTTTTCTTGTGGTGGGGGGGCTCTCTTATCCTCACCGGACAGG
GA

>CP8 *C. sativa* SAMPLE D
ATTCCTTACTTCTAATTAATATTATAATCATTAATTTTTGGATATGCATTTAGTTAATTCAACATTCTC
TTCTATATATTTTGGTTAATAGACAAATAATAACAATTGAATTTATTTTGTTTCTTTTATTTATTTACA
GGGGTTATTACAGATGTAGTAGTTCAAAAGGGTGTTTGGCCCGAAAACAAGTGGAGCGAAACAAAA
CCGACCCGGGAATGTTCATAGTGACATACACAGGAGAGCACAACCACCCAGCTCCCACGCACCGAA
ACTCACTAGCTGGGTCCACGCGTCAGAAGCCCTTGACACCCCAAAACGTCACCGCTGGTGACCCTAA
CAAAGCCCAAAAGCAGTACTCTTCCCCAGAAACCTCGTTCGAGGAAGAGCTTGTTGTACCTCAGACT
CAGACTCAGACTCAGACTCAGAGTACAACAACCGACAGCAAAGAAGAAGAAGAAGAAGAAGAAGA
AGAAGAAGAGAGAGAGGACTTGGTGGAGGAAGATGATGAAGAAGGGGACGAA

>Putative CA90 sample 25-primer CP8
TCACTCTTCTTCTTCTTCTTCTTCTTCTTCGTCGTTGCTGGCTGTTGTTGTACTCTGAGTCTGAGTCTGA
GTCTCAGTCTGAGGTCCTCTAGCTCTTCCTCGGGGGAAGATTCTGACTTTTAGTACTGGTTTTGGTCT
TTGTTAGGGACACCTTCGGTGACCTTTTGCTTCGTCCGGGGGTTCCCACCCGTGGACCCTCCTTGCGA
GTTTCCGGGCTGGGGATCTGGGTGGTTGTGCTCTCCTGTGTATGTCACTATGACCGTTCCGTGTTCGT
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AAATAAAAAAAAAAAAACAATGTAATTTCTTTTGTTTTTATTTGTCTATAAACCAAAAAATGTTGAAT
AAAATGTTGAATTATCTAAATGCTTATCATAAAATTAATGATTATAATATTAATTATAAATAAAGAA
TTTGAGAAACTAACCTTGCATATGGAGATTTTTTGATGGATTTTTGGGCCATATA

>CA90 control sample C-primer CP9
TTACGATGATTTCTTCATGGGTTTGGACGGCTTCGCCGGTCCGGGCACCGGAGACTACTTTCCCGACA
ACTTTCCGGCGAGCTTTGCTCCAATATCTAACTGTAGTAACTGGGTGGCCAACAATGCTGGCTGACTT
GAAGAAATTTCAAAATGCTGACGTGGATTATGCTTTTTGTGACACACACGTAGACACAGTTTTTAGA
GAGAGAGAGAGAGAGAGATGGTGTATCTGGGAGTGGTAACAATTGGGTGGAAATGGGTGGAAA

>*C. sativa* sample D-primer CP9

TCTCTCTCTCTCTCTCTAAAACTGTGTCTACGTGTGTGTCACAAAAAGCATAATCCACGTCAGCATTT
TGAAATTTCTTCAAGTCAGCCAGCATTGTTGGCCACCCAGTTACTACAGTTAGATATTGGAGCAAAG
CTCGCCGGAAAGTTGTCGGGAAAGTAGTCTCCGGTGCCCGGACCGGCGAAGCCGTCCAAACCCATG
AAGAAATCATCGTTAAGAACCATATCCGATATCCCAAACTCGTCCCCTTCTTCATCATCACA

>CA90 sample 2-primer CP9

TCTAAAAACTGTGTCTACGTGTGTGTCACAAAAAGCATAATCCACGTCAGCATTTTGAAATTTCTTCA
AGTCAGCCAGCATTGTTGGCCACCCAGTTACTACAGTTAGATATTGGAGCAAAGCTCGCCGGAAAGT
TGTCGGGAAAGTAGTCTCCGGTGCCCGGACCGGCGAAGCCGTCCAAACCCATGAAGAAATCATCGT
TAAGAACCATATCCGATATCCCAAACTCGTCCCCTTCTTCATCATCACA

>CA90 control sample B-primer CP10

CACCACCACCACCACCACCCACTGAAGAGGTGGTTGCTGTTGTTGCGGAAAAGGTGACCTCTATTGT
CGATGAGGACGGTGCGAAGACTGTTGAGGCAATCAAAGAGACCATAGTGGAAGTCTCGGCTCCTGC
TCCTCCTCCGTCCACAACAGAAACAGAAACAGAGGAACCTAAAGAAGAAGAAGAAGTCACTCCTCC
TCCAGCTCCCGAAGAAGTCTCAATCTGGGGAATCCCGCTTCTTCAGGACGAGAGAAGCGATGTAATT
CTCTTRAAATTCCTTAGAGCCAGGGATTACAAGGTGAAGGATGCATTCACTATGATAAAGAACACGG
TCCGTTGGCGCAAGGAATTCGAAATTGATGTTCTTCTTGAGCAAGATCATGGTAATGATTGGGACAA
GGTGGCTTTCACTCATGGATAA

>*C. sativa* sample D-primer CP10

CACCACCACCACCACCACCCACTGAAGAGGTGGTTGCTGTTGTTGTGGAAAAGGTGACCTCTATTGT
CGATGAGGACGGTGCGAAGACTGTTGAGGCAATCAAAGAGACCATAGTGGAAGTCTCGGCTCCTGC
TCCTCCTCCGTCCACAACAGAAACAGAAACAGAGGAACCTAAAGAAGAAGAAGAAGTCACTCCTCC
TCCAGCTCCCGAAGAAGTCTCAATCTGGGGAATCCCACTTCTTCAGGACGAGAGAAGCGATGTAATT
CTCTTAAAATTCCTTAGAGCCAGGGATTACAAGGTGAAGGATGCATTCACTATGATAAAGAACACGG
TCCGTTGGCGCAAGGAATTCGAAATTGATGTTCTTCTTGAGCAAGATCATGGTAATGATTGGGACAA
GGTGGCTTTCACTCATGGATA

>Putative CA90 sample 23-primer CP10

ATGAATCTTGCTCAGAAGAGCATCAATTTCGAATTCCTTGCGCCAACGGACCGTGTTCTTTATCATAG
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TCCTGAAGAAGCGGGATTCCCCAGATTGAGACTTCTTCGGGAGCTGGAGGAGGAGTGACCTCTTCTT
CTTCTTTAGGTTCCTCTGTTTCTGTTTCTGCTGTGGACGGAGGAGGAGCAGGAGCCGAGACTTCCACT
ATGGTCTCTTTGATTGCCTCAACAGTCTTCGCACCGTCCTCATCGACAATAGAGGTCACCTTTTCCAC
AACAACAGCAACCACCTCTTCAGTGGGTGGTGGTGGTGGTGGGGGAGCCTTCTCTTCTTCTTCTTCTT
CTTTTTTTGCTTCCACGTAATCGATGCTTGTTGTTACAA

>Putative CA90 sample 24-primer CP10

CATCTTGCTCAGAGAGCATCAATTTCGAATTCCTTGCGCCAACGGACCGTGTTCTTTATCATAGTGAA
TGCATCCTTCACCTTGTAATCCCTGGCTCTAAGGAATTTTAAGAGAATTACATCGCTTCTCTCGTCCT
GAAGAAGTGGGATTCCCCAGATTGAGACTTCTTCGGGAGCTGGAGGAGGAGTGACCTCTTCTTCTTC
TTTAGGTTCCTCTGTTTCTGTTTCTGTTGTGGACGGAGGAGGAGCAGGAGCCGAGACTTCCACTATGG
TCTCTTTGATTGCCTCAACAGTCTTCGCACCGTCCTCATCGACAATAGAGGTCACCTTTTCCACAACA
ACAGCAACCACCTCTTCAGTGGGTGGTGGTGGTGGTGGGGGAGCCTTCTCTTCTTCTTCTTCTTCTTT
TTTTGCTTCCATCGTAATCGATGCTTGTTGTTAC

>CA90 control sample A-primer CP11

ATGAAGAGACTGACACAATGGTCTCTTTAATGGCTTCCACAGTCTTTGCACCATCGTCATCTACAACT
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TCTTTTGCTGGTTCTGCTACTTTTTCTGATTCTGGGTTTGCAGATTCATCATCAGCAACCACTACGGAA
GGCTTTTCTTCAACTGGGTTCTTGTCCTTTTCCTCTAAAGCTTCTGGCTTTTCTTGATCTTCTTTGGCTG
GAGGTGGTGGTGGTGGTGGTGGTGAAGGCGGAGGTGGGAATTCATGCTTGTTGAGGGCTTCTTGGAT
GAAGAGGGCTTCTTGGATGAA

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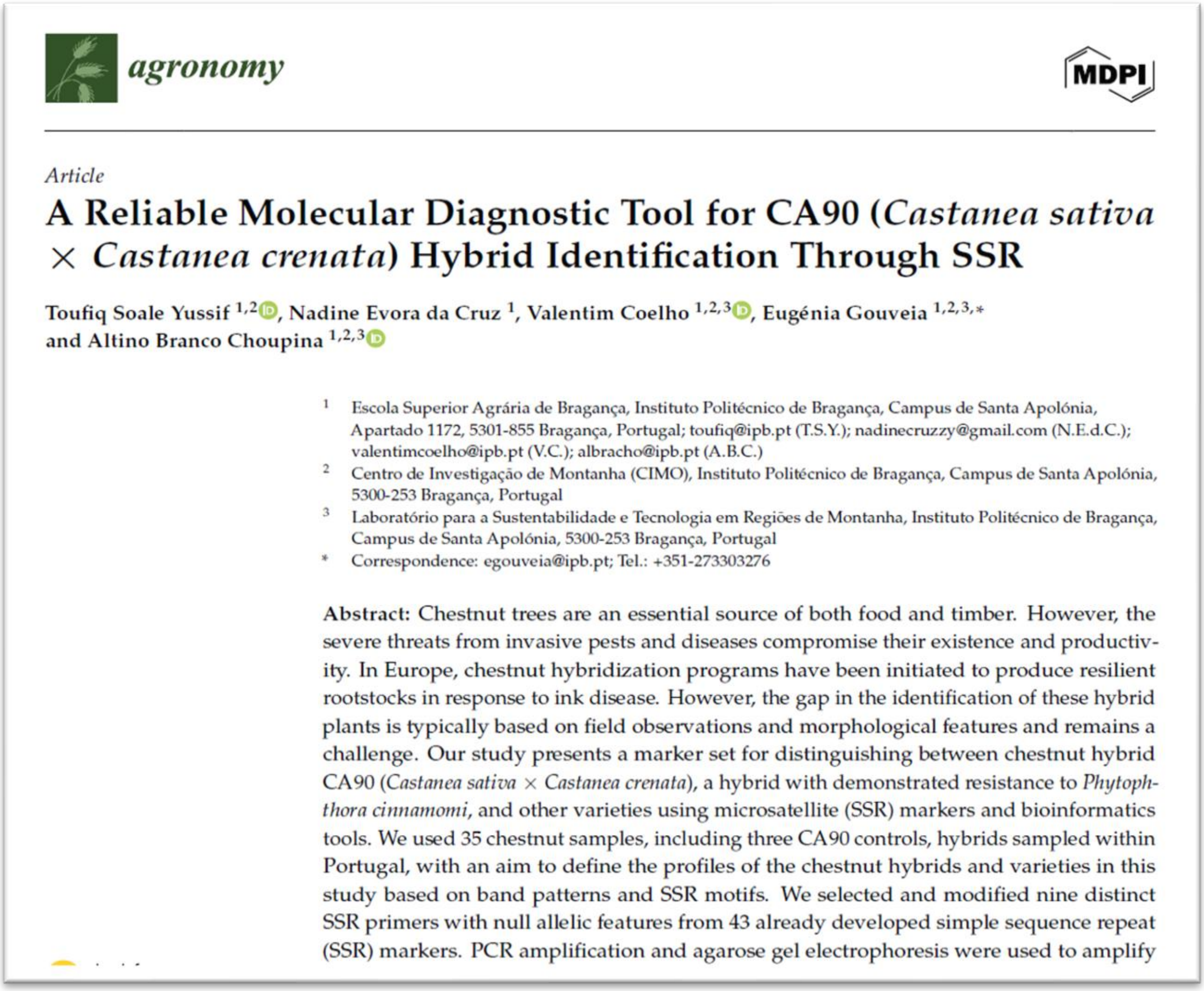

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Article

A Reliable Molecular Diagnostic Tool for CA90 (*Castanea sativa* × *Castanea crenata*) Hybrid Identification Through SSR

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Abstract: Chestnut trees are an essential source of both food and timber. However, the severe threats from invasive pests and diseases compromise their existence and productivity. In Europe, chestnut hybridization programs have been initiated to produce resilient rootstocks in response to ink disease. However, the gap in the identification of these hybrid plants is typically based on field observations and morphological features and remains a challenge. Our study presents a marker set for distinguishing between chestnut hybrid CA90 (*Castanea sativa* × *Castanea crenata*), a hybrid with demonstrated resistance to *Phytophthora cinnamomi*, and other varieties using microsatellite (SSR) markers and bioinformatics tools. We used 35 chestnut samples, including three CA90 controls, hybrids sampled within Portugal, with an aim to define the profiles of the chestnut hybrids and varieties in this study based on band patterns and SSR motifs. We selected and modified nine distinct SSR primers with null allelic features from 43 already developed simple sequence repeat (SSR) markers. PCR amplification and agarose gel electrophoresis were used to amplify

PRESENTATIONS AT SELECTED CONFERENCES

1.1 XI IMFAHE International Conference-Innovation Camp 2025 Confirmation -26th May 2025

--Oral- Presention

XI IMFAHE´s International Conference

Innovation Camp

The Impact of Global and Multidisciplinary Talent

May 26, 2025

Online

28	Melania Prieto Martín	URJC	Detecting anomalies in dense 3D crowds: are we safe?
29	Sheila Sousa Gomes Fortes	NSC	Identifying transcytotic targets and modeling the BBB to improve treatments for neurodegenerative diseases
30	Jose Bernardo González-Me	UVA	Generation of Spatially Explicit Tree Lists for Pinus pinaster Using Machine Learning and Remote Sensing in Central Spain
31	Leonardo Mendonça	IPB	Advanced Predictive Maintenance: A Hybrid Approach Combining AI-Driven Prediction with Huma
32	Toufiq Soale Yussif	IPB	Chestnut Hybrid, CA90(Castanea sativa x Castanea crenata) Identification Through Microsatellite Markers (SSR).
33	Elizandra Ardohain	IPB	SusBioBakery – Prospecting Sustainable Resources to Obtain Natural Bio-Based Ingredients for the Bakery Sector
34	Joana Dias	Minho	Sustainable production of functional red seaweed-based food ingredients using autohydrolysis
35	Ernesto Javier Delgado Pujol	US	Porous Ti6Al4V Scaffolds Produced by Laser/Metal Powder Bed Fusion: Enhanced for Therapeutic Activity Through Infiltration
36	Bárbara de Sena Nunes Men	IPB	ZEROfoodWASTE: Reuse of Agro-industrial Residues for the Production of Stale Bread-Based Products
37	Alicia Castillo Rivas	US	PRODUCTION OF CATALYSTS AND BIOFUELS FROM URBAN AND AGRIFOOD WASTE
38	Diana Gouveia Amaral	Minho	Rethinking collective spaces through the façade element: From formality to informality
39	Orestes Rivada-Wheelaghan	US	Molecular Tools to Promote Cooperativity During Electrochemical Carbon Dioxide Reduction Reaction
40	Cesar Ortiz	Malaga	DISE: A Dynamic Integrated Space-Economy Model for Orbital Debris Mitigation Policy Evaluation
41	Victor Hernández André	UVA	An analysis of the use of the economic resources of the “Comunidades de Villa y Tierra” at present
42	Sergio Valdelomar Muñoz	Jaen	The Power of Consciousness: Enhancing Consumer Attitudes Toward Sustainable Food Consumption
43	Cecilia López Boronat	Jaen	The impact of message framing on pro-environmental food communication: perceptions and valuations of the message.
44	Catarina Gomes Coelho	Minho	The Impact of Prefrontal Cortex tDCS and Cognitive Reappraisal on Psychophysiological Responses to Negative Emotions
45	Alejandro Araque,	Malaga	Protecting the creditor through the damages’ remedy
46	Carmen De Pablos Heredero	URJC	The effectiveness of deploying dialogic practices in healthcare organizations
47	Daniel Santos Carrasco	US	How stress affects the brain’s ability to tune out distractions: Insights from humans and rodents
48	Diego Hervella-Fariñas	ULL	Enhancing the Family-School Partnership: An Analysis of Primary Education Teachers' Training Needs
49	Rocío Caballero Díaz	US	Uncovering Hidden Brain Activity
50	Rebeca Company Almagro	US	“We appreciate your feedback”: How students of tourism respond to negative hotel reviews
51	Andrea Jiménez Cejas	ULL	Reshaping Education: Centring Marginalised Voices in Mainstream Curricula
52	Filipa Ferraz	Minho	In Silico Prediction of Candidiasis-Related microRNAs Using a Machine Learning Framework

1.2 African Plant Genomics Symposium
10-11 April 2025 United States International University - Africa Nairobi, Kenya

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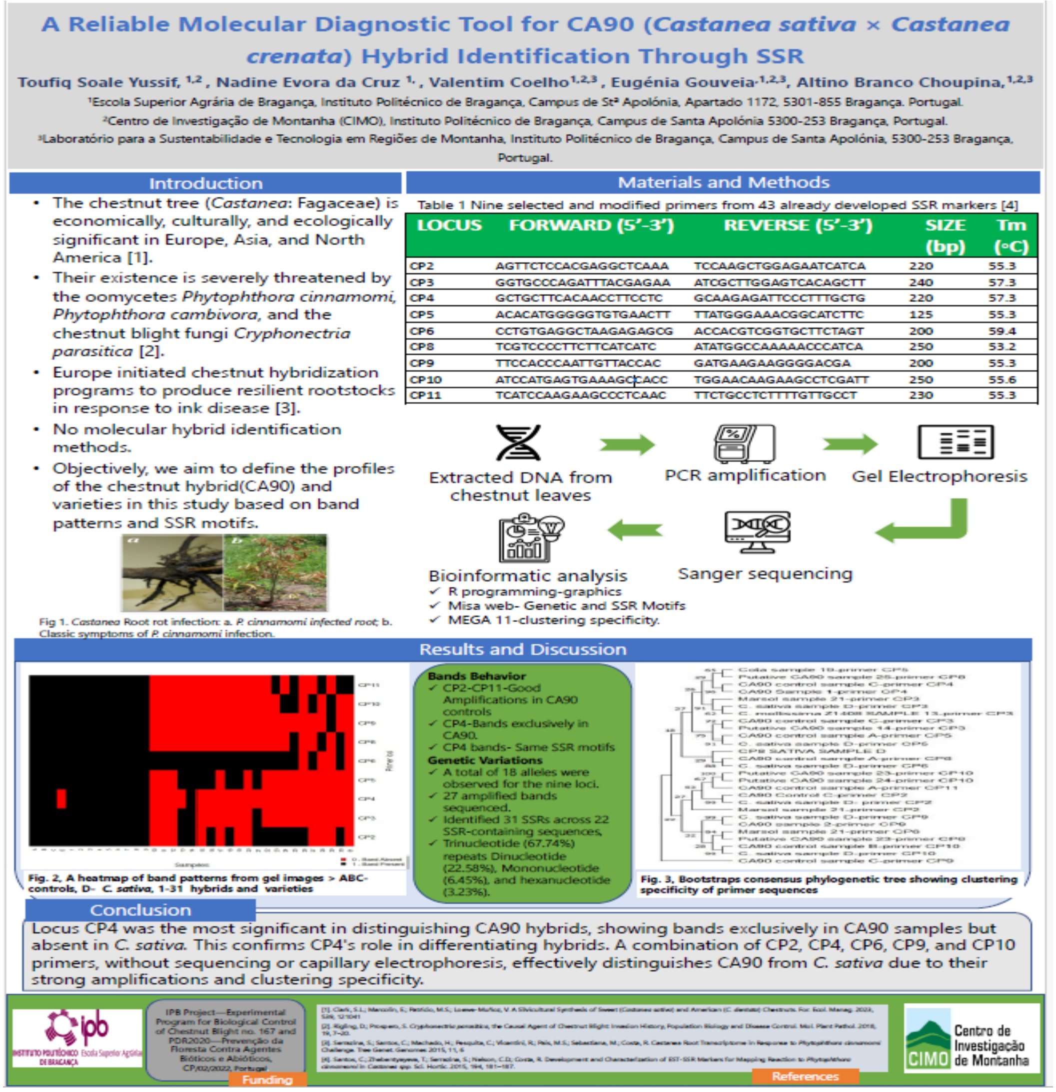
--Poster Presentation



A Reliable Molecular Diagnostic Tool for CA90 (*Castanea sativa* × *Castanea crenata*) Hybrid Identification Through SSR

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Introduction

- The chestnut tree (*Castanea*: Fagaceae) is economically, culturally, and ecologically significant in Europe, Asia, and North America [1].
- Their existence is severely threatened by the oomycetes *Phytophthora cinnamomi*, *Phytophthora cambivora*, and the chestnut blight fungi *Cryphonectria parasitica* [2].
- Europe initiated chestnut hybridization programs to produce resilient rootstocks in response to ink disease [3].
- No molecular hybrid identification methods.
- Objectively, we aim to define the profiles of the chestnut hybrid(CA90) and varieties in this study based on band patterns and SSR motifs.

Fig 1. *Castanea* Root rot infection: a. *P. cinnamomi infected root*; b. Classic symptoms of *P. cinnamomi* infection.

Materials and Methods

Table 1 Nine selected and modified primers from 43 already developed SSR markers [4]

LOCUS	FORWARD (5'-3')	REVERSE (5'-3')	SIZE (bp)	Tm (°C)
CP2	AGTTCTCCACGAGGCTCAAA	TCCAAGCTGGAGAATCATCA	220	55.3
CP3	GGTGCCCAGATTTACGAGAA	ATCGCTTGGAGTCACAGCTT	240	57.3
CP4	GCTGCTTCACAACCTTCCTC	GCAAGAGATTCCCTTTGCTG	220	57.3
CP5	ACACATGGGGGTGTGAACTT	TTATGGGAAACGGCATCTTC	125	55.3
CP6	CCTGTGAGGCTAAGAGAGCG	ACCACGTCGGTGCTTCTAGT	200	59.4
CP8	TCGTCCCCTTCTTCATCATC	ATATGGCCAAAAACCCATCA	250	53.2
CP9	TTCCACCCAATTGTTACCAC	GATGAAGAAGGGGACGA	200	55.3
CP10	ATCCATGAGTGAAAGCCACC	TGGAACAAGAAGCCTCGATT	250	55.6
CP11	TCATCCAAGAAGCCCTCAAC	TTCTGCCTCTTTTGTTGCCT	230	55.3

Extracted DNA from chestnut leaves → PCR amplification → Gel Electrophoresis → Sanger sequencing → Bioinformatic analysis

Bioinformatic analysis
- ✓ R programming-graphics
- ✓ Misa web- Genetic and SSR Motifs
- ✓ MEGA 11-clustering specificity.

Results and Discussion

Fig. 2, A heatmap of band patterns from gel images > ABC- controls, D- C. *sativa*, 1-31 hybrids and varieties

Bands Behavior
- ✓ CP2-CP11-Good Amplifications in CA90 controls
- ✓ CP4-Bands exclusively in CA90.
- ✓ CP4 bands- Same SSR motifs

Genetic Variations
- ✓ A total of 18 alleles were observed for the nine loci.
- ✓ 27 amplified bands sequenced.
- ✓ Identified 31 SSRs across 22 SSR-containing sequences,
- ✓ Trinucleotide (67.74%) repeats Dinucleotide (22.58%), Mononucleotide (6.45%), and hexanucleotide (3.23%).

Fig. 3, Bootstraps consensus phylogenetic tree showing clustering specificity of primer sequences

Conclusion

Locus CP4 was the most significant in distinguishing CA90 hybrids, showing bands exclusively in CA90 samples but absent in C. *sativa*. This confirms CP4's role in differentiating hybrids. A combination of CP2, CP4, CP6, CP9, and CP10 primers, without sequencing or capillary electrophoresis, effectively distinguishes CA90 from C. *sativa* due to their strong amplifications and clustering specificity.

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Funding

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Instituto Politécnico de Bragança Escola Superior Agrária

Centro de Investigação de Montanha CIMO

1.3 International Conference on April 14-15, 2025, Rome, Italy, Plant Science and Molecular Biology

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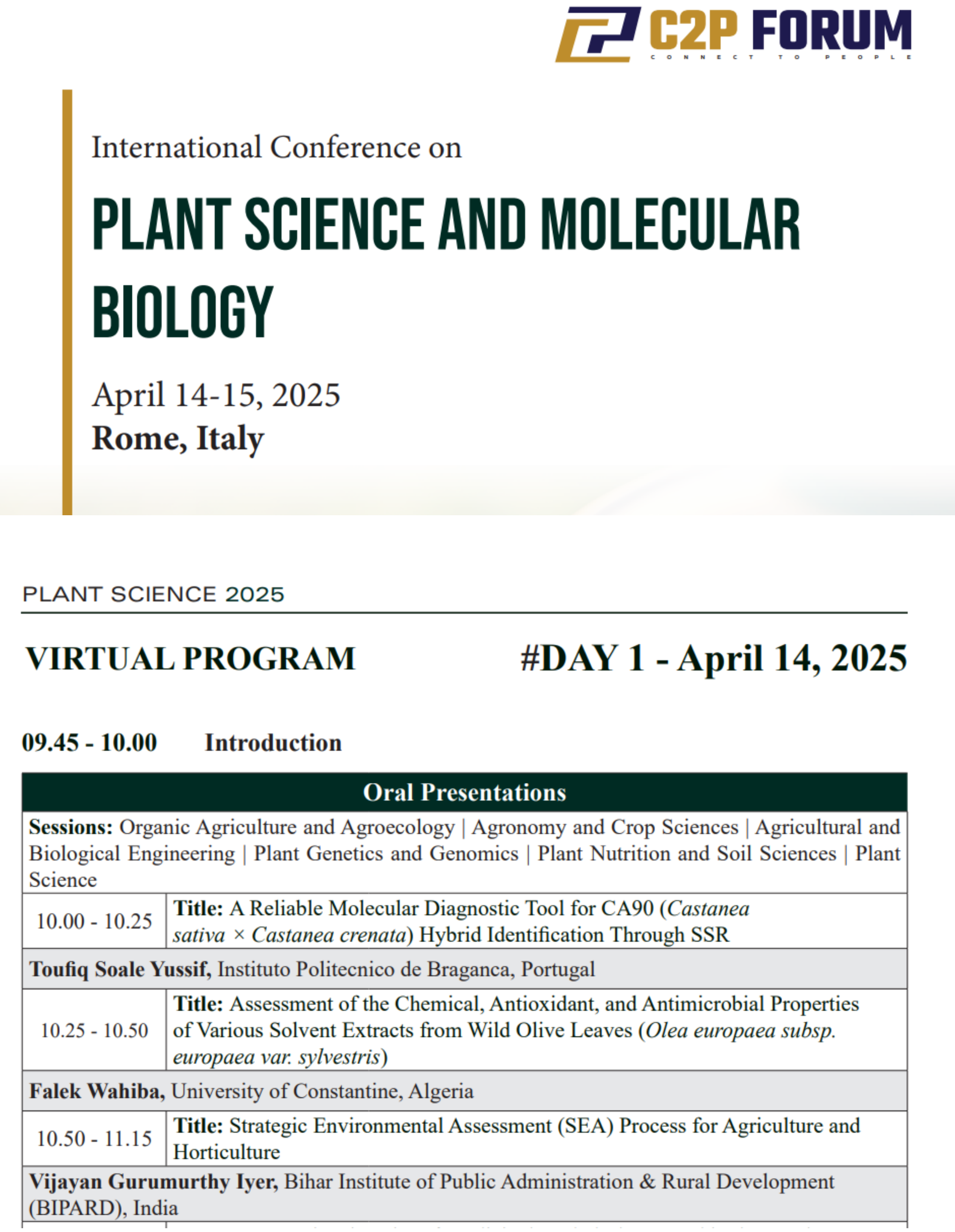
--Oral- Presentation



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VIRTUAL PROGRAM **#DAY 1 - April 14, 2025**

09.45 - 10.00 **Introduction**

Oral Presentations	
Sessions: Organic Agriculture and Agroecology | Agronomy and Crop Sciences | Agricultural and Biological Engineering | Plant Genetics and Genomics | Plant Nutrition and Soil Sciences | Plant Science	
10.00 - 10.25	**Title:** A Reliable Molecular Diagnostic Tool for CA90 (*Castanea sativa* × *Castanea crenata*) Hybrid Identification Through SSR
Toufiq Soale Yussif, Instituto Politecnico de Braganca, Portugal	
10.25 - 10.50	**Title:** Assessment of the Chemical, Antioxidant, and Antimicrobial Properties of Various Solvent Extracts from Wild Olive Leaves (*Olea europaea subsp. europaea var. sylvestris*)
Falek Wahiba, University of Constantine, Algeria	
10.50 - 11.15	**Title:** Strategic Environmental Assessment (SEA) Process for Agriculture and Horticulture
Vijayan Gurumurthy Iyer, Bihar Institute of Public Administration & Rural Development (BIPARD), India	