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PROGRAMA DE DOCTORADO EN RECURSOS Y TÉCNOLOGÍAS AGRARIAS, AGROAMBIENTALES Y ALIMENTARIAS

CARACTERIZACIÓN FENOLÓGICA, CLIMÁTICA, MOLECULAR, MORFOLÓGICA, FÍSICO-QUÍMICA Y SENSORIAL DEL "PERO DE CEHEGÍN" (*Malus domestica* Borkh)

TESIS DOCTORAL

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CARACTERIZACIÓN FENOLÓGICA, CLIMÁTICA, MOLECULAR, MORFOLÓGICA, FÍSICO-QUÍMICA Y SENSORIAL DEL "PERO DE CEHEGÍN" (*Malus domestica* Borkh)

Esta tesis, de acuerdo con la Normativa de Estudios de Doctorado de la Universidad Miguel Hernández de Elche, se presenta como un compendio de trabajos previamente publicados, cuyas referencias son las siguientes:

Trabajo publicado:

Martínez, R., Legua, P., Martínez, J.J., Melgarejo, P. 2019. Phenological growth stages of "Pero de Cehegín" (Malus domestica Borkh): Codification and description according to the BBCH scale. *Scientia Horticulturae*, 246, 826-834. doi: 10.1016/j.scienta.2018.11.067

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Esta memoria ha sido presentada por **D. Ramón Martínez García**, Ingeniero Agrónomo, para obtener el título de doctor.

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Orihuela, 9 de septiembre de 2020

Dr. Dña. Juana Fernández López, Catedrática de Universidad y Coordinadora del Programa de Doctorado en Recursos y Tecnologías Agrarias, Agroambientales y Alimentarias (ReTos-AAA) de la Universidad Miguel Hernández de Elche (UMH),

CERTIFICA:

Que la Tesis Doctoral titulada "***Caracterización fenológica, climática, molecular, morfológica, físico-química y sensorial del "pero de Cehegín" (Malus domestica Borkh*)"** de la que es autor el Ingeniero Agrónomo **D. Ramón Martínez García**, ha sido realizada bajo la dirección del **Dr. D. Pablo Melgarejo Romero (UMH)** y la codirección del **Dr. D. Juan José Martínez Nicolás (UMH)**, actuando como tutora de la misma la Dra. Dña. Pilar Legua Murcia (UMH). Considero que la Tesis es conforme, en cuanto a forma y contenido, a los requerimientos del Programa de Doctorado ReTos-AAA por tanto, apta para su exposición y defensa pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a catorce de noviembre de dos mil veinte.

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Coordinadora del Programa Doctorado ReTos-AAA

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A todos, muchas gracias.

ÍNDICE

ABREVIACIONES Y SÍMBOLOS

ABREVIACIONES

FW	Peso del fruto
D1	Diámetro ecuatorial 1 del fruto
D2	Diámetro ecuatorial 2 del fruto
AD	Diámetro medio ecuatorial del fruto
A1	Altura 1 del fruto
A2	Altura 2 del fruto
AH	Altura media del fruto
PCw	Anchura de la cavidad peduncular
PCd	Profundidad de la cavidad peduncular
CCw	Anchura de la cavidad calicina
CCd	Profundidad de la cavidad calicina
Sv	Semillas viables
Sa	Semillas abortivas
F	Dureza
TSS	Total solidos solubles
TA	Acidez total
MI	Índice de madurez
AA	Actividad antioxidante
Moisture	Humedad
TPC	Contenido de polifenoles totales
BBCH	Biolo-gischeBundesantalt, Bundessortenamt y Chemische Industrie
GDH	Grados horas de crecimiento
SE	Error estándar

SÍMBOLOS

ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic)
DPPH	(2,2-difenil-1-picrilhidrazil)
FRAP	Poder antioxidante para reducir iones férricos
L*	Luminosidad
a*	Coordenadas rojo/verde (+a indica rojo, -a indica verde)
b*	Coordenadas amarillo/azul (+b indica amarillo, -b indica azul)
C*	Croma o saturación
Hº	Ángulo de matiz

PRODUCCIÓN CIENTÍFICA DURANTE EL PERIODO PREDOCTORAL

Calidad del compendio de cada publicación:

PUBLICACIÓN 1

Phenological growth stages of "Pero de Cehegín" (*Malus domestica* Borkh): Codification and description according to the BBCH scale

Autores: Ramón Martínez, Pilar Legua, Juan J. Martínez-Nicolás, Pablo Melgarejo

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Categoría JCR	Categoría del cuartil	Rango	Factor de impacto	Factor de impacto de los últimos 5 años
Horticulture	**Q1**	**5/36**	**2,769 (2019)**	**2,844**

PUBLICACIÓN 2

Molecular, Physico-Chemical and Sensory Characterization of the traditional Spanish apple variety "Pero de Cehegín"

Autores: Ramón Martínez, Pilar Legua, Francisca Hernández, Ángel A. Carbonell-Barrachina, Yolanda Gogorcena, Juan J. Martínez-Nicolás, Pablo Melgarejo

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Ambito de la publicación : Agronomía

Categoría JCR	Categoría del cuartil	Rango	Factor de impacto	Factor de impacto de los últimos 5 años
Agronomy	**Q1**	**18/91**	**2,603 (2019)**	**n/a**

ESTRUCTURA DE LA TESIS

Para la elaboración de la presente Tesis Doctoral se ha seguido la metodología basada en compendio de publicaciones. Para la redacción de la misma, se ha seguido la normativa vigente en la Universidad Miguel Hernández de Elche.

La Tesis Doctoral se estructura en las siguientes partes:

1. Introducción
2. Objetivos
3. Resumen de la Metodología
4. Publicaciones Científicas
5. Resumen de los Resultados, Discusión y Conclusiones
6. Conclusiones Generales e Investigaciones Futuras
7. Referencias Bibliográficas

La **introducción** contiene una breve revisión bibliográfica sobre el *"Pero de Cehegín"*. En la segunda parte se describen los **objetivos** estimados en la presente Tesis Doctoral. En la tercera parte se detalla un **resumen de la metodología** utilizada para la recopilación de datos y los programas informáticos utilizados para el tratamiento estadístico de los datos. En el apartado de **publicaciones científicas** se recogen cada una de las dos siguientes publicaciones que componen esta Tesis Doctoral. El **resumen de los resultados, discusión y conclusiones** muestra un resumen de los resultados más interesantes e importantes conseguidos en esta tesis doctoral, una discusión general de los mismos y las conclusiones de cada publicación. Seguidamente se indican las **conclusiones generales** obtenidas con los estudios realizados en esta Tesis Doctoral y las **investigaciones futuras.** Y por último se recogen las **referencias bibliográficas** consultadas para la elaboración de esta memoria, y citadas fuera de las publicaciones científicas.

RESUMEN

La recuperación de variedades locales y tradicionales, es una acción muy importante en el ámbito de la conservación de los recursos fitogenéticos. Estas variedades antiguas y locales, a menudo tienen propiedades específicas y poco comunes que las pueden hacer interesantes en comparación con las variedades comerciales, por lo que es prioritario caracterizarlas adecuadamente y poner en valor las propiedades que poseen, bien para su comercialización o bien para la mejora genética de otras variedades o de ellas mismas.

La presente Tesis Doctoral tiene como objetivo principal dar a conocer las características de la variedad tradicional conocida como "Pero de Cehegín". Para alcanzar este objetivo se plantean los siguientes objetivos específicos: (I) documentación sistemática y gráfica de los estados fenológicos de desarrollo por medio de la codificación BBCH; (II) cálculo de unidades frio necesarias, desde el comienzo de la caída de hojas hasta el final de la latencia invernal y cálculo de calor como grados horas de crecimiento (GDH) hasta la plena floración; (III) caracterización genética y comparación con las bases de datos nacionales; (IV) caracterización morfológica, físico-química y sensorial de algunos de sus clones conservados en los bancos de germoplasma; (V) comparación del "Pero de Cehegín" con variedades estándar en el mercado para conocer sus principales características distintivas.

ABSTRACT

The recovery of local and traditional varieties is a very important action for the conservation of phylogenetic resources. These old and local varieties often have specific and rare properties that can make them interesting compared to commercial varieties, so it is a priority to properly characterize them and value the properties they possess, either for their classification or for the genetic improvement of other varieties or of themselves.

The main objective of this Doctoral Thesis is to present the characteristics of the traditional variety known as "Pero de Cehegín". To achieve this objective, the following

specific objectives are proposed: (I) systematic and graphic documentation of the phenological stages of development by means of BBCH coding; (II) calculation of necessary cold units, from the beginning of leaf fall to the end of winter dormancy and calculation of heat as degrees hours of growth (GDH) until full flowering; (III) genetic characterization and comparison with national databases; (IV) morphological, physico-chemical, and sensory characterization of some of its clones preserved in the germplasm banks; (V) comparison of "Pero de Cehegín" with standard varieties in the market to know its main distinctive characteristics.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

El manzano (*Malus* domestica Borkh.), uno de los frutales más cultivados en el Mundo, es el resultado de una hibridación interespecífica (Janick *et al*., 1996; Forsline *et al*., 2003) y aunque la familia de las Rosaceae, a la que pertenece el género *Malus*, está ricamente representada en la flora europea (Strasburger *et al*, 1990), parece que es resultado de un largo proceso evolutivo cuyo centro de origen se considera en Asia Central (Velasco *et al*., 2010; Cornille *et al*., 2014) e hibridaciones posteriores con otras especies de manzanos como *Malus dasyphylla* Borkh (Chevalier, 1953), *Malus orientalis* Uglitz, *Malus baccata* (L.) Borkh. y *Malus silvestris* (L.) Miller, que han contribuido a su diversidad actual (Vavilov, 1926; Harris *et al*., 2002; Cornille *et al*., 2012, 2014; Gross *et al*., 2014).

En 2018, los manzanos han sido el tercer cultivo mundial de frutas más importante después de todos los tipos de cítricos y plátanos (FAO, 2020) y el tercero más importante en España después de todos los cítricos y grupo de melocotones y nectarinas (MAPA, 2020a), si bien también es una de las frutas que más se importa en España junto con la piña, el plátano y el kiwi (MAPA, 2020b). La producción global de manzana se basa principalmente en variedades descubiertas de forma espontánea (por ejemplo, "Golden Delicious", "Red Delicious", "Braeburn", "McIntosh" o "Jonathan" y algunos de sus mutantes) o en selecciones derivadas principalmente de estos cultivares a través de hibridación controlada (Laurens *et al*., 2012).

Como en otros países europeos, la producción española tradicionalmente se ha basado en un número muy limitado de cultivares, existiendo una polarización muy manifiesta en el grupo "Golden" que sigue siendo hoy en día, la más producida, por ser también la más conocida por el consumidor. En el periodo 2008-2018, la variedad "Golden Delicious" acaparó el 45 % de la producción nacional (MAPA, 2020a). Esta excesiva uniformidad de los cultivos ha sido una tendencia que ha venido caracterizando la agricultura desde la segunda mitad del siglo XX (Rivera *et al*., 1997), ya que la mayoría de las variedades cultivadas se obtuvieron de un número reducido de linajes progenitores y, por lo tanto, comparten un alto grado de identidad (Noiton y Alspach, 1996). Como consecuencia, en este último siglo, muchos de los cultivares

tradicionales o localmente adaptados han sido considerados obsoletos y reemplazados, lo que irremediablemente lleva a una dramática pérdida de diversidad genética.

En el caso del manzano, los primeros esfuerzos encaminados a preservar los cultivares de manzano autóctonos españoles ya comenzaron a finales de la década de 1950, cuando se estableció una colección nacional en la Estación Experimental Aula Dei en Aragón. Posteriormente, los recursos genéticos de manzanos españoles se han conservado en colecciones regionales, ubicadas principalmente en la zona norte de España (Galicia, Asturias, Navarra, Aragón y Cataluña), que representan casi la totalidad de los recursos genéticos de la manzana española. Estas colecciones contienen principalmente cultivares locales recolectados de sus respectivas regiones y que coinciden con los principales centros de producción de manzana nacional.

Sin embargo, otras zonas de España, como el Sudeste, no fueron lo suficientemente prospectadas, y muchos cultivares tradicionales de manzana no fueron considerados e introducidos en esas colecciones. Uno de estos cultivares, es el conocido como "Pero de Cehegín", que a pesar de ser conocido localmente desde hace siglos no fue tenido en cuenta por el Departamento de Pomología de la Estación Experimental de Aula Dei, en el estudio de variedades de frutales de hueso y de pepita de toda España para el establecimiento de las colecciones que inició en 1950 y que continuó hasta 1975; en este estudio fue nombrado únicamente como variedad no bien definida recogida en Murcia con el nombre de "Pero de Cehegín". Por ser una variedad autóctona apenas conocida fuera de la comarca, tampoco fue tenida en cuenta en 1960 por la Comisión Nacional de Estimación y Expertos frutales dentro de la lista de variedades de cultivo autorizadas en los viveros.

Consciente de ello, y dada la gran erosión genética a la que estaba sometida la variedad, y el cada vez menor número de ejemplares cultivados, a finales del siglo pasado inicié junto con el profesor D. Pablo Melgarejo (Departamento de Producción Vegetal de la Universidad Miguel Hernández de Elche), un trabajo de recuperación del "Pero de Cehegín", lo que permitió la creación de dos colecciones, una ubicada en la Escuela Politécnica superior de Orihuela y otra en el propio municipio de Cehegín. Estas colecciones se han mantenido y ampliado desde entonces, permitiendo la conservación

a día de hoy de un total de 27 accesiones de "Pero de Cehegín", y evitando muy probablemente, la desaparición definitiva de muchas de ellas, dado el estado de abandono en el que se encontraban. Las colecciones, y continuando con el trabajo inicial comenzado con el "Pero de Cehegín", las he ido ampliando a los largo del tiempo con la introducción de ejemplares de variedades tradicionales de la Cuenca del Río Segura, muchas de ellas en peligro de extinción. Algunas de las accesiones incluidas en los bancos corresponden a las variedades locales conocidas con los nombres de "Pero de mata", "Pero de Alguazas" "Manzano de la Cuesta de Gost", "Pero de Abanilla", "Manzana del tío Caenas" ó "Pero de Blanca", si bien estas accesiones no son objeto de esta tesis.

El municipio de Cehegín se encuentra en el Noroeste de la provincia de Murcia y principalmente en éste, así como en los municipios cercanos es donde esta variedad tradicional, el "Pero de Cehegín", encuentra un hábitat adecuado para su cultivo, con buena adaptación al suelo y a las condiciones climáticas, encontrándose en la comarca casi la totalidad de la diversidad genética que presenta. No ha sido cultivado de forma intensiva, por el contrario, esta variedad local se ha cultivado tradicionalmente en pequeños huertos, asociado a otros cultivos y en número reducido, siendo el principal destino de la cosecha el consumo propio, o su venta en los mercados locales. Conservada durante siglos gracias a esta agricultura "tradicional", es ya citado en la Edad Media como unos de los cultivos más abundantes del municipio con el nombre de "peral invernisco" en una descripción de la economía de Cehegín, entre los años 1344-1507 (de Maya, 1996). Todavía hoy es posible encontrar algunos pocos ejemplares antiguos en lugares donde ha existido huerta tradicionalmente.

Los "Peros de Cehegín", también llamados "Peros de Alcuza", por su peculiar forma semejante a la "Al-kuza", palabra de origen árabe que definía a la vasija que contenía el aceite, o "Peros de Invierno", por su excepcionales condiciones naturales de conservación, que lo mantenían comestible durante todo el invierno hasta comienzos de la primavera, han sido durante mucho tiempo un emblema identificativo del municipio. Cualidades como el dulzor y el fragante aroma, le han hecho además acreedor de una merecida fama.

El "Pero de Cehegín" se encuentra enmarcado dentro de los "Manzanos cultivados comunes", en el género y especie *Malus domestica* Borkh. (Rivera *et al*., 1997).

La sistemática de este cultivo queda como sigue:

División: Fanerógamas

Subdivisión: Angiospermas

Clase: Dicotiledóneas

Subclase: Rosidae

Orden: Rosales

Familia: Rosaceae

Género: *Malus*

Especie: *M. domestica* Borkh

Junto a la recuperación de variedades locales y tradicionales, la caracterización de las mismas, son dos de las actividades prioritarias en el ámbito de la conservación de los recursos fitogenéticos (Urrestarazu, *et al*., 2012a). Las variedades antiguas y locales, a menudo tienen propiedades específicas y poco comunes que las pueden hacer interesantes en comparación con las variedades comerciales. De hecho, la variabilidad genética y la diversidad alélica presente en variedades tradicionales pueden ser de gran interés en términos de respuesta a la selección en adaptación hacia un entorno cambiante (Caballero y García-Dorado, 2013). Por lo tanto, su diversidad alélica podría ser esencial para la mejora de los cultivos, proporcionando la presencia de rasgos interesantes para el desarrollo de nuevas variedades.

Para poner en valor estas variedades, es importante dar a conocer las propiedades que poseen y que a la vez las distinguen de las variedades estándar en el mercado (Mitre *et al*., 2009; Dan *et al*., 2015), por lo que es extremadamente importante evaluar sus características. Conocidas sus características, la reintroducción de estas variedades y su uso sostenible, representa la mejor manera de preservar este germoplasma para las generaciones futuras (Bignami *et al*., 2003).

Para una caracterización y evaluación precisas, un primer paso es definir, mediante marcadores fenotípicos, rasgos visibles que son altamente heredables y se expresan en todos los entornos (Morico *et al*., 1998). El estudio del comportamiento fenológico de los cultivos como parte de un ambiente bien caracterizado es importante, tanto para obtener resultados de producción satisfactorios como para determinar las técnicas agronómicas más adecuadas (Rea y Eccel., 2006), además de ayudar en la correcta caracterización de las accesiones presentes en los bancos. Las etapas fenológicas de las plantas se describen utilizando la escala BBCH (Biolo-gische Bundesantalt, Bundessortenamt, y Chemische Industrie) con su sistema uniforme de codificación y descripción (Lancashire *et al*., 1991; Hack *et al*., 1992; Meier, 1997). La escala básica de BBCH está representada por dos dígitos que representan las etapas de crecimiento primario y secundario. Esta escala consta de 10 etapas principales (0–9), que se dividen en 10 etapas de crecimiento secundarias (0–9). Cada etapa representa fases de desarrollo claramente reconocibles y distinguibles de la planta.

Junto a la caracterización fenológica, una buena caracterización climática, podemos considerarlas como dos herramientas básicas para dar información importante sobre las variedades a elegir y cuáles son las mejores prácticas de cultivo a seguir (Rea y Eccel., 2006). En las especies caducifolias, para salir del reposo, tanto las yemas florales como vegetativas, deben primeramente estar expuestas a temperaturas bajas (período considerado de acumulación de frío), y posteriormente a temperaturas cálidas, (período de acumulación de calor). Estas dos etapas diferenciadas, son conocidas como endodormancia y ecodormancia, respectivamente (Lang *et al*., 1987). La determinación de los requerimientos de frío, calculados como unidades frio (UF) y de calor, calculados como GHC (Grados horas de crecimiento) puede realizarse a través de la relación entre la temperatura media promedio de cada periodo.

Igualmente importante para una buena caracterización, es el estudio de los rasgos morfológicos, los cuales se han utilizado ampliamente para discriminar entre variedades de la misma especie (Cantini *et al*., 1999; Barranco y Rallo, 2000) y moleculares por medio de los marcadores de ADN desarrollados para genotipar germoplama. Entre estos, las repeticiones de secuencia simple (SSR) o microsatélites son los marcadores de elección en muchas especies de frutas (Bouhadida *et al*., 2010; Martín *et al*., 2011; Öz *et*

al., 2013). Para el caso de las manzanas, una gran cantidad de marcadores SSR han sido descriptos (Gianfranceschi *et al*., 1998; Hokanson *et al*., 1998; Liebhard *et al*., 2002; Silfverberg-Dilworth *et al*., 2006) y proporcionan una herramienta muy válida para evaluar la diversidad genética, descubrir duplicados o posibles sinonimias y homonimias, y ayudar en la gestión de las colecciones.

Por otro lado, la caracterización físico-química y sensorial aporta información respecto a la calidad del producto. Schuphan (1961) definió la calidad como un conjunto de factores que consisten en características externas (valor de mercado), características tecnológicas (valor de transformación), características internas (valor nutricional), valor de imagen (basado en conceptos psicológicos e irracionales) y valor sensorial (rasgos organolépticos). En la calidad externa de la fruta influyen valores como el color, forma, tamaño, mientras que la calidad interna (que determina la calidad de la alimentación) incluye valores como el sabor, textura, valor nutricional, dulzura y acidez (Shewfelt, 1999; Kingston, 2010). En general, la calidad y la aceptabilidad del consumidor de las frutas de manzana están asociadas con su atractivo sensorial y composición química (Alberti *et al*., 2017; Musacchi y Serra, 2018).

En conjunto, la caracterización y estudio de todos los parámetros señalados nos ayudarán a conocer y cuantificar objetivamente las características de esta peculiar variedad local conocida como "Pero de Cehegín"

2. OBJETIVOS

2. OBJETIVOS

El objetivo general de esta tesis fue poner en conocimiento las características principales del "Pero de Cehegín", por lo que para alcanzar dicho objetivo, se han planteado los siguientes objetivos específicos:

I. Documentación sistemática y gráfica de los estados fenológicos de desarrollo por medio de la codificación BBCH.

II. Cálculo de unidades frio necesarias, desde el comienzo de la caída de hojas hasta el final de la latencia invernal y cálculo de calor como grados horas de crecimiento (GDH) hasta la plena floración.

III. Caracterización genética y comparación con las bases de datos nacionales.

IV. Caracterización morfológica, físico-química y sensorial de algunos de sus clones conservados en los bancos de germoplasma.

V. Comparación del "Pero de Cehegín" con variedades estándar ampliamente comercializadas para conocer algunas de sus características distintivas.

3. RESUMEN DE LA METODOLOGÍA

3. RESUMEN DE LA METODOLOGÍA

3.1 Condiciones Experimentales y Material Vegetal

Para el estudio de la codificación BBCH, cálculo de unidades frío y cálculo de grados horas de crecimiento, los datos fueron obtenido de árboles adultos (20 años) de "Pero de Cehegín" *Malus domestica* Borkh., cultivados en Cehegín (Murcia), con coordenadas UTM X: 608020 Y: 4217507, bajo condiciones homogéneas y riego por goteo.

Para el análisis de las características físico-químicas, componentes bioactivos y análisis sensorial, se seleccionaron 5 clones de "Pero de Cehegín", uno de manzano Golden Delicius y otro de Fuji, del banco de germoplasma localizado en Cehegín (Murcia). Los árboles, injertados sobre el patrón M9, tenían 12 años de edad, estaban plantados a un marco de 4x2 m, con fertirrigación y cultivados en condiciones homogéneas. En noviembre de 2017, por cada clon, se recolectaron en tres árboles, 60 frutos (20 por árbol), evitando frutos dañados, o con cualquier fisiopatía.

Para el análisis molecular, se recolectaron hojas de estos 5 clones de "Pero de Cehegín" en el banco de germoplasma de Cehegín. Se congelaron doscientos miligramos en nitrógeno líquido y se molieron hasta un polvo fino usando el molino mezclador MM400 (Retsh, Haan Alemania) y se almacenaron a -20 °C hasta su posterior procesamiento y análisis.

3.2 Codificación BBCH y Cálculo de Horas Frío y GDH

Las fotografías se tomaron con una cámara digital (Canon EOS 350D), y las mediciones se realizaron con un calibrador digital (Mitutoyo modelo CD-15DC Digimatic). Los datos climáticos fueron tomados de una estación meteorológica del sistema de información agrícola de Murcia, identificado como C52-Cehegín, con coordenadas UTM X: 606972 Y: 4218110. Para determinar las unidades frio necesarias, desde el comienzo de la caída de hojas hasta el final de la latencia invernal, se utilizó el método de Utah (Richardson *et al*., 1974). A partir de ese momento, se calculó la acumulación de calor como grados horas de crecimiento (GDH), utilizando el Modelo de Carolina del Norte (Shaltout y Unrath, 1983) hasta la plena floración.

3.3 Análisis de Marcadores Moleculares SSR

El ADN genómico se aisló con el kit Qiagen Dneasy Plant Mini (Qiagen, Hilden, Alemania) de acuerdo con las instrucciones del fabricante. La concentración de ADN se determinó en un NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, EE. UU.) y la concentración de ADN se ajustó a 5 ng μL^{-1} para PCR multiplex. Las reacciones de PCR se llevaron a cabo con 13 SSR, 11 recomendadas por el Grupo de Trabajo ECP / GR *Malus* / *Pyrus* (Programa Cooperativo Europeo para Recursos Fitogenéticos; Lateur *et al*., 2013) (CH04e05, CH01h10, CH02d08, CH01f02, CH02c11, CH04c07, CH02c09 y CH01h01; GD12, GD147, Hi02c07) más dos debido a su utilidad para discriminar los cultivares españoles (CH05f06 y CH03d07) (Pereira *et al*., 2017). La amplificación se realizó en un termociclador 2.700 (Applied Biosystems®), utilizando tres PCR multiplex (es decir, A, B y C; ver detalles Urrestarazu *et al*., 2012b) en un volumen final de 10 µL utilizando 10 ng de plantilla de ADN, 0,10 -015 µM de cada cebador y 1X PCR Master mix de QIAGEN kit multiplex PCR (Qiagen, Hilden, Alemania). Las condiciones de ciclo de PCR para los tres múltiplex fueron las siguientes: preincubación durante 15 minutos a 95 °C, seguido de cinco (series A y B) o siete (serie C) ciclos de toma de contacto a 95 °C durante 30s, 65 °C –1 °C / ciclo durante 1 minuto y 72 °C durante 1 minuto, seguido de 30 ciclos a 95 °C durante 30 s, 60 °C (conjuntos A y B) o 58 °C (conjunto C) durante 1 minuto, 72 °C durante 1 min y el ciclo final de 30 min de extensión a 72 °C. Para evaluar los resultados de la amplificación, 1 µl del producto de PCR se separó por electroforesis en un gel de agarosa al 2%. Las bandas de amplificación se detectaron utilizando el transiluminador GelDoc 2000 (Bio-Rad Laboratories, CA, EE.UU.) y se visualizaron mediante el software de análisis de Cantidad Uno (Bio-Rad Laboratories, CA, EE. UU.). Luego, los productos diluidos mezclados con formamida se analizaron en el secuenciador de ADN ABI PRISM (3130XL; Applied Biosystems, Foster City, CA, EE.UU.). El estándar GeneScan 500 LIZ Size se utilizó para determinar el tamaño del alelo. El análisis de fragmentos y el dimensionamiento se llevaron a cabo utilizando el software Peak Scanner versión 1.0 (Applied Biosystems).

3.4 Parámetros Físicos del Fruto

Para evaluar los atributos de calidad del fruto, se midieron en 20 frutos por clon las siguientes variables físicas: peso del fruto (FW) (g), diámetro ecuatorial (D1) (mm), diámetro ecuatorial (D2) (mm), media del diámetro ecuatorial (DM=(D1+D2)/2) (mm), longitud del fruto (A1) (mm), longitud (A2) (mm), media de la longitud del fruto (Am=(H1/H2)/2) (mm), anchura de la cavidad del pedúnculo (PCw) (mm), profundidad de la cavidad del pedúnculo (PCd) (mm), anchura de la cavidad calicina (CCw) (mm), profundidad de la cavidad calicina (CCd) (mm), apertura de lóculos (OL), número de semillas viables (Sv), número de semillas abortivas (Sa) y Firmeza (F) (Kg cm^{-2}). Las mediciones de longitud se realizaron con un calibrador digital electrónico Mitutoyo (modelo CD-15 DC, Inglaterra, con una precisión de 0,01 mm). El peso se midió usando una balanza digital Sartorius (modeloBL-600, con una precisión de 0,01 g). La firmeza se midió con un penetrómetro Bertuzzi (modelo FT 327). El color se determinó utilizando el sistema CIE L * a * b * y un colorímetro Minolta modelo C-300 con iluminante D65 y 10° de error de observación (Minolta Corp., Osaka, Japón) acoplado a un procesador de datos Minolta DP-301. El ángulo Hue (matiz) se calculó [H^{o} = arctan (b*/a*)] y croma [C* = $(a^{*2} + b^{*2})^{1/2}$]. La medición del color se hizo en la superficie del fruto en cuatro caras opuestas en la zona ecuatorial. Para el contenido de humedad, las muestras se secaron a 60 °C hasta peso constante (Horwitz y Latimer, 2005).

3.5 Sólidos Solubles Totales, Acidez e Indice de Madurez

Los sólidos solubles totales (TSS) se evaluaron con un refractómetro digital Atago N1 (Atago Co. Ltd., Tokio, Japón) a 20 °C, y se expresaron como un porcentaje (°Brix). La acidez titulable total (TA) se determinó por triplicado usando un dispositivo de titulación automático (877 Titrino plus, análisis de iones Metrohm CH9101, Herisau, Suiza) con NaOH 0,1 N hasta pH 8,1, usando 1 ml de zumo diluido en 25 ml de agua destilada, y los resultados expresados en gramos de ácido málico por litro. El índice de madurez (MI) se calculó como la relación entre TSS / TA. Todos los análisis se realizaron por triplicado para garantizar la precisión, y los resultados se expresaron como media ± error estándar (SE).

3.6 Actividad Antioxidante (AA) y Contenido de Polifenoles Totales (TPC)

La actividad antioxidante (AA) se evaluó mediante tres métodos analíticos diferentes: $ABTS^{+}$, DPPH• y FRAP. Los métodos $ABTS^{+}$ y FRAP se aplicaron según Re *et al*. (1999) y Benzie y Strain (1996), respectivamente. El método DPPH• se aplicó según lo descrito por Brand-Williams *et al*. (1995) con una modificación en el tiempo de reacción (Nuncio-Jáuregui *et al*., 2015). Los resultados (media ± SE) fueron expresados como mmoles Trolox kg^{-1} fw. El TPC se midió utilizando el método colorimétrico Folin - Ciocalteu descrito por Singleton *et al*. (1999). La absorbancia del color azul resultante se midió a 765 nm usando un espectrofotómetro visible UV (Termospectromic Helios Gamma UVG 1002 E, Cambridge, Reino Unido). Todas las determinaciones se realizaron por triplicado y los resultados se expresaron como equivalentes de ácido gálico (GAE), mg 100 g^{-1} fw.

3.7 Extracción de Azúcares

El perfil azúcares se realizó según lo descrito por Hernández *et al.* (2016) usando un equipo de HPLC con alguna modificación. La extracción consistió en la homogeneización de 1 ml de muestra con 5 ml de tampón fosfato seguido de filtración e inyección. Se utilizaron una columna (columna Supelcogel TM C-610H de 30 cm × 7,8 mm) y una columna previa (Supelguard 5 cm × 4,6 mm, Supelco, Inc., Bellefonte, PA). Los azúcares se detectaron utilizando el detector de índice de refracción (RID).

Los azúcares de Sigma (Poole, Dorset, Reino Unido) se utilizaron para las curvas de calibración y cuantificación, y mostraron buena linealidad ($R^2 \geq 0,999$). Los análisis se realizaron por triplicado y los resultados se expresaron como %.

3.8 Análisis Sensorial

El análisis sensorial fue realizado por cinco panelistas capacitados (de 40 a 60 años; 2 mujeres y 3 hombres), con más de 500 h de capacitación en pruebas sensoriales, del departamento de Tecnología Agroalimentaria (UMH). El estudio se realizó en una

sala de degustación normalizada de la UMH (21 ± 2ºC y 55 ± 5% de humedad relativa) con 15 cabinas sensoriales. Las muestras se sirvieron aleatoriamente en vasos de plástico cubiertos de 90 ml, desechables, libres de azúcar, a temperatura ambiente, y se codificaron con números de 3 dígitos. Galletas sin sal y agua destilada se pusieron a disposición de los panelistas o catadores para limpiar sus paladares entre muestras. Cada panelista disponía de un cuestionario para evaluar los siguientes atributos: color (color de la piel, color de la pulpa, homogeneidad del color de la piel), olor y sabor (olor a manzana, olor afrutado, olor a piña, olor a membrillo, olor a pera, acidez, dulzor, amargor, astringencia, sabor afrutado, sabor a manzana, sabor a piña, sabor a manzana-pera, postgusto) textura (dureza, crocancia, jugosidad, acorchado, granulosidad, fibrosidad). Los panelistas usaron una escala numérica y lineal de 0 a 10 para cuantificar la intensidad de los atributos, donde 0 representa nada y 10 extremadamente fuerte, con incrementos de 1. Únicamente para el atributo "color de la piel" se utilizó una escala diferente, utilizando una escala numérica de 0 a 20, donde 0 representa verde, 10 representa amarillo y 20 representa rojo.

3.9 Tratamiento Estadístico

Los resultados se analizaron mediante el programa estadístico SPSS 22.0 para Windows (SPSS Science, Chicago, IL, ESTADOS UNIDOS). Las diferencias entre cultivares ($P \leq 0.05$) fueron evaluadas por análisis de varianza (ANOVA). El método utilizado para discriminar entre medias (Prueba de rango múltiple) fue la prueba HSD de Tukey con un nivel de confianza del 95%. También se realizaron análisis de componentes principales (PCA) y análisis de conglomerados (CA). El análisis de conglomerados se aplicó a datos estandarizados para asociaciones jerárquicas empleando el método de Ward para la aglomeración y la distancia Euclidiana al cuadrado como medida de disimilitud.

4. PUBLICACIONES

PUBLICACIÓN 1

Phenological growth stages of "Pero de Cehegín" (*Malus domestica* Borkh): Codification and description according to the BBCH scale

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PUBLICACIÓN 1: TRANSCRIPCIÓN LITERAL

Phenological growth stages of "Pero de Cehegín" (*Malus domestica* Borkh): Codification and description according to the BBCH scale

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ABSTRACT

Phenological stages of the "Pero de Cehegín" (*Malus domestica* Borkh) are described here according to the BBCH scale. Based on this general scale, the phenology of "Pero de Cehegín" showed 8 of the 10 main stages (0–9): bud development, leaf development, shoot development, inflorescence emergence, flowering, fruit development, fruit maturity and senescence. The correct identification of the phenological stages in plants is greatly important for the characterization of the variety, the management of the crop and the management of diseases and plagues as well. Thus, this study will provide knowledge and will help in the dissemination of knowledge of this peculiar apple variety among growers and scientists.

Keywords: Apple tree, BBCH scale, Growth stage, Minor fruit tree, Phenological stages

1. Introduction

The "Pero de Cehegín" (*Malus domestica* Borkh) is a variety also known as "Pero de Alcuza" or "Pero de Invierno". It is mainly grown in the Southeastern region of Spain, where this variety has an adequate habitat for its cultivation, and where most of its genetic diversity is located. Also in recent years have a growing demand from the market with high prices.

The family of the *Rosaceae*, where the *Malus* belongs to, is richly represented in the European flora (Strasburger *et al*., 1993). Although wild apples (*Malus sylvestris* Miller) spontaneously grow in some places in the Iberian Peninsula, it seems that most of the cultivated apples, for the table or for cider, come from hybrids that involve other species such as *Malus dasyphylla* Borkh. that arrived from Asia (Chevalier, 1953). The "Pero de Cehegín" is found framed, according to this classification, within the "Common cultivated apples", in the genus and specie *Malus domestica* Borkh (Rivera *et al*., 1997).

The tree is very large in size, very vigorous and with vertical growth, with extensive and superficial roots. It has deciduous leaves, simple, large, pointed ovals, serrulated, with herbaceous consistency, dark-green in color and pubescent on the abaxial side, with a more or less light green color on the adaxial side, and a dense covering of hair, not easily removed. The flowers are found in an umbel arrangement, held up by regular short, thick and hairy pedicels up to 6 cm in diameter (Rivera *et al*., 1997). It usually flowers in mid-April, but it does so in a staggered manner, continuing until the mid-May or so. The full-bloom stage usually takes place between the second and third weeks of April (Martínez and Melgarejo, 2008).

The fruits are medium sized, longer than wide, and flattened on the edges of its axis. Before maturation, their color is dark green, and once mature, the color is greenish-yellow, with more color on the sun-exposed part. Its flesh is firm and of great quality, with an exquisite taste and a fragrant aroma (Martínez and Melgarejo, 2008). The maturation of the fruit takes place in the month of November (Rivera *et al*., 1997), although due to the long period of flowering, this maturation is staggered, with some fruits remaining in the tree until the first or second week of December (Martínez and Melgarejo, 2008).

Although the "Pero" denomination has enjoyed a certain esteem in the area of its cultivation, the name is used with little precision, so that for some, the name implies fruits that are characterized by their intense aroma, while for others, by the peculiar texture of their flesh, and yet for another set, the name implies an elongated, oval or conical shape. The following table shows the differences between "Pero" and "Apple" (Rivera *et al*., 1997).

Table 1. Differences between Apples and Pears.

Apples	Peros
Spherical or flattened fruit	Oval, cone-shaped or heart-shaped fruit
Stylar end more or less smooth	Stylar end surrounded by various bumps
Thin or medium skinned fruits	Thick-skinned fruit
Aromatic fruit	Very aromatic fruit
Relatively sweet	Very sweet
Flesh has a soft, juicy consistency	Very hard, compact and starchy
Difficult to conserve without refrigeration	Relatively good for storage without refrigeration

The excessive uniformity of the crops has been a tendency that has characterized agriculture in the second half of 20th century (Rivera *et al*., 1997) and the substitution of many traditional cultivars for improved foreign ones has led to the strong genetic erosion of a given country's agricultural heritage. Therefore, the conservation of genetic diversity is very important for preventing its disappearance. To a large extent, the conservation of this diversity has depended, in many occasions, and without being conscious about it, on the growers themselves, who have made a direct selection on the specimens that are better adapted to their growing conditions. The local varieties are the result of the selection through climactic and soil adaptations and human action (Harlan, 1975a, b).

The first work on the selection and characterization of the "Pero de Cehegín" was conducted by Martínez (2000), and allowed for the characterization of a few individuals, and a first identification of the phenological stages of the variety according to the

combination of letters by Fleckinger (Martínez, 2000). But overall, this study was the start of two species collections that are found in two very different areas, one at the Polytechnic School of Orihuela (Alicante, Spain) (Miguel Hernández University) and the other in the Northwestern region of Murcia (Spain). These collections have been widened since then, with the introduction of a great number of clones, selected in the past few years, which allowed for the obtaining of a germplasm bank with 30 accessions.

The correct identification of the phenological stages of the plants is of great importance for the characterization of the variety, the management of the crop, as well as the management of diseases and plagues.

Until the beginning of the nineties, there was no uniform coding system to describe the stages of development of the main cultivated plants and weeds. In 1945, Fleckinger defined «Phenological stages» using a combination of letters and numbers (Fleckinger, 1948). However, this scale did not meet the requirements desired for the growth of fruit trees. Zadoks *et al*. (1974) published the first decimal coding system to standardize the description of the growth stages of different crops, using the same codes. Based on the descriptions of the cereals (Zadoks *et al*., 1974), a scale known as the BBCH scale (Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie), was proposed by Bleiholder *et al*. (1991), and Lancashire *et al*. (1991). A more advanced scale, the extended BBCH, was posteriorly proposed by Hack *et al*. (1992) and Hess *et al*. (1997). Later, the "BBCH monograph", which showed the phenological stages of a group of 27 crops and weeds, was published (Meier, 1997). Many researchers have used the BBCH scale to describe the phenological stages of different trees, such as the pomegranate (Melgarejo *et al*., 1997), Citrus (Agustí *et al*., 1997), Japanese loquat (Martínez-Calvo *et al*., 1999), quince (Martínez-Valero *et al*., 2001), olive (Sanz-Cortés *et al*., 2002), kaki (García-Carbonell *et al*., 2002), Búlida apricot (Pérez-Pastor *et al*., 2004), cherimoya (Cautín and Agustí, 2005), guava (Salazar *et al*., 2006), kiwi (Salinero *et al*., 2009), mango (Hernández *et al*., 2011), jujube trees (Hernández *et al*., 2015), nashi tree (Martínez-Nicolás *et al*., 2016), Indian currant (Kishore, 2017) and mulberry trees (Sánchez-Salcedo *et al*., 2017).

The study of the phenological behavior of the crops as part of a well characterized area, is important for obtaining satisfactory production results as well as for determining

the most adequate agronomical aspects (Valentín *et al*., 2001). There are numerous research studies on the interactions between climate and crops that have involved the creation of specific bio-climactic indices, such as the calculation of the end of dormancy of deciduous species, with chilling hours below 7 °C (Weinberger, 1950), the Utah model (Richardson *et al*., 1974), the North Carolina model (Shaltout and Unrath, 1983), the dynamic model (Fishman *et al*., 1987; Erez *et al*., 1988) or the modified Utah model (Linsley-Noakes *et al*., 1994). This study will help with the characterization of clones found in the germplasm banks, as well as the diffusion and knowledge of the variety among growers and scientists.

The present research is focused on describing the phonological growth stages of the ¨Pero de Cehegín¨ tree according to the BBCH scale, following the classification keys for monocot and dicot plants (Hack *et al*., 1992). Also, an initial climactic characterization of the variety will be conducted, based on the determination of the Chilling Units according to the Utah model (Richardson *et al*., 1974), from the start of leaf drop until the end of dormancy, as well as the growing degree hours (GDH), according to the North Carolina model (Shaltout and Unrath, 1983), from the end of dormancy until full bloom.

2. Materials and methods

Data were collected from adult trees (20 years old) of *Malus domestica* Borkh., grown under homogeneous conditions in Murcia (Spain) at coordinates UTM X: 608020 Y: 4217507 and 521m of altitude above sea level. A drip irrigation system was used for fertigation purposes.

The area's climate is characterized by having scarce precipitation, and a noticeable annual thermal oscillation. The average amount of rainfall in the last 20 years (from 1997 to 2016) has been 368.51 mm, with an average annual temperature of 16.01 °C (SIAM, 2017).

The plot where the selected specimens were located was visited periodically to take pictures and to observe each of the stages identified. The frequency of visits varied depending on the phenological stage studied, sometimes the plot was visited every day for the identification of the phenological stages of flowering, and in other occasions a

weekly visit was conducted to identify the phenological stage of leaf fall or fruit maturation.

Representative parts of the plants were photographed to describe and identify the different stages of phenological growth.

The study for the identification of the phenological stages was conducted for two years, in the period Jan 2015 to December 2016, with the mean of the two years used, as there were no significant differences between them. The photographs were taken with a digital camera (Canon EOS 350D), and the measurements were taken with a digital caliper (Mitutoyo model CD-15DC Digimatic) (Table 1).

The climate data was taken from a meteorological station from the agricultural information system of Murcia, identified as C52-Cehegín, with coordinates UTM X: 606972 Y: 4218110, located at 1100m from the study site. The climactic data taken into account in the study were from dates November 2014 to December 2016. For determining the chilling units needed, the Utah method was used (Richardson *et al*., 1974), which calculated the Chilling Units (CU) needed (according to the scale detailed in Table 2).

Table 2. Corresponding temperature and chill unit (CU) value of the Utah Model.

Temperature (ºC)	Chill unit (CU)
< 1.4	0.0
1.5–2.4	0.5
2.5–9.1	1.0
9.2–12.4	0.5
12.5–15.9	0.0
16.0–18.0	−0.5
> 18.0	−1.0

The calculation of the Chilling Units was conducted from the start of leaf fall until the end of the winter dormancy period with the production of buds. At this time, the accumulation of heat as growing degree hours (GDH), using the North Carolina Model (Shaltout and Unrath, 1983) until full-bloom were calculated. The following mathematical expression was used:

$$GDH = \sum_{1}^{24} (T_{mh} - 4.5)$$

where T_{mh} is average hourly temperature.

3. Results

The principal phenological stages of "Pero de Cehegín" tree are described according to the growth stage identification keys for mono and dicotyledonous plants (Hack *et al*., 1992). The extended BBCH scale considers 10 main growth stages, numbered from 0 to 9. This study handled 8 of the 10 principal stages, starting at bud development (stage 0) and ending at the senescence and beginning of the rest period stage (stage 9).

The secondary stages are also numbered from 0 to 9, and refer to either a percentage of growth value, or to different qualitative stages within the main stage. In this study, 41 secondary stages were defined.

The different phenological stages are described below:

3.1. Principal growth stage 0: bud development (Fig. 1)

00: Dormancy. Leaf and flower buds, closed and covered by darkbrown scales.

01: Beginning of leaf bud swelling. Buds visibly swollen, red elongated scales, with slightly green patches.

03: End of leaf bud swelling: greenish-brown scales slightly separated. Bud scales colored, with slightly green patches and some parts densely covered with hairs.

07: Beginning of bud burst: first green leaf tips just visible.

09: Green leaf tip about 5mm above bud scales.

3.2. Principal growth stage 1: leaf development

10: First leaves separating: Green leaf tips about 10mm above the bud scales; first leaves separating.

11: First leaves unfolded. The first young leaves unfolded, the rest of the leaves have not completely unfolded yet.

15: More leaves unfolded, but not yet at full size; petioles visible. Most leaves are unfolded, but they have not reached their final size.

19: All leaves completely unfolded and expanded. All the leaves are unfolded, and the first ones that unfolded have reached their final size.

3.3. Principal growth stage 3: shoot development

31: Beginning of shoot growth: The shoot develops, it is light green in color and has lots of hair; about 10% of final length.

33: Shoots about 30% of final length.

35: Shoots about 50% of final length.

37: Shoots about 70% of final length.

39: Shoots about 90% of final length.

3.4. Principal growth stage 5: inflorescence emergence

51: Inflorescence buds swelling; Buds are visibly swollen, elongated

red scales, with slightly green patches.

52: End of bud swelling: colored bud scales, with slightly green patches and some areas densely covered with hairs.

53: Bud burst: the green tips of the leaves are observed, although they still enclose the flowers.

54: Sepals visible, but still united (green bud). Green leaf tips protrude 10mm above the bus scales; first leaves, separating.

55: Flower buds, visible: Flower buds are observed, they are covered in hair.

56: Green bud stage: Simple flowers separating, still closed, they are covered in hair.

57: Pink bud stage: Sepals lightly open, visible red flower petals, elongating.

59: Bud stage: most of the flowers with petals that are between red and pink, forming a ball.

3.5. Principal growth stage 6: flowering

60: First flowers open. Flowers are white to pink-white.

62: Beginning of flowering: about 20% of flowers open.

64: Beginning of flowering: about 40% of flowers open.

65: Full flowering: 50% of flowers open.

67: Flower fading. Most petals falling or dry.

69: End of flowering. All petals fallen or dry. Fruit set.

3.6. Principal growth stage 7: fruit development

71: Fruit set: The diameter of the fruit is up to 10 mm; the fruits are not set yet, they fall.

72: Diameter of the fruit up to 20 mm: The diameter of the fruit measures up to 20 mm, elongated fruit.

74: Diameter of the fruit up to 40 mm: The diameter of the fruit measures up to 40 mm, the fruit are erect and elongated.

75: The fruit reach about 50% of the varietal's final size: Fruit are dark green, the typical olive oil bottle (pear-like) shape can be clearly observed.

77: The fruit reach about 70% of the variety's final size.

79: The fruit reach about 90% of the variety's final size.

3.7. Principal growth stage 8: maturity of fruit

81: Beginning of skin color change. The yellow color that is typical of the variety begins to show.

85: Advanced ripening; increase in intensity of variety-specific yellow color.

89: Fruit color fully developed. Fruit ripe for consumption, the flesh is crisp and sweet with typical taste and correct firmness. Fruit is suitable for harvesting and consumption,

and also has good conditions for storage. It has the typical yellow color with red areas on the surface that receives direct light from the sun.

3.8. Principal growth stage 9: senescence and beginning of the rest period

91: Shoot growth complete; foliage fully bright yellow-greenish. Terminal bud developed; foliage completely green still.

93: Beginning of senescence of old leaves; leaves fall.

95: 50% of leaves fallen.

97: Winter rest period. All leaves fallen.

Fig. 2 shows a schematic representation of the chronological progression of the principal growth stages of "Pero de Cehegín" (Table 3).

The climacteric characterization of the experimental plot, which can be observed in Table 4, determines 1372.75 Chilling Units according to the Utah model (Richardson *et al*., 1974) for the 2015 and 2016 growing seasons and from the start of leaf fall, until the moment previous to bud swelling. The calculation of GDH, according to the North Carolina model (Shaltout and Unrath, 1983) gave a mean result of 5818 GDH for the 2015 and 2016 growing seasons, from bud swelling until full flowering, which coincides with the phenological stage 65 according to the BBCH code described previously.

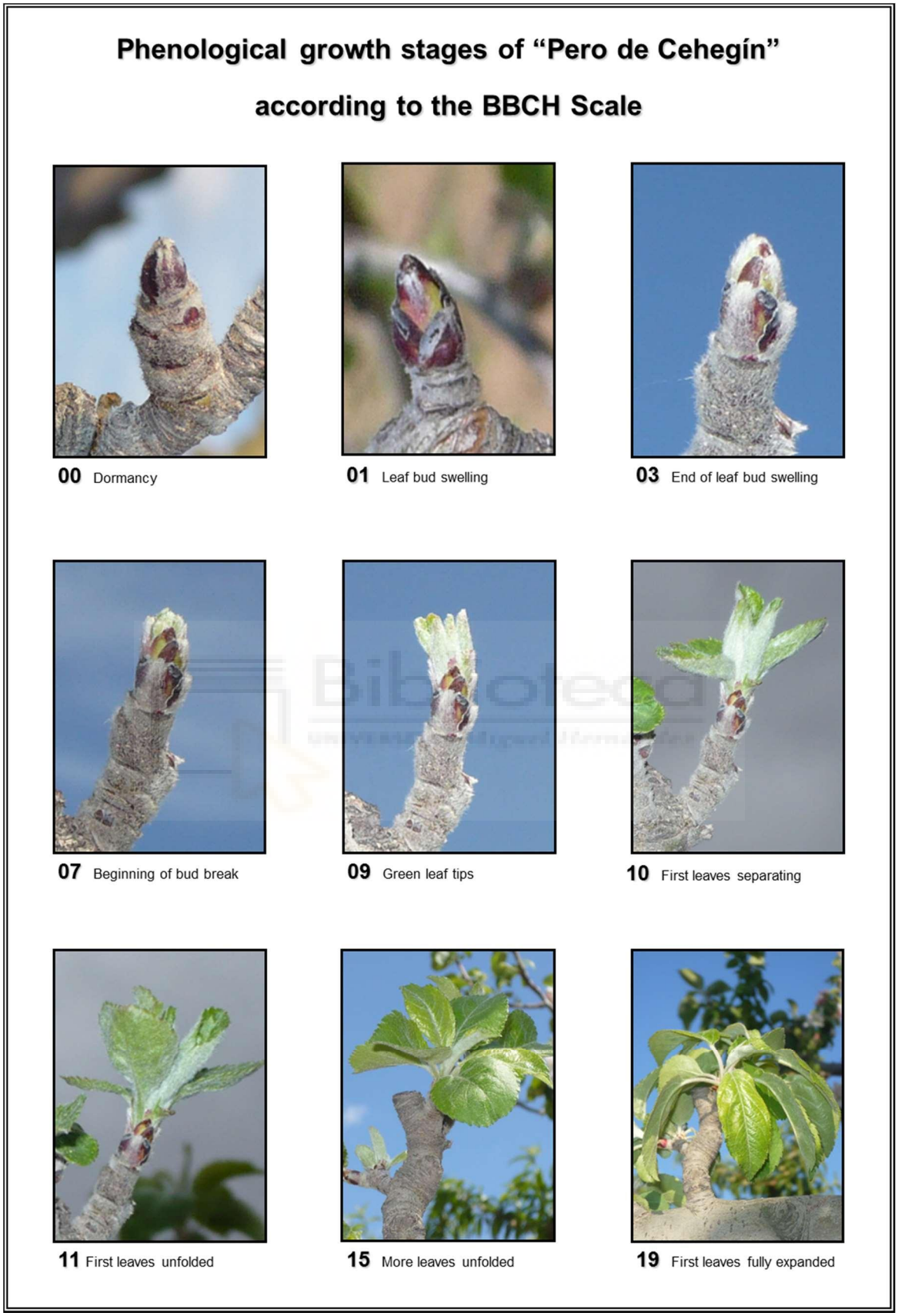

Fig. 1. Phenological growth stages of "Pero de Cehegín" tree according to BBCH-scale.

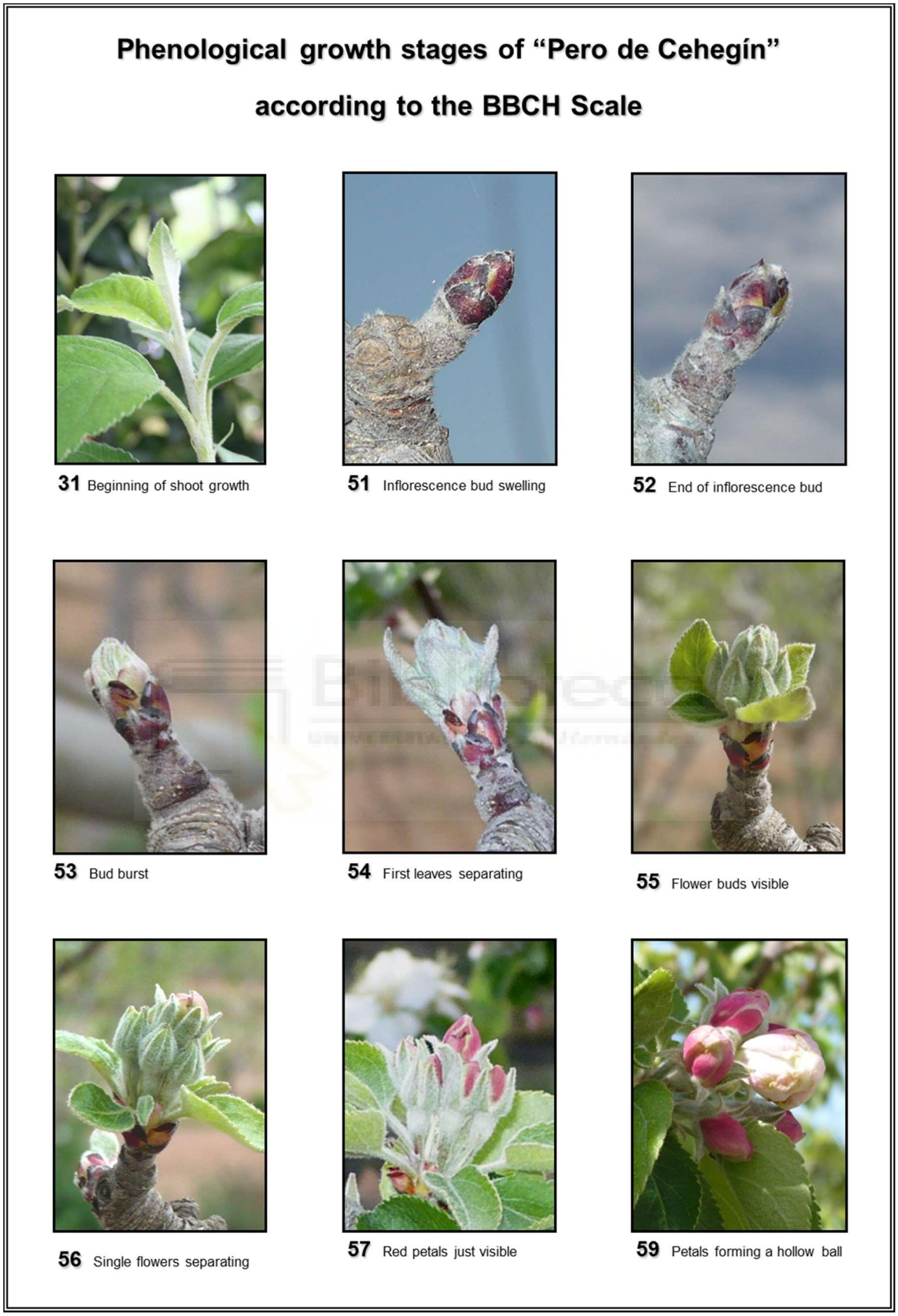

Fig. 1. (continued)

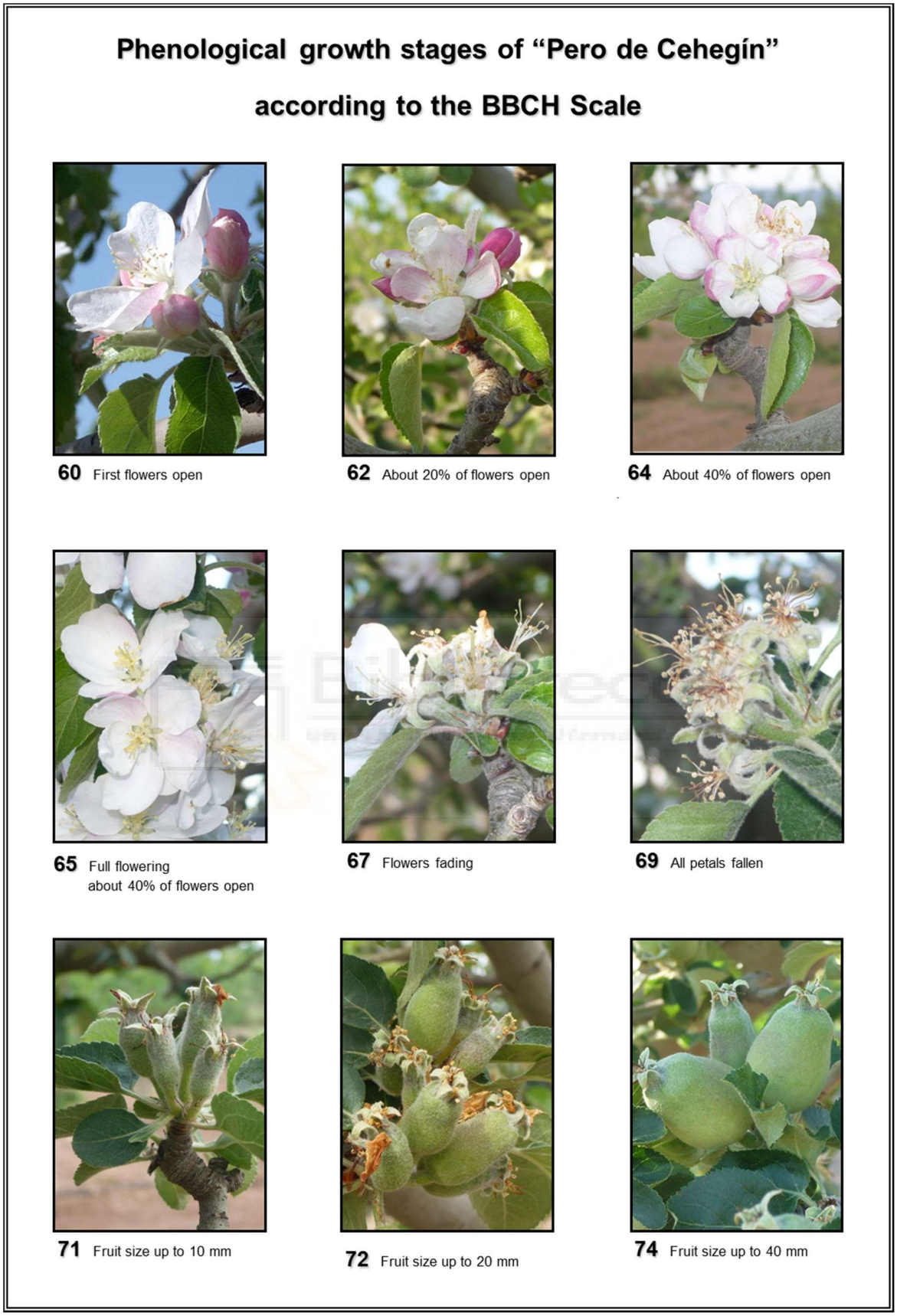

Fig. 1. (continued)

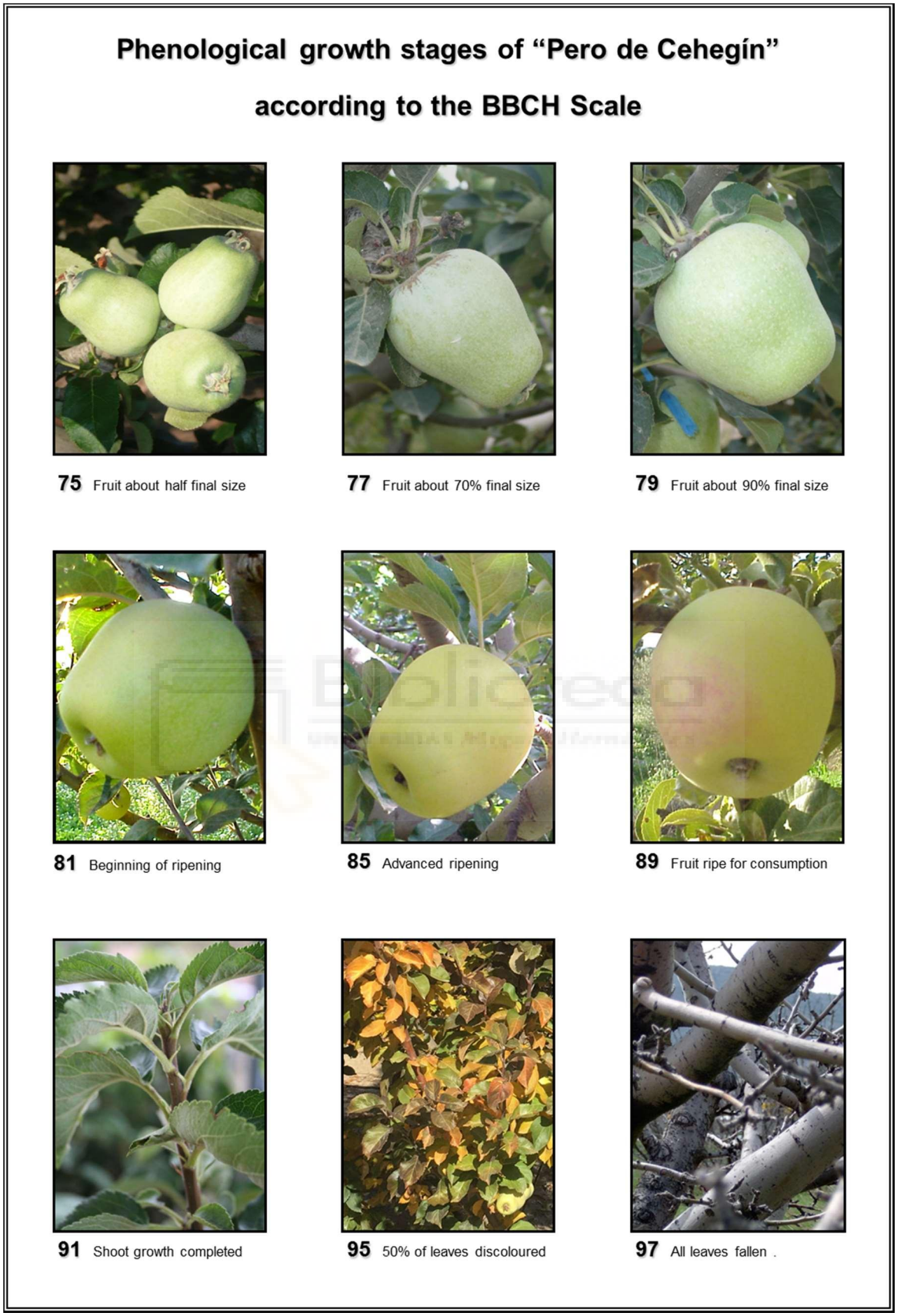

Fig. 1. (continued)

Table 3. Description of the phenological stages of Pero de Cehegín (*Malus domestica* Borkh.) according to the BBCH scale.

BBCH code	Description
Principal growth stage 0: bud development (Fig. 1) *(24 days)*	
00	Dormancy
01	Beginning of leaf bud swelling
03	End of leaf bud swelling
07	Beginning of bud burst
09	Green leaf tip about 5 mm above bud scales
Principal growth stage 1: leaf development (45 days)	
10	First leaves separating
11	First leaves unfolded
15	More leaves unfolded, but not yet at full size
19	All leaves completely unfolded and expanded
Principal growth stage 3: shoot development (110 days)	
31	Beginning of shoot growth
33	Shoots about 30% of final length
35	Shoots about 50% of final length
37	Shoots about 70% of final length
39	Shoots about 90% of final length
Principal growth stage 5: Inflorescence emergence (28 days)	
51	Inflorescence buds swelling
52	End of bud swelling
53	Bud burst
54	Sepals visible, but still united
55	Flower buds, visible
56	Green bud stage
57	Pink bud stage
59	End of bud development
Principal growth stage 6: flowering (35 days)	
60	First flowers open
62	20% of flowers open
64	40% of flowers open
65	50% of flowers open
67	Flower fading
69	End of flowering
Principal growth stage 7: fruit development (164 days)	
71	Fruit set: The diameter of the fruit is up to 10 mm
72	Diameter of the fruit up to 20 mm
74	Diameter of the fruit up to 40 mm
75	The fruit reach about 50% of the varietal's final size
77	The fruit reach about 70% of the variety's final size
79	The fruit reach about 90% of the variety's final size
Principal growth stage 8: maturity of fruit (43 days)	
81	Beginning of skin color change
85	Advanced ripening
89	Fruit color fully developed
Principal growth stage 9: senescence and beginning of the rest period (118 days)	
91	Shoot growth complete
93	Beginning of senescence of old leaves
95	50% of leaves fallen
97	Winter rest period

Fig. 2. Schematic representation of the chronological progression of the principal growth stages of "Pero de Cehegín".

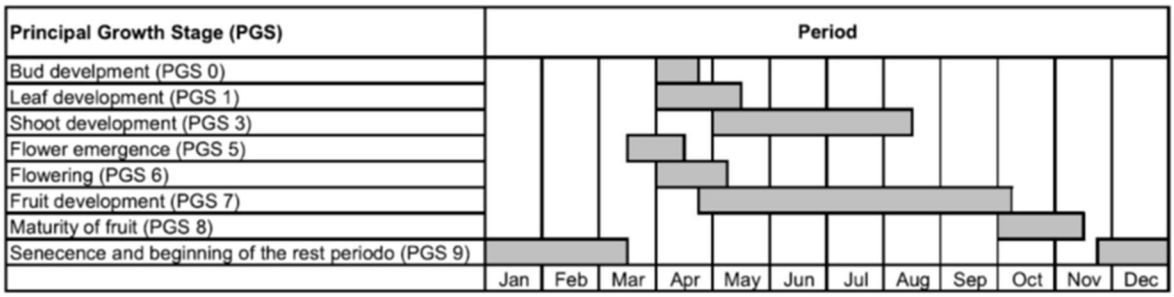

Table 4. Chilling Units and GDH values.

	Crop cycle 2015	Crop cycle 2016	Average Value
Start of leaf fall	11/22/2014	11/17/2015	19-nov
End of winter dormancy	03/14/2015	03/18/2016	16-mar
CHILLING UNITS	**1389.50**	**1356.00**	**1372.75**
Start of bud swelling	03/05/2015	03/19/2015	17-mar
Full-bloom	04/14/2015	04/13/2016	14-abr
GDH	**5739.00**	**5897.00**	**5818.00**

4. Discussion

The BBCH scale (Hack *et al*., 1992) provides a precise description of this unknown and peculiar variety of apple tree, both vegetative and reproductive, which is important for the correct programing of agricultural practices. The results of this study, despite the differences shown between the apple fruit and Peros, essentially coincide with those obtained by Meier (1997) for the phenological stages of pome fruits (apples and pears). The sequential progression of the main stages of growth indicates that the phases of reproductive growth have overlap with the vegetative one (Fig. 2). This overlap is

observed in other fruit trees such as pomegranate (Melgarejo *et al*., 1997), in Indian gooseberry (*Phyllanthus emblica* L.) (Kishore, 2017).

The knowledge on the phenology of fruit trees is absolutely essential for the precise programming of horticultural practices, such as pest management, physiological disorders, post-harvest management, and nutrients and water management. Also is important for leaf sampling for determining the seasonal demand of nutrients in agreement with the different vigor of the "Pero de Cehegín" (Mao *et al*., 1991). To correctly manage the agricultural fields of the "Pero de Cehegín" has been one of the key operations for improving performance and quality (Singh *et al*., 2014).

The scale proposed will be a good reference for growers and scientists. This coding and identification of the different phonological stages will allow for the characterization of each of the selected clones, provide data on the flower biology, facilitate of data on the influence of the environmental factors, help in the improvement of growing techniques (nutrition, thinning, treatments against plagues and diseases, the application of growth regulators, etc.) and the detection of anomalies of physiological character or any other kind.

Phenological and climatic characterizations are two basic tools for providing growers with important information on the varieties to be chosen, and the best growing practices to be followed (Valentín *et al*., 2001). In this study, the first climatic characterization of the "Pero de Cehegín" variety was conducted according to the results obtained in the study plot. This will allow us to conduct comparisons with other clones found in the germplasm banks. The response of plant phenology to climate variability varies according to the location and the crop species. The extent to which plants are influenced by climate and their capacity for intrinsic adaptation ultimately determines their production potential and ecological stability (Tang *et al*., 2016; Fitchett *et al*., 2015).

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PUBLICACIÓN 2

Molecular, Physico-Chemical and Sensory Characterization of the traditional Spanish apple variety "Pero de Cehegín"

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PUBLICACIÓN 2: TRANSCRIPCIÓN LITERAL

Molecular, Physico-Chemical and Sensory Characterization of the traditional Spanish apple variety "Pero de Cehegín

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ABSTRACT

The “Pero de Cehegín” is an ancient local variety of apple grown in Murcia (Spain). In this study, microsatellites markers showed evidence of a unique profile that has never been reported before in other Spanish apple germplasm collections. Five “Pero de Cehegín” clones were evaluated and compared with two commercial apple varieties, “Fuji” and “Golden Delicious”, to assess its marketing potential. For this, the physical (weight, height, and width of the fruit, moisture content, firmness, and color of the fruit, among others), and chemical (total soluble solids, total acidity, and maturity index) properties of the fruits were evaluated. In addition, the content of bioactive compounds such as total polyphenol content, total antioxidant activity using the ABTS+, DPPH•, and FRAP methods, and the sugar profile were analyzed, and their sensory profile was also evaluated. Physico-chemical differences were found within the “Pero de Cehegín” clones and between the commercial varieties. “Pero de Cehegín” had a high firmness, high total soluble solids, very low total acidity, high FRAP antioxidant capacity, and more sucrose content in comparison with “Fuji” and “Golden Delicious”. These distinctive characteristics and the good appearance of the fruit make this variety a marketable product that will increase the offering of traditional, local, but underutilized fruit varieties.

Keywords: bioactive compounds; diversity; genetic resources; malus × domestica borkh; SSR markers

1. Introduction

The Rosaceae family, to which the genus Malus belongs, is widely represented in the European flora [1]. It seems that most of the cultivated apple trees grown for the production of both table and cider apples come from hybrids from different species, such as Malus dasyphylla Borkh that originally came from Asia [2] and the European wild apple (Malus sylvestris Mill.); both species could be considered as the origin of the cultivated or domestic apple tree (Malus domestica Borkh.) [3,4] and some gene flow between them has been described [5].

Apple was the third-most important fruit produced and eaten around the world with a production of 86.1 million t in 2018, of which 19.6 million t corresponded to Europe. The largest producer was China, representing around 45.5% of the world's total production. The US was the second-largest producer with 5.4%, followed by Poland (4.6%), Turkey (4.2%), Iran (2.9%), and Italy (2.8%). Spain, with 0.56 million tons was the 10th European producer [6]. In Spain, the apple represents the third-most important crop after citrus fruits and the group of peaches and nectarines [7], and is also one of the most imported fruits along with pineapple, banana, and kiwi [8]. The main apple production areas are the regions of Catalonia and Aragon, with a very important polarization in the production of the group "Golden". In the 2008–2018 period, the "Golden Delicious" variety accounted for 45% of the national production [7].

The "Pero de Cehegín", framed within the "common cultivated apple trees" in the genus and species Malus domestica Borkh [9], is a local variety of apple tree, cultivated mainly in the northwest of the Murcia province (Spain). The reason for its name is that its cultivation has historically been carried out almost exclusively in the municipality of Cehegín, which has a suitable habitat for this apple variety. The fruits (Figure 1) are medium size, longer than wide, and flat at the ends of their axis. Before ripening, its color is dark green, and once ripe its changes to a greenish-yellow, being more colored in the sun-exposed area. Its pulp is firm and of great quality, and has an exquisite flavor and a fragrant aroma [10]. The fruit ripening occurs in November [9].

Figure 1. Fruit of "Pero de Cehegín".

Although the denomination of "Pero" has enjoyed some esteem in the cultivation region, the name is usually applied with little precision. Thus, for some local farmers, the "Pero" fruits are characterized by their intense aroma, for others, by a peculiar texture of its pulp, and for others by an elongated, oval, or conical shape, almost resembling a pear. Table 1 shows the differences between "Pero" and "apple" [9].

Table 1. Differences between apple and "Pero de Cehegín".

Attribute	Apple	*"Pero de Cehegín"*
Shape	Spherical or flattened	Oval, conical or heart-shaped
Stilar cavity	More or less smooth	Surrounded by several gibbosities
Peel thickness	Thin or medium	Thick
Aroma	Aromatic	Very aromatic
Sweetness	Sweet	Very sweet (acid when grown in the Middle European Region)
Pulp	Consistent to soft pulp, juicy	Pulp very hard, compact and mealy
Storage	Refrigerated	Without room temperature

Local varieties are the result of selection through climatic and edaphic adaptation and human action [11,12]. However, these traditional varieties have been replaced with

new ones of foreign origin, which causes a strong genetic erosion in the agricultural heritage. However, these old and local varieties in terms of biodiversity have an extremely high value [13], and as opposed to commercial varieties, often carry specific and uncommon properties that could be interesting to different consumers. Therefore, consumers must understand the kind of properties that distinguish them from standard varieties in the market [14,15]. Due to these reasons, the conservation of the local genetic diversity to prevent its disappearance is urgent. Along this line, Martínez [16] carried out the first prospection and selection of the "Pero de Cehegín" in several areas of Cehegín (Murcia, Spain); this work was the beginning of two germplasm collections. Germplasm banks, in their current conception, constitute essential systems for avoiding the loss of genetic biodiversity and, therefore, to guarantee the future of endangered species. The collections are located in two different areas, one in the Higher Polytechnic School of Orihuela, EPSO (UMH) in the province of Alicante, and the other in Cehegín, in the northwest of Murcia region. These collections were extended with the introduction of new clones selected in recent years from different geographic origins, and now total 27 accessions.

The genetic and phenotypic characterization of these traditional varieties will help in the conservation and use in future breeding programs. The molecular characterization of apple collections has been performed in the past using Simple Sequence Repeats (SSR). A high number of microsatellites have been described and used to identify different varieties [17,18,19,20] and to assess the genetic diversity in core collections [21,22,23,24]. In particular, there are several studies in Spanish germplasm collections [23] but to our knowledge, the "Pero de Cehegín" has not been included yet.

At present, several studies have been published about apple variety characterization from China [25], Portugal [26,27], Romania [14], Argentina [28], Brazil [29], and India [30,31].

Generally, the definition of quality and acceptability of apple fruits by consumers are associated with their sensory appeal and chemical composition [32,33]. The concept of "appearance" includes several external fruit characteristics such as size, shape, absence of defects, and color [33]. The rescue of traditional varieties, with distinctive

characteristics and good appearance, will help diversify and enrich the offerings for the consumers' demand for new apples or traditional and local varieties with good nutritional, organoleptic, and morphological characteristics.

Consequently, the present study aims to determine the genetic identity of "Pero de Cehegín" using microsatellite markers and to evaluate the main physicochemical characteristics, bioactive compounds, and sensory profile of five selected clones of "Pero de Cehegín" fruits. The "Pero de Cehegín" properties were compared with those of two commercial varieties ("Fuji" and "Golden Delicious"). The marketing potential of these five clones is also discussed.

2. Materials and Methods

2.1. Experimental Conditions and Plant Material

For the physicochemical characteristics, bioactive compounds and sensory profile, five clones of "Pero de Cehegín" (identified as P1, P2, P3, P4, P5), Malus domestica, the "Golden Delicious" variety and the "Fuji" variety, were collected from the germplasm bank located in the municipality of Cehegín (Murcia, Spain). Furthermore, the five "Pero de Cehegín" clones were also used for molecular analysis. Aiming to base the molecular characterization of "Pero de Cehegín" and its comparison, two reference varieties were taken, "Fuji" and "Granny Smith", due its international importance as commercial cultivars.

The apple trees were 12 years old and spaced following a 4 × 2 m pattern. All the clones were grafted onto the M9 rootstock and grown under standard conditions (with a loam-type soil, fertilizing with a quantity of 2 kg of organic matter and 2 kg of complex fertilizer 15% N-15% P2O5-15% K2O per tree and year, and according to Papadakis [34] for a Mediterranean climate). For each clone, 20 fruit samples were randomly collected from three trees (for a total of 60 fruits per clone) avoiding damaged fruits. Harvest was done manually in all clones at their commercial maturity stage, during November 2017, 10 days after finishing its development and reaching its characteristic color. The fruits were stored refrigerated at 3 °C until their study a few days later.

2.2. Polymerase Chain Reaction Amplification and SSR Analyses

Leaves were collected from the trees of the five "Pero de Cehegín" clones in the Cehegín germplasm bank. Two hundred milligrams were frozen in liquid nitrogen and ground to a fine powder using the mixer mill MM400 (Retsh, Haan Germany) and stored at −20 °C until further processing. Genomic DNA was isolated with Qiagen Dneasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined with a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and adjusted to 5 ng μL−1 for multiplex PCR.

PCR reactions were carried out with 13 SSRs, including 11 recommended by the ECP/GR Malus/Pyrus Working Group (European Cooperative Programme for Plant Genetic Resources; Lateur *et al.* [35] (CH04e05, CH01h10, CH02d08, CH01f02, CH02c11, CH04c07, CH02c09, and CH01h01; GD12, GD147, Hi02c07) plus another two due to their usefulness in discriminating the Spanish varieties (CH05f06 and CH03d07) [23].

The amplification was performed in a 2700 thermal cycler (Applied Biosystems®), using three multiplex PCRs A, B, and C; see details [36] in a final volume of 10 μL using 10 ng of DNA template, 0.10–015 μM of each primer, and 1X PCR Master mix from the QIAGEN multiplex PCR kit (Qiagen, Hilden, Germany). The PCR cycling conditions for the three multiplexes were as follows: pre-incubation for 15 min at 95 °C, followed by five (sets A and B) or seven (set C) touchdown cycles at 95 °C for 30 s, 65–1 °C/cycle for 1 min and 72 °C for 1 min, followed by 30 cycles at 95 °C for 30 s, 60 °C (set A and B) or 58 °C (set C) for 1 min, 72 °C for 1 min and the ending cycle 30 min extension at 72 °C. To evaluate the amplification results, 1 μL of the PCR product was separated by electrophoresis on a 2% agarose gel. The amplification bands were detected using the GelDoc 2000 (Bio-Rad Laboratories, Hercules, CA, USA) transilluminator and visualized with the Quantity One Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA). Then, the diluted products mixed with formamide were analyzed on ABI PRISM DNA sequencer (3130XL; Applied Biosystems, Foster City, CA, USA). The GeneScan 500 LIZ Size standard was used to determine allele size. Fragment analysis and sizing were carried out using the Peak Scanner Software version 1.0 (Applied Biosystems).

2.3. Physical Parameters

For each clone, 20 fruits were taken from the 60 collected and the following physical parameters were measured: fruit weight (FW) (g), equatorial diameter 1 (D1) (mm), equatorial diameter 2 (D2) (mm), average equatorial diameter (AD = (D1 + D2)/2) (mm), fruit height 1 (H1) (mm), fruit height 2 (H2) (mm), average fruit height (Am = (H1/H2)/2) (mm), width of the pedicle cavity (PCw) (mm), depth of the pedicle cavity (PCd) (mm), width of the calycine cavity (CCw) (mm), depth of the calyx cavity (CCd) (mm), aperture of locules (in transverse section) (OL) [37], number of viable seeds (Sv), number of abortive seeds (Sa), and firmness (F) (kg cm−2).

Fruit weight was measured with a Sartorius digital bench scale (model BL-600, 0.01 g accuracy). Fruit equatorial diameter, length, and depth of the pedicle cavity and the width and depth of the calycine cavity were measured with a Mitutoyo CD-15DC Digimatic caliper (0.01 mm accuracy). Pulp firmness was assessed with a Bertuzzi penetrometer (model FT 327, with an 8 mm deep probe). Apple peel color (four different measurements at four equidistant points on the equatorial region of each fruit) was assessed using a Minolta C-300 Chroma Meter (Minolta Corp., Osaka, Japan) coupled to a Minolta DP-301 data processor, and the results were expressed in the CIELab system. The mean values for lightness (L *), red-greenness (a *), and blue-yellowness (b *) coordinates for each fruit were reported. In addition, the objective color was calculated as chromaticity or chroma [C * = (a *2 + b *2)1/2] and hue angle [Hº = arctan (b */a *)]. For the moisture content, samples were dried at 60 °C for 48 h until a constant weight was reached [38].

2.4. Total Soluble Solids, Acidity, and Maturity Index

Total soluble solids (TSS) were assessed in triplicate with a digital refractometer Atago N1 (Atago Co. Ltd., Tokyo, Japan) at 20 °C, and expressed as °Brix. Total titratable acidity (TA) was determined in triplicate using an automatic titration device (877 Titrino plus, Metrohm ion analyses CH9101, Herisau, Switzerland) with 0.1 N NaOH up to pH 8.1, using 1 mL diluted juice in 25 mL distilled H2O, and the results expressed as g malic acid L−1. Once the TSS and TA contents were assessed, the maturity index (MI) of the evaluated varieties was determined (TSS/TA).

2.5. Antioxidant Activities (AA) and Total Polyphenol Content (TPC)

There are different methods for evaluating fruit antioxidant activity. This diversity of methods is because none of them are able to exactly determine the total antioxidant potential in a food system by themselves. For this reason, the antioxidant activity of apple fruits was evaluated using three different assays: (i) the free radical scavenging activity was tested using ABTS+ and DPPH• assays and (ii) the ferric reducing ability (FRAP assay). The ABTS+ (2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation and ferric-reducing antioxidant power (FRAP) methods were applied according to [39] and [40], respectively. The radical scavenging activity was evaluated using the DPPH• radical (2, 2-diphenyl-1-picrylhydrazyl) method, as described by [41] with a modification in the reaction time [42]. Calibration curves, in the 0.5–5.0 mmol Trolox L−1 range, were prepared for all three methods and showed good linearity (R2 = 0.998). Results were expressed as mmoles Trolox kg−1 fresh weight (fw).

The TPC was measured using the Folin–Ciocalteu colorimetric method described by Singleton *et al.* [43]. The absorbance of the resulting blue color was measured at 765 nm using a UV–visible spectrophotometer (Termospectromic Helios Gamma UVG 1002 E, Cambridge, UK). Calibration curves with concentrations of gallic acid as the standard were used for quantification. All determinations were performed in triplicate and the results were expressed as gallic acid equivalents (GAE) in, mg 100 g−1 fw.

2.6. Sugar Profile

Sugar profiles were obtained following the method described by Hernández *et al.* [44] using HPLC, with some modifications. The extraction consisted in the homogenization of 1 mL of sample juice with 5 mL of phosphate buffer followed by filtration and injection. A column (Supelcogel TM C-610H column 30 cm × 7.8 mm) and a pre-column (Supelguard 5 cm × 4.6 mm, Supelco, Inc., Bellefonte, PA, USA) were used for the analysis. Sugars were detected using a refractive index detector (RID).

Sugars from Sigma (Poole, Dorset, UK) were used for calibration curves and quantification and showed good linearity (R2 ≥ 0.999). Analyses were run in triplicate and results were expressed as %.

2.7. Sensory Evaluation

Seven trained panelists (aged 40–60 years; four female and three male) with more than 500 h of training in sensory testing from the Department of Agrofood Technology (UMH) participated in this study. The study was conducted in a normalized tasting room of UMH (21 ± 2 °C and 55% ± 5% relative humidity) with 15 normalized sensory cabins. Samples were randomly served into odor-free, disposable 90 mL covered plastic cups, at room temperature, and coded using 3-digit numbers. Unsalted crackers and distilled water were provided to panelists to clean their palates between samples.

Each panelist filled a questionnaire evaluating the following attributes: color (peel color, pulp color, homogeneity of peel color), flavor ((apple, fruity, pineapple, quince, and pear odor (perception of volatile compounds with the fruit outside the mouth), sourness, sweetness, bitterness, astringency and fruity, apple, pineapple, and apple-pear aroma (perception of volatile compounds with the fruit outside the mouth), aftertaste) and texture (hardness, crispness, juiciness, crispy, granularity, fibrousness). The attributes description and the reference materials used are shown in Table 2.

The panelists used a numerical scale from 0 to 10 with increments of 0.5 points, to rate the attribute's intensity, where 0 represented "no intensity" or "no perceptible intensity" and 10 "extremely strong". Spanish trained panels commonly use this scale because it is intuitive and very easy to understand. A different scale was used only for the "peel color" attribute, with a numeric scale from 0 to 20, where 0 represents green, 10 represents yellow, and 20 represents red.

Table 2. List of appearance, flavour and texture attributes used to describe apple fruits and their definitions

Attribute	Definition	References
Peel colour	Colour of the fruit peel	0=green. 10=yellow . 20=red.
Pulp colour	Colour of the fruit pulp	0=green. 10=yellow
Homogeneity peel colour	Homogeneity of colour in the fruit peel	0= none 10: very strong
Apple smell	Sweet. light fruity. Somewhat floral aromatic, associated with apple juice and apples	Hacendado* mango-apple nectar=5.5
Fruity smell	Fresh. Fruity aromatics associated with undefined fruits	Hacendado* yogurt "Macedonia"=8.5
Pineapple smell	Sour. Fresh. fruity aromatics associated with ripe pineapple	Hacendado* 100% pineapple juice=7.5
Quince smell	Floral. Fresh. and fruity aromatics associated to quince	Freshly harvested quinces. cv. Vranja=6.5
Pear smell	Sweet. Slightly musty. Floral. honey/caramel-like. Fruity aromatic associated with ripe pears	Hacendado* pear nectar=6.5
Sourness	Fundamental taste factor of which citric acid is typical	0.05 % Citric acid solution = 3.5
Sweetness	Fundamental taste factor of which sucrose is typical	2% Sucrose solution=2.0
Bitterness	Fundamental taste factor of which caffeine or quinine is typical	0.020% Caffeine solution = 3.5
Astringency	Dry puckering mouthfeel associated with an alum solution	0.05% Alum solution=2.5
Fruity flavour	A flavour blend which is sweet and reminiscent of a variety of fruits	0= none. 10=very strong
Apple flavour	A flavour commonly associated with apple juice and apples	0= none. 10=very strong
Pineapple Flavour	A flavour commonly associated with pineapple juice and pineapples	0= none. 10=very strong
Apple-Pear Flavour	Flavour reminiscent of a variety of different apples and pears	0= none. 10=very strong
Aftertaste	Remaining desirable flavour after swallowing	5s= 1; 20 s=10
Hardness	Force required to bite through	Fresh unpeeled carrot=8.5
Crunchiness	The intensity of audible noise at first chew with molars	Fresh unpeeled carrot=7.5
Juiciness	Amount of water released from the sample during chewing	Raw peeled carrot=1.5
Corkiness	Puffiness of the cross section	Marshmallow=1.0
Graininess	Amount of woody particles remaining in the mouth just before swallowing the sample	Over-ripe pear. cv. Alejandrina=6.0
Fibrosity	Stringy particles (size and amount)	Fresh strawberry=3.0

** Spanish commercial mark*

2.8. Statistical Analysis

The results were analyzed using the SPSS v.22.0 statistical program for Windows (SPSS Science, Chicago, IL, USA). The differences among varieties ($p < 0.05$) were evaluated with an analysis of variance (ANOVA). The method used to discriminate among means (Multiple Range Test) was Tukey's test ($p < 0.05$), and all the results were shown as mean values ± SE (standard error). Principal component analysis (PCA) and cluster analysis (CA) were also performed. The cluster analysis was applied to standardized data for hierarchical associations, employing Ward's method for agglomeration and the squared Euclidean distance as the dissimilarity measure.

3. Results and Discussion

3.1. Molecular Characterization

The five clones of "Pero de Cehegín" shared the same SSR profile at the 13 microsatellite markers studied. The size of the 26 alleles obtained for "Pero de Cehegín" (clone 1), "Fuji", and "Granny Smith" (references varieties) are shown in Table 3. The two reference varieties were used for size alignment and comparison with other studies.

To our knowledge, this is the first molecular study for the identification of the "Pero de Cehegín". The profile of "Pero de Cehegín", did not show correspondence with profiles of diploid accessions named "Pero" in other Iberian collections conserved at different institutions. No correspondence was found with "Pero Rufino", "Pero Rosa", and other "Pero" accessions from local and traditional apples located in Andalucía [19], "Peruco Lardero" from the germplasm bank at the University of Lleida [48] and its synonym "Pero Mingan" at Aula Dei-CSIC [36], "Pero de Coura", and other "Pero" from three Portuguese germplasm banks [49]. Additionally, correspondence was neither found with other triploid accessions such as "Pero Pardo", "Pera de Sangüesa" (syn. Moceta) at Aula Dei-CSIC, all characterized in larger studies including 498 and 1453 accessions [23] from Spanish apple germplasms. Therefore, this is the first molecular study that identifies "Pero de Cehegín" as a new genotype, not present in other Spanish germplasm banks.

Table 3. Molecular profiles (allele sizes in base pairs) of "*Pero de Cehegín*" and two commercial varieties used as reference (Fuji and Granny Smith) obtained with 13 microsatellites markers.

	Locus												
	CH01f02 [b]	CH01h01 [b]	CH01h10 [b]	CH02c09 [b]	CH02c11 [b]	CH02d08 [b]	CH03d07 [b]	CH04c07 [b]	CH04e05 [b]	CH05f06 [b]	GD12 [a]	GD147 [a]	Hi02c07 [c]
Reference													
Fuji AD 3488 *	182	116	91/97	231/243	230/234	211	227	106/118	175/202	164/174	148/154	139/153	116/116
Granny Smith AD 3196 *	180/206	112/130	97	231/241	228/232	211/250	193/227	106/112	175	170/174	150/154	133/139	114/118
Accession													
Pero de Cehegín (P1) *	168/182	112/118	101/119	251/255	210/224	211/254	193/193	98/108	175/203	174/180	133/153	152/152	114/116

[a] [45], [b] [46], [c] [47], * Alleles sizes were aligned as profiles published in Germplasm Bank at the University of Lleida [48].

3.2. Physical parameters

Table 4 shows the results obtained for the physical evaluation of five "Pero de Cehegín" clones studied. Important variability was found for fruit weight, diameter, and height among the different "Pero de Cehegín" clones. The smallest fruits were those produced by the P3 clone with a FW = 148.41 g, which was not significantly different with "Golden Delicious", while the P2 clone, with a mean weight of 219.43 g, had the largest fruits. The rest of the clones and "Fuji" apples had intermediate values.

As for the AD, we found two groups. Group 1, with the higher values, included two "Pero de Cehegín" clones (P1, P2) and "Fuji" and group 2, with lower values, included the rest of clones and "Golden Delicious". For the average height (AH) of the fruit, the P2 clone had the highest value (81.77 mm), and P3 (68.33 mm), "Fuji" (68.25 mm), and "Golden Delicious" (64.23 mm) with the lowest values. These results highlight the morphometric differences shown by "Pero de Cehegín" as compared to common commercial varieties such as "Fuji" and "Golden Delicious".

Fruit size plays an important role in consumer preferences when buying fruit because different fruit sizes can satisfy different types of consumers [33]. The literature has reported varieties with an average fruit weight above 300 g [50] but also values below 100 g [31]. The present results show that the "Pero de Cehegín" clones have an adequate size for commercialization, with values even higher than "Fuji" and "Golden Delicious" varieties, which are widely accepted by consumers. In general, large fruits are preferred by most consumers, but for fresh consumption, only up to a certain size limit [51,52]. Hampson *et al*. [53] indicated that for the dessert apples, the ideal size was between 74 and 76 mm in diameter. In the "Pero de Cehegín" clones studied the average diameter of the fruit ranged between 66 (P3) and 75 mm (P2), which agrees with the range of acceptance.

On the other hand, the PCw of the "Pero de Cehegín" P3 clone (27.86 mm) has a lower value than the rest of the clones (between 30.53 and 31.11 mm), similar to "Fuji" (28.69 mm) and "Golden Delicious" (27.15 mm). The PCd values oscillated between 14.68 and 17.16 mm, with significant differences only between the P1 and P5 clones but without differences observed with commercial varieties. The CCw varied between 25.30

and 29.89 mm. Only clones P3, P4, and P5 were non-significant with "Fuji" and "Golden Delicious". The calicine depth (CCd) varied between 12.48 and 15.91 mm, and it was only significantly different between P2 and P3 clones.

For the locule opening characteristic (in a cross-section) (OL) [37], all the studied samples had a level 2 without significant differences among them.

The number of viable seeds (Sv) for "Pero de Cehegín" clones were similar (average values between 1.05 units for P3 and 2.65 units for P5). The maximum Sv value was found for the "Golden Delicious" variety (3.75). More variability was found for abortive seeds (Sa), with mean values ranging from 0.8 units (P3) to 4.15 units (P4).

Fruit firmness in "Pero de Cehegín" clones had higher values than those of the commercial varieties, ranging from 3.65 (P1) to 4.44 kg cm−2 (P3). Firmness is one of the most important parameters perceived by consumers [54], as a high fruit firmness is often related to freshness, while fruit flouriness can be a deterrent to the increase in fresh fruit consumption [55]. Therefore, the firmness found in "Pero de Cehegín" is a positive characteristic that may help with its consumption. Firmness, juiciness, and no flouriness are the textural features preferred by consumers [56].

For moisture content, all the clones showed similar values ranging from 77.92% (P5) to 79.72% (P1). The "Fuji" variety had a value of 78.85% and the highest value was observed for the "Golden Delicious" variety (82.53%). In the literature, different varieties have been reported with values between 78.12% and 95.00% [27,29,57,58]. The low moisture content for "Pero de Cehegín" could be directly related to their good natural properties for conservation.

Similarly, the color directly affects the appearance and the acceptability of the fruit by the consumer [29]. The green, yellow, and red apple peel colors can help to distinguish the cultivar [33]. Therefore, color can be considered one of the most important fruit characteristics, aside from being one of the indicators of maturity.

The color parameters for the different studied clones are shown in Table 5. For all color traits, "Fuji" was significantly different to "Pero de Cehegín" clones and the "Golden Delicious" variety. For all the parameters, except L * and a *, all the "Pero de

Cehegín" clones were not significantly different. For luminosity (L *), the results indicated that the peel of "Pero de Cehegín" fruits had light colors. Four clones (P1, P2, P3, and P4) were not significantly different amongst themselves. The "Fuji" variety (bicolor apple) showed the lowest luminosity and the "Golden Delicious" variety was the most luminous fruit, although no significant differences between P2, P3, and P5 "Pero de Cehegín" clones were observed. The results of the a * index, which represents the red-green color scale, showed small differences among the "Pero de Cehegín" clones and Golden Delicious. As expected, only Fuji showed a high value of the colorimetric variable a * (24.52, reddish color).

Table 4. Mean values of the morphological parameters measured in five clones of "Pero de Cehegín" and two varieties of reference ("Fuji" and "Golden Delicius") harvested in 2017

Parameters	"Pero de Cehegín"					Commercial varieties	
	P1	P2	P3	P4	P5	Fuji	Golden Delicius
FW (g)	195.18 ± 4.14 c	219.43 ± 8.20 d	148.41 ± 3.52 a	170.75 ± 4.12 b	181.39 ± 4.70 bc	190.79 ± 4.25 bc	149.68 ± 2.78 a
AD (mm)	72.80 ± 0.62 bcd	75.43 ± 1.10 d	66.24 ± 0.57 a	69.86 ± 0.71 ab	70.85 ± 0.71 abc	72.94 ± 0.67 cd	68.02 ± 0.45 ab
AH (mm)	77.40 ± 0.91 d	81.77 ± 1.32 e	68.33 ± 0.86 b	73.48 ± 0.64 c	75.19 ± 0.93 cd	68.25 ± 0.91 b	64.23 ± 0.73 a
PCw (mm)	30.53 ± 0.33 b	31.39 ± 0.48 b	27.86 ± 0.38 a	31.11 ± 0.46 b	30.62 ± 0.37 b	28.69 ± 0.44 a	27.15 ± 0.37 a
PCd (mm)	14.68 ± 0.99 a	14.94 ± 0.55 ab	15.65 ± 0.53 ab	15.74 ± 0.44 ab	17.16 ± 0.55 b	14.98 ± 0.40 ab	15.77 ± 0.35 ab
CCw(mm)	29.89 ± 0.68 c	27.77 ± 0.47 bc	25.77 ± 0.38 ab	25.72 ± 0.44 ab	26.67 ± 0.36 ab	27.64 ± 0.68 b	25.30 ± 0.45 a
CCd (mm)	14.02 ± 0.60 ab	15.91 ± 0.67 b	12.87 ± 0.37 a	14.15 ± 0.40 ab	13.58 ± 0.63 ab	12.48 ± 0.68 a	13.61 ± 0.67 ab
Sv	1.40 ± 0.43 a	1.85 ± 0.59 ab	1.05 ± 0.47 a	1.45 ± 0.43 a	2.65 ± 0.40 ab	2.00 ± 0.63 ab	3.75 ± 0.64 b
Sa	3.40 ± 0.64 cd	1.00 ± 0.46 ab	0.80 ± 0.41 a	4.15 ± 0.46 d	2.95 ± 0.48 bcd	2.20 ± 0.46 abcd	1.80 ± 0.36 abc
OL	2.00 ± 0.01	2.00 ± 0.02	2.00 ± 0.01	2.00 ± 0.03	2.00 ± 0.01	2.00 ± 0.01	2.00 ± 0.01
F (kg cm^{-2})	3.65 ± 0.05 b	3.96 ± 0.08 c	4.44 ± 0.10 d	4.00 ± 0.06 c	3.93 ± 0.05 bc	2.61 ± 0.07 a	2.65 ± 0.07 a
Moisture content (%)	79.72 ± 1.27 ab	79.76 ± 0.67 ab	78.24 ± 0.93 a	78.45 ± 1.09 ab	77.92 ± 0.82 a	78.85 ± 0.28 ab	82.53 ± 0.61 b

Values (means ± SE n=3) followed by the same letter, within the same file, are not significant different according to Tukey's test procedure at 5% significance level: Abbreviations: FW: fruit weight; AD: average equatorial diameter; AH: average fruit height; PCw: width of the pedicle cavity; PCd: depth of the pedicle cavity; CCw: width of the calycine cavity ; CCd: depth of the calyx cavity; Sv: number of viable seeds; Sa: number of abortive seeds; OL: aperture of locules; F: Firmness.

Table 5. Colour coordinates (CIEL*a*b*) in the five clones of "Pero de Cehegín" and varieties of reference ("Fuji" and "Golden Delicius") harvested in 2017

Parameters	"Pero de Cehegín"					Commercial varieties	
	P1	P2	P3	P4	P5	Fuji	Golden Delicius
L*	72.84 ± 0.45 b	74.18 ± 0,50 bc	74.76 ± 0.39 bc	72.73 ± 0.58 b	75.70 ± 0.54 c	51.57 ± 0.95 a	76.09 ± 0.29 c
a*	-4.87 ± 0.40 a	-3.71 ± 0.42 ab	-2.93 ± 0.42 abc	-4.84 ± 0.35 ab	-2.81 ± 0.52 bc	24.52 ± 0.81 d	-1.03 ± 0.28 c
b*	42.31 ± 0.52 b	44.09 ± 0.66 bc	44.39 ± 0.52 bc	43.51 ± 0.63 bc	43.13 ± 0.60 b	18.31 ± 0.66 a	45.95 ± 0.47 c
C*	42.67 ± 0.49 b	44.34 ± 0.64 bc	44.58 ± 0.51 bc	43.84 ± 0.60 bc	43.35 ± 0.58 b	31.16 ± 0.45 a	46.00 ± 0.47 c
Hº	96.72 ± 0.56 c	95.06 ± 0.58 c	93.96 ± 0.56 bc	96.54 ± 0.51 c	93.95 ± 0.71 bc	37.15 ± 1.75 a	91.34 ± 0.36 b

*Values (means ± SE of n=3) followed by the same letter, within the same file, are not significant different according to Tukey's test procedure at 5% significance level.

Table 6. Quality parameters, antioxidant activity, contents of total phenolic and sugars in the five clones of "Pero de Cehegín" and varieties of reference ("Fuji" and "Golden Delicius") harvested in 2017.

Parameters	"Pero de Cehegín" Clones					Commercial varieties	
	P1	P2	P3	P4	P5	Fuji	Golden Delicius
Total soluble solids (◦Brix)	15.70 ± 0.38 ab	17.10 ± 0.21 b	17.20 ± 0.17 b	15.90 ± 0.50 b	15.40 ± 0.12 ab	16.63 ± 0.65 b	13.90 ± 0.40 a
Total acidity (g of malic acid L^{-1})	1.70 ± 0.04 a	1.72 ± 0.11 a	1.44 ± 0.16 a	1.83 ± 0.06 a	1.68 ± 0.22 a	3.97 ± 0.34 b	3.30 ± 0.22 b
Maturity index	92.41 ± 2.23 b	100.10 ± 5.51 b	122.30 ± 13.88 b	86.98 ± 4.62 b	94.56 ± 11.90 b	42.21 ± 2.10 a	42.47 ± 2.95 a
$ABTS^{+}$ (mmol Troloxkg^{-1} fw)	1.95 ± 0.33 a	1.48 ± 0.03 a	1.46 ± 0.25 a	1.67 ± 0.11 a	1.76 ± 0.22 a	1.08 ± 0.37 a	0.86 ± 0.54 a
$DPPH^{\bullet}$(mmol Troloxkg^{-1} fw)	2.83 ± 0.17 a	2.51 ± 0.31 a	2.88 ± 0.33 a	3.20 ± 0.23 a	3.22 ± 0.17 a	2.89 ± 0.08 a	2.58 ± 0.43 a
FRAP (mmol Troloxkg^{-1} fw)	0.66 ± 0.06 bc	0.52 ± 0.06 abc	0.60 ± 0.03 abc	0.41 ± 0.00 abc	0.69 ± 0.08 c	0.32 ± 0.10 ab	0.25 ± 0.13 a
TPC (mg GAE 100 g^{-1} fw)	199.44±38.05ab	180.66±0.62ab	197.56±13.90 ab	117.04±20.38 ab	157.18±20.80 ab	102.72±38.60 a	282.07±71.71b
Sucrose (g 100 mL^{-1})	6.58 ± 0.15b	7.24 ± 0.13b	6.61 ± 0.66b	6.99 ± 0.20b	5.85 ± 0.07b	3.96 ± 0.17a	3.17 ± 0.21a
Glucose (g 100 mL^{-1})	2.54 ± 0.22a	2.50 ± 0.23a	2.58 ± 0.26a	2.00 ± 0.33a	2.53 ± 0.07a	3.32 ± 0.48a	2.35 ± 0.15a
Fructose (g 100 mL^{-1})	6.52 ± 0.20a	6.43 ± 0.20a	5.93 ± 0.36a	5.59 ± 0.22a	5.88 ± 0.01a	8.85 ± 0.10b	8.40 ± 0.48b

Values (means ± SE of n=3) followed by the same letter, within the same file, row are not significant different according to Tukey's test procedure at 5% significance level.
Abbreviations: TPC: total phenolic; GAE: gallic acid equivalents; fw: fresh weight.

3.3. Total Soluble Solids, Acidity, and Maturity Index

"Pero de Cehegín" did not show significant differences among clones for any of the traits evaluated (Table 6). However, among commercial varieties ("Fuji" and "Golden Delicious") differences were also found in TSS and TA. These differences could be due to the genetic variation among the plant material studied since based on the literature, the composition of apple fruits will be affected by the genotype [25,59].

The total soluble solids (TSS) parameter varied from 15.40 °Brix for the P5 clone up to 17.20 °Brix for the P3 clone, without significant differences with the rest of the clones and "Fuji" (16.63 °Brix). Clones P1 and P5 were not different from "Golden Delicious" (13.9 °Brix). Previous studies on different varieties have reported values between 9.99 and 18.1 °Brix [25,27,28,29,60].

The total acidity (TA) mean values for the different "Pero de Cehegín" clones ranged from 1.44 (P3) to 1.83 g L−1 (P4) without significant differences between them, but these values were significantly lower than in the "Fuji" and "Golden Delicious" varieties (3.97 and 3.30 g L−1, respectively). The total acidity value (TA) of the clone P3 of "Pero de Cehegín" was lower than reported in other studies of apple varieties [25,28,29,60,61,62]. For apples, the total acidity is a very important parameter for the quality of fruit and consumption, particularly in Europe [63]. Some research studies [52,64] have indicated that total acidity values below 3 mg mL−1 and above 10 mg mL^{-1} can be undesirable. This statement could be in contradiction with the results of this work, as the acidity value for the "Pero de Cehegín" clones resulted below 3 mg mL−1 and this traditional Spanish apple fruit has been historically considered as appealing and of high quality.

The maturity index (MI) for the "Pero de Cehegín" clones ranged from 86.98 (P4) to 122.30 (P3), significantly higher as compared with "Golden Delicious" (MI: 42.47) and "Fuji" (MI: 42.21) varieties. Generally, the maturity index is considered as responsible for the fruit's taste, thus, based on these results, it could be affirmed that the "Pero de Cehegín" clones are sweet tasting.

3.4. Antioxidant Activities (AA) and Total Polyphenol Content (TPC)

To evaluate the potential antioxidant activity of apple fruit, three in vitro assays, based on the scavenging activity (DPPH•), radical scavenging capacity (ABTS+), and ferric reducing antioxidant potential (FRAP) were used and the results are reported in Table 6. The results did not indicate significant differences between "Pero de Cehegín" clones and commercial varieties ("Golden Delicious" and "Fuji") for ABTS+ and DPPH• (Table 6). Differences were only obtained by using the FRAP method, with higher values for the "Pero de Cehegín" clones than for "Fuji" and "Golden Delicious" observed. A previous study [65] indicated the importance of the genotype in the antioxidant activity, and this could be reason for the differences observed.

In the present study, the highest TCP concentration was obtained by the "Golden Delicious" variety (282.07 mg GAE 100 g^{-1} fw). This value was significantly different when compared with the "Fuji" variety, which had the lowest TCP value (102.72 mg GAE 100 g^{-1} fw). However, the "Pero de Cehegín" clones did not show significant differences between them and the values for "Fuji" and "Golden Delicious", ranging from 117.04 (P4) to 199.44 mg GAE 100 g^{-1} fw (P1). Different studies for apple varieties have shown total polyphenol values between 66.2 and 290 mg GAE 100 g^{-1} fw [66,67,68,69].

3.5. Sugar Profile

Significant differences among the "Pero de Cehegín" clones and "Fuji" and "Golden Delicious" varieties were found for sucrose and fructose. Sucrose content in the "Pero de Cehegín" was higher than in commercial varieties, while fructose was the opposite. For glucose, no differences were observed. In apples, D-fructose has been reported as the principal sugar, independently of the variety [25,27,70,71]. Conversely, "Pero de Cehegín" had similar, and even higher, values of sucrose than fructose as the main sugars, well above glucose. This sucrose:fructose ratio can be defined as a distinctive characteristic for this variety.

3.6. Sensory Evaluation

The mean values obtained in the sensory evaluation of the different organoleptic attributes of "Pero de Cehegín" clones are presented in Figure 2. For "Pero de Cehegín" appearance attributes, the panelists provided scores ranging from 5.00 (P1) to 8.00 (P3) for peel color, but notably lower than the Fuji variety (14.20). The homogeneity for peel color in "Pero de Cehegín" ranged between 6.2 (P3 and P4) and 8.4 (P2). For odor related attributes, P3 clone obtained the highest value for apple and fruity odor (the most characteristic odor of this fruit), while P1 and P2 clones showed the highest pineapple odor values. The highest values of quince and pear notes were provided for the "Golden Delicious" variety. Additionally, related to the other attributes, the "Pero de Cehegín" clones showed low sourness, high astringency, corking and fibrosity, and medium crispy when compared with Golden "Delicious" and "Fuji" varieties. The sourness perception in the sensory analysis was in agreement with the total titratable acidity (TA) (Table 6), that is, smaller values for "Pero de Cehegín" clones (P1–P5) than "Golden Delicious" and "Fuji" varieties (used as controls). The high value of two attributes (corkiness and fibreness) of the "Pero de Cehegín" clones must be highlighted. The high intensity of these attributes could be a disadvantage for fresh fruit consumption, such as apple or quince [72]. On the other hand, these values can be related to the highest values of firmness (F) (Table 4) for "Pero de Cehegín" clones. However, in the sensory analysis, a higher level of hardness for "Pero de Cehegín" clones and "Golden Delicious" and "Fuji" samples was not observed. In this sense, the firmness test can be considered as an indirect measurement to predict the consumer's perception on the texture of the fruit, and contradictory results have been published [73], indicating the importance of comparing the analytical methods with the sensory ones.

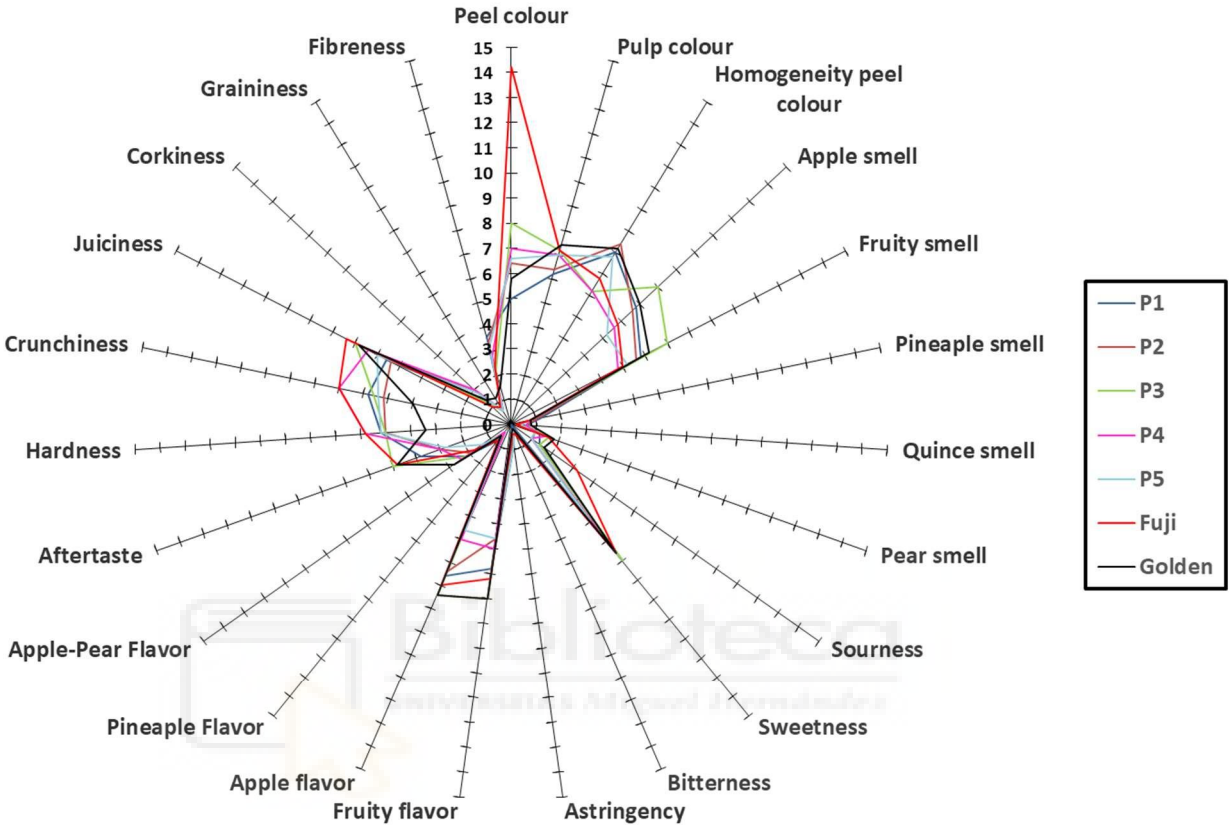

Figure 2. Sensory scores the five clones of "Pero de Cehegín" and two varieties of reference ("Fuji" and "Golden Delicius") harvested at maturity in 2017. Sensory scores ranged from 0 (not detectable) to 10 (strong), except colour which ranges between 0 and 20.

3.7 Principal component analysis

Aiming to easily explain the trends and relationships among the measured variables (32) for all clones and varieties (7), a principal component analysis (PCA) was performed. The first five components explained 96.12% of the variation, while the first three components explained 82.43% of the total observed variability (Table 7). The first component, PC1 (39.54% of the total variance), was related to TA, MI, F, H1, H2, AH, sucrose, and fructose. (Table 7, Figure 3). PC2 (27.76% of the total variance) was related with the color coordinates L *, a *, b * C *, and Hº (color), and FW, D1, D2, AD (weight and fruit size), CCw. PC3 (15.13% of the total variance) was related to polyphenols, moisture, and CCd. The rest of the PCs were related to the traits highlighted in Table 7.

The PC1-PC2 biplot (Table 7, Figure 3), separated "Fuji" and "Golden Delicious" varieties, located on the negative X-axis (PC1), from the "Pero de Cehegín" clones, located on the positive X-axis. In the same way, and for the "Pero de Cehegín" clones, the results separated the P1 and P2 clones, placed on the negative Y-axis (PC2) and very close to each other, from the rest of the clones, placed on the positive Y-axis.

The results obtained from the hierarchical cluster analysis, using the linkage method between groups, were shown as a dendrogram (Figure 4), with two branches separating "Pero de Cehegín" clones from commercial varieties. Then, "Pero de Cehegín" clones were grouped into two groups. The first group consisted of clones P1 and P2, while the second group include clones P3, P4, and P5. The dendrogram obtained by the cluster analysis matches with results of the principal component analysis (PCA).

Table 7. Eigenvalues, proportion of variation and eigenvectors associated with principal components of the PCA.

Principal Components (axes)	1	2	3	4	5	6
Proportion of variation	39.54	27.76	15.13	7.97	5.72	3.88
Cumulative proportion of variation	39.54	67.30	82.43	90.40	96.12	100
Variable	**Eigenvectors**					
FW (Fruit weight)	0.147	−0.241	0.148	0.008	−0.235	−0.168
D1 (Equatorial diameter 1)	0.175	−0.221	0.203	0.064	0.024	0.007
D2 (Equatorial diameter 2)	0.068	−0.271	0.250	0.068	−0.066	−0.026
AD (Average equatorial diameter)	0.135	−0.242	0.234	0.068	0.028	0.018
H1 (Fruit height 1)	0.262	−0.058	0.120	0.029	−0.159	−0.123
H2 (Fruit height 2)	0.237	−0.134	0.108	0.041	−0.226	−0.089
AH (Average fruit height)	0.253	−0.093	0.115	0.035	−0.191	−0.109
PCw (Width of the pedicle cavity)	0.233	−0.090	0.030	0.239	−0.258	0.004
PCd (Depth of the pedicle cavity)	−0.008	0.191	−0.167	0.299	0.003	−0.528
CCw (Width of the calycine cavity)	0.135	−0.238	0.180	−0.020	0.274	0.030
CCd (Depth of the calyx cavity)	−0.193	0.158	0.263	0.026	−0.032	−0.013
OL (Aperture of locules)	0.142	−0.078	0.158	0.037	0.539	0.227
Sv (Number of viable seeds)	−0.180	0.085	0.215	0.210	−0.084	−0.412
Sa (Number of abortive seeds)	0.062	−0.032	−0.026	0.564	0.113	0.333
F (Firmness)	0.233	0.134	−0.168	−0.134	−0.029	0.014
TSS (Total soluble solids)	0.129	−0.138	−0.228	−0.373	−0.150	0.047
TA (Total titratable acidity)	−0.253	−0.150	0.043	0.064	−0.047	0.000
MI (Maturity index)	0.236	0.114	−0.129	−0.218	0.063	−0.034
Moisture	−0.151	0.068	0.374	−0.057	−0.041	0.108
L * (Lightness) (D65)	0.124	0.286	0.148	0.001	0.002	−0.075
a * (Red-greenness) (D65)	−0.173	−0.250	−0.135	−0.026	0.001	−0.083
b * (Blue-yellowness) (D65)	0.123	0.286	0.153	−0.014	−0.030	0.019
C * (Chromaticity) (D65)	0.101	0.295	0.157	−0.035	−0.056	0.019
Hº (Hue angle) (D65)	0.165	0.257	0.139	0.022	0.001	0.062
$ABTS^{+}$	0.229	−0.043	0.046	−0.024	0.369	−0.275
$DPPH^{\bullet}$	0.066	0.027	−0.320	0.415	0.141	0.001
TPC (Total phenolic)	−0.068	0.202	0.304	−0.190	0.186	−0.077
FRAP	0.239	0.028	−0.058	−0.035	0.310	−0.298
Sucrose	0.268	0.030	−0.080	−0.088	−0.119	0.174
Glucose	−0.102	−0.258	−0.091	−0.190	0.189	−0.298
Fructose	−0.242	−0.149	0.131	−0.054	0.036	−0.051

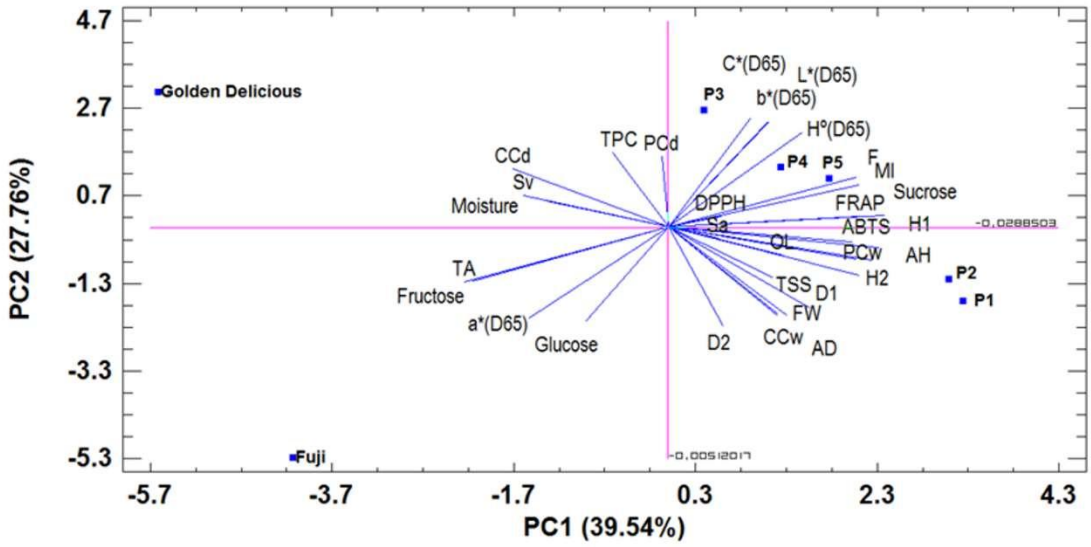

Figure 3. PCA of the measured parameters. Biplot PC1-PC2

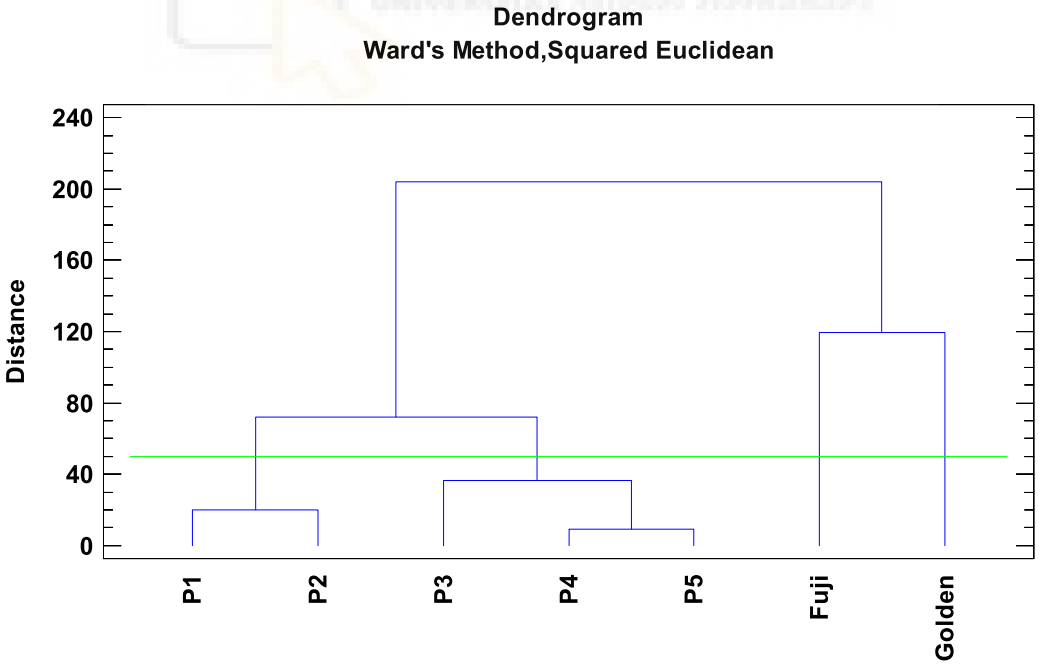

Figure 4. Dendrogram constructed with the number physicochemical traits evaluated following Ward´s method. Squared Euclidean distance represents dissimilarity.

4. Conclusions

This study allowed identifying the "Pero de Cehegín" as a unique and new genotype, not reported in the Spanish germplasm banks and revealed the interest in continuing with apple prospection work in Spanish areas of the Southeast which has not been sufficiently explored.

The study of the physicochemical characteristics, bioactive compounds, and sensory profile of five clones of "Pero de Cehegín" has allowed observing the existence of variability between clones. An important variability was found for fruit weight, diameter, and height between the different "Pero de Cehegín" clones. In comparison with "Fuji" and "Golden Delicious", "Pero de Cehegín" showed high firmness, high total soluble solids, very low total acidity, high FRAP antioxidant capacity, and more sucrose content. These distinctive characteristics and its good appearance and special odor make this variety a marketable product that with an adequate evaluation and diffusion can increase the market offering and complement the demand for new products, thereby opening the possibility of spreading its cultivation in areas where it can be adapted. This is a preliminary study, so to confirm the phenotypic characterization, this study will continue for two more years.

Author Contributions

Conceptualization and methodology, R.M. and P.M.; formal analysis, data collection and writing—original draft preparation, R.M.; validation and writing—review and editing, P.L., F.H., Á.C.-B., Y.G., J.J.M.-N. and P.M.; experimental analysis, R.M. and Y.G.; supervision, P.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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5. RESUMEN DE RESULTADOS, DISCUSIÓN Y CONCLUSIONES

5.1 PUBLICACIÓN 1

Martínez, R., Legua, P., Martínez, J.J., Melgarejo, P. 2019. **Phenological growth stages of "Pero de Cehegín" (Malus domestica Borkh): Codification and description according to the BBCH scale.** *Scientia Horticulturae*, 246, 826-834. doi: 10.1016/j.scienta.2018.11.067

5.1.1. Resumen de resultados y discusión

Las principales etapas fenológicas del "Pero de Cehegín" han sido descritas según las claves de identificación de la etapa de crecimiento para mono y plantas dicotiledóneas (Hack *et al*., 1992). La escala BBCH extendida considera 10 etapas principales de crecimiento, numeradas del 0 al 9. Este estudio manejó 8 de las 10 etapas principales, comenzando en el desarrollo del brote (etapa 0) y terminando en la senescencia (etapa 9). Las etapas secundarias también están numeradas del 0 al 9, y se refieren a diferentes porcentajes del valor de crecimiento o a diferentes etapas cualitativas dentro del escenario principal. En este estudio, se definieron 41 etapas secundarias, y se presentaron fotográficamente un total de 36 etapas.

La caracterización climática determinó una media de 1372,75 Unidades Frío de acuerdo con el modelo de Utah (Richardson *et al*., 1974) para las temporadas de 2015 y 2016, desde el inicio de la caída de las hojas, hasta el momento anterior a la hinchazón de las yemas. El cálculo de GDH, según el modelo de

Carolina del Norte (Shaltout y Unrath, 1983) arrojó un resultado medio de 5818 GDH para 2015 y 2016, desde la hinchazón de las yemas hasta la plena floración, que coincide con la etapa fenológica 65 según al código BBCH descrito.

5.1.2. Resumen de las conclusiones.

La escala BBCH (Hack *et al*., 1992) proporciona una descripción precisa de esta variedad desconocida y peculiar de manzano, tanto vegetativa como reproductiva, que es importante para la correcta programación de las prácticas agrícolas. Los resultados de este estudio, a pesar de las diferencias mostradas entre la manzana y Peros, coinciden esencialmente con los obtenidos por Meier (1997) para las etapas fenológicas de los principales frutales de pepita (manzanas y peras).

La escala propuesta será una buena referencia para productores y científicos. Esta codificación e identificación de las diferentes etapas fonológicas nos permitirá la correcta y precisa caracterización de cada uno de los clones seleccionados y conservados en los bancos de germoplasma, proporcionará datos sobre la biología floral, facilitará los datos sobre la influencia de los factores ambientales, ayudará a mejorar las técnicas de cultivo (nutrición, riego, tratamientos contra plagas y enfermedades, la aplicación de reguladores de crecimiento, etc.) así como nos facilitará la detección de anomalías de carácter fisiológico o de cualquier otro tipo.

Este estudio también realiza la primera caracterización fenológica de la variedad "Pero de Cehegín", que proporcionará información importante sobre el comportamiento de esta variedad y las mejores prácticas de cultivo a seguir.

5.2 PUBLICACIÓN 2

Ramón Martínez, Pilar Legua, Francisca Hernández, Ángel A. Carbonell-Barrachina, Yolanda Gogorcena, Juan J. Martínez-Nicolás, Pablo Melgarejo. 2020. **Molecular, Physico-Chemical and Sensory Characterization of the traditional Spanish apple variety "Pero de Cehegín"**. *Agronomy*, 10, 1093. doi: 10.3390/agronomy10081093

5.2.1. Resumen de resultados y discusión

Los cinco clones de "Pero de Cehegín" compartieron el mismo perfil de SSR en los 13 marcadores microsatélite estudiados. El perfil de "Pero de Cehegín" no mostró correspondencia con perfiles de accesiones llamadas "Pero" en otras colecciones ibéricas (España y Portugal) conservadas en diferentes instituciones consultadas. Por lo tanto, este es el primer estudio molecular que pone de manifiesto al "Pero de Cehegín" como un nuevo genotipo, no presente en otros bancos españoles de germoplasma.

Respecto a los parámetros físicos medidos en los frutos, se encontró una variabilidad importante para el peso, diámetro y altura de la fruta entre los diferentes clones de "Pero de Cehegín" estudiados. Las frutas más pequeñas fueron las producidas por el clon P3 con un peso medio (FW) de 148,41 g mientras que el clon P2 con 219,43 g presentó los frutos más grandes. Estos resultados destacan las diferencias morfométricas presentadas por el "Pero de Cehegín" en comparación con variedades comerciales comunes como "Fuji" y "Golden Delicius". En valores como el ancho de la cavidad pedicular (PCw), profundidad de la cavidad peduncular (PCd), anchura de la cavidad calicina (CCw) y profundidad de la cavidad calicina (CCd), se observaron diferencias significativas entre clones, pero en menor medida que para el peso, diámetro y altura de la fruta. Características como número de semillas viables (Sv) y contenido en humedad, no mostraron diferencias significativas entre los distintos clones de "Pero de Cehegín". La firmeza de los frutos en los clones de "Pero de Cehegín" presentó valores más altos que los de las dos variedades comerciales incluidas en este ensayo, oscilando entre 3,65 (P1) y 4,44 kg cm^{-2} (P3), con diferencias significativas entre clones y las variedades comerciales "Fuji" y "Golden Delicius".

Para los rasgos de color, para todos los parámetros, excepto L* y a*, todos los clones "Pero de Cehegín" no fueron significativamente diferentes. Para la luminosidad (L*), los resultados indicaron que la piel de los frutos de "Pero de Cehegín" tenían colores claros. Cuatro clones (P1, P2, P3 y P4) no fueron significativamente diferentes entre ellos. La variedad Fuji (manzana bicolor) mostró la luminosidad más baja y la variedad "Golden Delicius" fue la fruta más luminosa, aunque no hubo diferencias significativas entre los clones P2, P3 y P5 "Pero de Cehegín". Los resultados del índice a*, que representa la escala rojo-verde, mostraron pequeñas diferencias entre los clones "Pero de Cehegín" y "Golden Delicius". Como se esperaba, solo Fuji mostró un alto valor de la variable colorimétrica a* (24,52, color rojizo).

El "Pero de Cehegín" no mostró diferencias significativas entre los clones para TSS, TA, MI, TPC, actividad antioxidante y azúcares.

Sin embargo, con las variedades comerciales ("Fuji" y "Golden Delicius") se encontraron diferencias en TSS y TA. El parámetro de sólidos solubles totales (TSS) varió desde 15,40 ºBrix para el clon P5 hasta 17,20 ºBrix para el clon P3, sin diferencias significativas con el resto de los clones y "Fuji" (16,63 ºBrix). Los valores medios de acidez total (TA) para los diferentes clones "Pero de Cehegín" variaron de 1,44 gL^{-1} (P3) a 1,83 gL^{-1} (P4) sin diferencias significativas entre ellos, pero estos valores fueron significativamente más bajos que en "Fuji" y "Golden Delicius" 3,97 gL^{-1} y 3.30 gL^{-1}, respectivamente. El valor de acidez total (TA) del clon P3 de "Pero de Cehegín" fue inferior al reportado en otros estudios de cultivares de manzana (Hagen *et al*., 2007; Wu *et al*., 2007; Drogoudi *et al*., 2008; Seipel *et al*., 2009; Vieira *et al*., 2009; Reig *et al*., 2015). El índice de madurez (MI) para los clones "Pero de Cehegín" varió de 86,98 (P4) a 122,30 (P3), significativamente más alto en comparación con las variedades "Golden Delicius" (MI: 42,47) y "Fuji" (MI: 42,21). En general, el índice de madurez se considera responsable del sabor de la fruta, por lo que, según los resultados, se podría afirmar que los clones "Pero de Cehegín" tienen un sabor dulce.

La actividad antioxidante (AA) se estudió mediante los métodos DPPH•, $ABTS^{+}$ y FRAP. Los resultados no mostraron diferencias significativas entre los clones de "Pero de Cehegín" y las variedades comerciales ("Golden Delicius" y "Fuji") para $ABTS^{+}$ y

DPPH•. Sí se observaron diferencias utilizando el método FRAP con valores más altos para los clones "Pero de Cehegín" que para “Fuji” y “Golden Delicius”. Scalzo y col. (2005) indicaron la importancia del genotipo para la actividad antioxidante. En el presente estudio, la mayor concentración de polifenoles totales (TPC) se obtuvo para la variedad “Golden Delicius” (282,07 mg GAE 100 g^{-1} fw), siendo significativamente diferente a los resultados para la variedad Fuji, que fue la más baja (102,72 mg GAE 100 g^{-1} fw). Los "Peros de Cehegín" no mostraron diferencias significativas entre ellos, mostrando valores que oscilaron entre los obtenidos en “Fuji” y “Golden Delicius”, variando desde 117,04 mg GAE 100 g^{-1} fw (P4) y 199,44 mg GAE 100 g^{-1} fw (P1).

Se encontraron diferencias significativas entre los clones "Pero de Cehegín" y las variedades “Fuji” y “Golden Delicius” para sacarosa y fructosa. La sacarosa en el "Pero de Cehegín" fue mayor que en las variedades comerciales, mientras que la fructosa fue todo lo contrario. Para la glucosa, no se observaron diferencias. En manzanas, se ha informado que la D-fructosa es el azúcar principal, independientemente de la variedad (Karadeniz y Ekşi, 2002; Sanz *et al*., 2004; Wu *et al*., 2007; Feliciano *et al*., 2010). Por el contrario, "Pero de Cehegín" presentó valores similares e incluso más altos de sacarosa que de fructosa como azúcares principales, y muy por encima de la glucosa. Esta relación sacarosa: fructosa puede indicarse como una característica distintiva de esta variedad.

En la evaluación sensorial, la homogeneidad del color de la piel en el "Pero de Cehegín" varió entre 6,2 (P3 y P4) y 8,4 (P2). Para los atributos relacionados con el olor, el clon P3 presentó el valor más alto de olor a manzana y a fruta (el olor más característico de esta fruta), mientras que los clones P1 y P2 mostraron los valores más altos de olor a piña. Los valores más altos de notas de membrillo y pera se presentaron en la variedad “Golden Delicius”. Además, en relación con los otros atributos, los clones "Pero de Cehegín" mostraron baja acidez, alta astringencia, acorchado y fibrosidad, así como crocancia media en comparación con las variedades “Golden Delicius” y “Fuji”. La percepción de acidez en el análisis sensorial estuvo de acuerdo con la acidez total (TA), es decir, valores más pequeños para los clones "Pero de Cehegín" (P1-P5) que para las variedades “Golden Delicius” y “Fuji” (utilizadas como controles). Es resaltable el valor elevado de 2 atributos (acorchado y fibrosidad) de los clones "Pero de Cehegín". Estos valores pueden estar relacionados con sus altos valores de firmeza. Sin embargo, en el

análisis sensorial, no se apreció un mayor nivel de dureza para los clones "Pero de Cehegín" y las muestras de “Golden Delicius” y “Fuji”.

El dendrograma obtenido por el análisis de conglomerados y coincidente con los resultados del análisis de componentes principales (PCA) mostró que por un lado se agrupan los clones de “Pero de Cehegín” y por otro las variedades comerciales “Golden Delicius” y “Fuji”. A su vez, los clones de “Pero de Cehegín” se separaron en dos grupos. El primer grupo incluye los clones P1 y P2, mientras que el segundo grupo incluye los clones P3, P4 y P5.

5.2.2 Resumen de las conclusiones

Se ha identificado al "Pero de Cehegín" como un genotipo único y nuevo, no reportado en los bancos españoles de germoplasma y reveló el interés de continuar con el trabajo de prospección de manzanas en áreas españolas del Sudeste no suficientemente exploradas.

El estudio de las características físico-químicas, compuestos bioactivos y perfil sensorial de cinco clones de "Pero de Cehegín" ha permitido observar la existencia de variabilidad entre clones. Se encontró una variabilidad importante para el peso, el diámetro y la altura de la fruta entre los diferentes clones "Pero de Cehegín". En comparación con “Fuji” y “Golden Delicius”, el "Pero de Cehegín" mostró alta firmeza, alto contenido de sólidos solubles totales, muy baja acidez total, alta capacidad antioxidante FRAP y mayor contenido de sacarosa.

6. CONCLUSIONES GENERALES E INVESTIGACIONES FUTURAS

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6.1 Conclusiones Generales

En general, los resultados obtenidos en esta Tesis muestran que:

- La variedad tradicional "Pero de Cehegín", posee un genotipo único y diferente a los presentes en los bancos de germoplasma españoles. Lo que demuestra la importancia de los trabajos previos para la recuperación y conservación, y el interés de continuar con la prospección de manzanas en zonas del Sudeste español no suficiente explotadas en peligro de erosión genética, así como la continuación de la caracterización de las accesiones de variedades tradicionales rescatadas y presentes en los bancos.
- Se han descrito y fotografiado de forma precisa según la escala BBCH las principales etapas fenológicas del "Pero de Cehegín", y se ha realizado la primera caracterización fenológica y climática de unidades frío según el modelo de Utah (Richardson *et al*., 1974) y el cálculo de GDH según el modelo de Carolina del Norte (Shaltout y Unrath, 1983), proporcionando información importante sobre la variedad que podrá ser utilizada por productores y científicos.
- El estudio de las características físico-químicas, compuestos bioactivos y perfil sensorial de cinco clones de "Pero de Cehegín" nos ha permitido determinar la existencia de variabilidad entre clones, principalmente en caracteres morfométricos (peso, diámetro, altura).
- Del mismo modo y en comparación con las variedades comerciales "Fuji" y "Golden Delicius", el "Pero de Cehegín" ha mostrado características distintivas como una elevada firmeza, un alto contenido de solidos solubles totales, muy baja acidez total, alta capacidad antioxidante FRAP y más contenido en sacarosa. Estas características unidas a su buen aspecto y aroma hacen a esta variedad un producto comercializable, que con una adecuada evaluación y difusión puede aumentar la oferta en el mercado e incrementar la demanda de nuevos productos.

6.2 Investigaciones Futuras

Esta tesis deja abiertos campos de actuación, entre los que cabe destacar:

- La prospección en la Región de Murcia y el Sudeste español para la selección y recuperación de variedades tradicionales del género *Malus*.
- La caracterización de todos los clones de "Pero de Cehegín" recuperados, así como otras accesiones de variedades tradicionales rescatadas y presentes en la colecciones.
- La publicidad de las peculiares características del "Pero de Cehegín", para contribuir a su cultivo y a la diversificación de la oferta de pomáceas en el mercado.

7. REFERENCIAS BIBLIOGRÁFICAS

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