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Desarrollo de herramientas morfológicas y genómicas para el estudio del pepino dulce (*Solanum muricatum*) y especies relacionadas. Caracterización de su valor nutracéutico

TESIS DOCTORAL
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Valencia, diciembre de 2015

RESUMEN

La reducción en la diversidad agrícola es un problema grave en nuestra agricultura. Cada vez se cultiva un menor número de especies distintas, y estas además son cada vez más homogéneas. Una de las actividades que se pueden llevar a cabo para minimizar el impacto de esta pérdida de variabilidad es la introducción de nuevos cultivos para la diversificación hortícola. En este sentido, el pepino dulce (*Solanum muricatum*) es un cultivo que puede tener interés para nuestra horticultura y para los mercados vecinos. Se trata de una especie de origen andino que normalmente se propaga vegetativamente, y que se puede cultivar en el área mediterránea. El pepino dulce se aprovecha por sus frutos jugosos, dulces y muy aromáticos, además de ello presenta cantidades relevantes de compuestos beneficiosos para la salud.

El banco de germoplasma del COMAV conserva una colección de entradas de pepino dulce y de especies silvestres relacionadas. En la actualidad se disponen de descriptores morfológicos para la correcta caracterización de estos materiales, pero se hacía necesario disponer descriptores fenológicos como los desarrollados en este trabajo. En particular, la escala BBCH desarrollada es una clave que permite describir y delimitar los distintos estadios de desarrollo fenológico en la especie, presentando numerosas aplicaciones.

Por otro lado era necesaria una correcta caracterización de estas entradas conservadas en el banco de germoplasma, para ello y como complemento a la realización de una caracterización morfológica, se llevó a cabo una caracterización molecular empleando marcadores SSR derivados de tomate. En este caso, se sacó beneficio de la proximidad genética entre ambas especies para transferir los marcadores de tomate (especie ampliamente estudiada) a pepino dulce, permitiendo diferenciar tanto morfológicamente, como molecularmente las especies silvestres de la cultivada, y dentro de esta los tipos modernos de los tradicionales.

Una limitación en el estudio de la genética del pepino dulce era el número reducido de secuencias de ADN disponibles en las bases de datos, razón por la cual se proyectó la secuenciación del transcriptoma de una variedad de pepino dulce (Sweet Long) y de una entrada de la especie que se considera el ancestro silvestre del pepino dulce *S. caripense*. Esta secuenciación y posterior ensamblaje del transcriptoma ha permitido realizar un estudio inicial donde se ha realizado un análisis comparativo

entre pepino dulce y sus especies cercanas tomate y patata, un estudio filogenético entre Solanáceas cultivadas, un análisis comparativo de algunos genes de interés agronómico, así como el desarrollo masivo de marcadores moleculares.

Debido a las potenciales propiedades nutracéuticas de los frutos de pepino dulce, se decidió realizar una caracterización de la misma en la colección de entradas estudiada anteriormente. Por un lado se ha evaluado el contenido en materia seca, proteínas, antioxidantes, pigmentos y minerales; por otro lado, teniendo en cuenta que los polifenoles son unos de los compuestos con mayor poder antioxidante, se llevó a cabo un estudio que pretendió dilucidar el perfil de polifenoles en cuatro entradas de pepino dulce y una entrada de *S. caripense*, así como su poder antioxidante. Como complemento a esto último se evaluó el efecto de extractos de pepino dulce sobre células de macrófagos sometidas a estrés oxidativo, observándose una reducción significativa en la producción de óxido nítrico, lo cual indica la existencia de un efecto antiinflamatorio. Estas propiedades beneficiosas del pepino dulce son su mayor virtud, y junto con una elevada calidad organoléptica y una buena promoción, pueden favorecer la introducción y desarrollo de este cultivo.

En definitiva, esta tesis supone la obtención de información relevante sobre la diversidad del pepino dulce, además de un estudio en distintos aspectos, como el fenológico, morfológico, molecular, genómico, nutricional y nutracéutico. Consideramos que esta información será de gran utilidad en el desarrollo y valorización de este cultivo marginado.

RESUM

La reducció en la diversitat agrícola és un problema greu en la nostra agricultura. Cada vegada es cultiva un menor nombre d'espècies diferents, i aquestes a més són cada vegada més homogènies. Una de les activitats que es poden dur a terme per a minimitzar l'impacte d'aquesta pèrdua de variabilitat és la introducció de nous cultius per a diversificació hortícola. En aquest sentit, el cogombre dolç (*Solanum muricatum*) és un cultiu que pot tenir interès per a la nostra horticultura i per als mercats veïns. Es tracta d'una espècie d'origen andí que normalment es propaga vegetativament, i que es pot cultivar en l'àrea mediterrània. El cogombre dolç s'aprofita pels seus fruits sucosos, dolços i molt aromàtics, a més d'això presenta quantitats rellevants de compostos beneficiosos per a la salut.

El banc de germoplasma del COMAV conserva una col·lecció d'entrades de cogombre dolç i d'espècies silvestres relacionades. En l'actualitat es disposen de descriptors morfològics per a la correcta caracterització d'aquests materials, però es feia necessari disposar descriptors fenològics com els desenvolupats en aquest treball. En particular, l'escala BBCH desenvolupada és una clau que permet descriure i delimitar els diferents estadis de desenvolupament fenològic en l'espècie, presentant nombroses aplicacions.

D'altra banda era necessària una correcta caracterització d'aquestes entrades conservades en el banc de germoplasma, per a això i com a complement a la realització d'una caracterització morfològica es va dur a terme una caracterització molecular emprant marcadors SSR derivats de tomaca. En aquest cas, es va traure benefici de la proximitat genètica entre ambdues espècies per a transferir els marcadors de tomaca (espècie àmpliament estudiada) a cogombre dolç, permetent diferenciar tant morfològicament, com molecularment les espècies silvestres de la cultivada, i dins d'aquesta els tipus moderns dels tradicionals.

Una limitació en l'estudi de la genètica del cogombre dolç era el nombre reduït de seqüències d'ADN disponibles en les bases de dades, raó per la qual es va projectar la seqüenciació del transcriptoma d'una varietat de cogombre dolç (Sweet Long) i d'una entrada de l'espècie que es considera l'ancestre silvestre del cogombre dolç, *S. caripense*. Aquesta seqüenciació i posterior assemblatge del transcriptoma ha permès realitzar un estudi inicial on s'ha realitzat una anàlisi comparativa entre

cogombre dolç i les seues espècies properes tomaca i creïlla, un estudi filogenètic entre solanàcies cultivades, una anàlisi comparativa d'alguns gens d'interès agronòmic, així com el desenvolupament massiu de marcadors moleculars.

A causa de les potencials propietats nutracèutiques dels fruits de cogombre dolç, es va decidir realitzar una caracterització de la mateixa en la col·lecció d'entrades estudiada anteriorment. D'una banda s'ha avaluat el contingut en matèria seca, proteïnes, antioxidants, pigments i minerals; d'altra banda, tenint en compte que els polifenols són uns dels compostos amb major poder antioxidant, es va dur a terme un estudi que va pretendre dilucidar el perfil de polifenols en quatre entrades de cogombre dolç i una entrada de *S. caripense*, així com el seu poder antioxidant. Com a complement a això últim es va avaluar l'efecte d'extractes de cogombre dolç sobre cèl·lules de macròfags sotmeses a estrès oxidatiu, observant-se una reducció significativa en la producció d'òxid nítric, la qual cosa indica l'existència d'un efecte antiinflamatori. Aquestes propietats beneficioses del cogombre dolç són la seua major virtut, i juntament amb una elevada qualitat organolèptica i una bona promoció, poden afavorir la introducció i desenvolupament d'aquest cultiu.

En definitiva, aquesta tesi suposa l'obtenció d'informació rellevant sobre la diversitat del cogombre dolç, a més d'un estudi en diferents aspectes, com el fenològic, morfològic, molecular, genòmic, nutricional i nutracèutic. Considerem que aquesta informació serà de gran utilitat en el desenvolupament i valorització d'aquest cultiu marginat.

SUMMARY

The reduction of agricultural biodiversity is a serious problem for our agriculture. Increasingly fewer species are cultivated, and these are also increasingly homogeneous. The introduction of new crops for the horticultural diversification is one of the activities that can be implemented to minimize the impact of this variability loss. In this regard, pepino (*Solanum muricatum*) is a crop that may be of interest to our horticulture and neighboring markets. It is a species of Andean origin, usually propagated vegetatively, and that can be grown in the Mediterranean area. The pepino is used for its juicy, sweet and aromatic fruits, which in addition presents significant amounts of beneficial compounds for health.

The COMAV genebank has a collection of pepino and wild relatives accessions. At present, morphological descriptors are available for proper characterization of these materials, but it was necessary to develop phenological descriptors as the ones presented in this work. In particular, the BBCH scale developed in this thesis is a key that allow us to describe and define the various stages of phenological development in the species, displaying numerous applications.

On the other hand, a correct characterization of the accessions preserved in the genebank was necessary. For this reason, and in addition to carrying out a morphological characterization, a molecular characterization using SSR markers derived from tomato was performed. In this case, the benefit of the genetic proximity between the two species allowed us to transfer the tomato markers (widely studied species) to pepino, allowing us to differentiate morphologically and molecularly the wild species from the cultivated one, and within the latter, differentiate modern and traditional types.

A limitation in the study of the genetics of pepino was the small number of DNA sequences available in the databases. For this reason, we sequenced the transcriptome of a variety of pepino (Sweet Long) as well as the species that is considered the wild ancestor of the pepino, *S. caripense*. The sequencing and subsequent assembly of the transcriptome has allowed an initial comparative analysis between pepino and its closely related species, tomato and potato, a phylogenetic study of cultivated Solanaceae, a comparative analysis of some genes of agronomic interest, and the massive development of molecular markers.

Because of the potential nutraceutical properties of the pepino fruit, we decided to perform a characterization using the previously studied collection. We have evaluated the dry matter, protein, antioxidants, pigments and minerals contents. On the other hand, considering that polyphenols are one of the most important antioxidant compounds, we conducted a study trying to elucidate the profile of polyphenols and their antioxidant activity in four pepino and one *S. caripense* entries. We also measured the effect of the pepino extracts on macrophage cells subjected to oxidative stress. The results obtained revealed a significant reduction in nitric oxide production, which indicates the existence of an anti-inflammatory effect. These beneficial properties of pepino are its main strength and, together with a high organoleptic quality and good promotion can encourage the introduction and development of this crop.

In summary, in this thesis we have obtained relevant information about the diversity of pepino and we have studied its phenological, morphological, molecular, genomic, nutritional and nutraceutical characteristics. We believe that this information will be useful in the development and recovery of this neglected crop.

INDICE

INTRODUCCIÓN

1.- INTRODUCCIÓN

1.1.- Domesticación y diversidad de cultivos

Se estima que la agricultura y con ella la domesticación de los cultivos, surgió hace aproximadamente 12,000 años (Dirzo y Raven, 2003). Este hecho ocurrió tras la finalización de la última era glaciar, y tuvo lugar simultáneamente e independientemente en dos territorios. Por un lado en la denominada "creciente fértil" o "media luna fértil" (zona de Oriente Próximo desde Mesopotamia al Antiguo Egipto) y por otro lado en la zona llamada Chogha Golan (en el moderno Irán). En los sucesivos milenios la agricultura comenzó a desarrollarse también de manera independiente en otras zonas del planeta (Figura 1). A lo largo de este tiempo, aproximadamente 2,500 especies vegetales, en un total de 160 familias, han sufrido algún proceso de domesticación y 250 de estas especies se consideran totalmente domesticadas (Gepts, 2012).

Valilov, tras unos viajes alrededor del mundo propuso ocho áreas, donde situó los centros de origen de los distintos cultivos (Vavilov, 1951). Estos centros incluían todos los continentes con la excepción de Oceanía, donde sus pobladores fueron recolectores hasta tiempos modernos. Valilov determinó estos centros de origen teniendo en cuenta varios criterios. Por un lado encontró una alta variabilidad de tipos cultivados, por otro lado también encontró que en esas zonas convivían tipos domesticados con sus ancestros silvestres, y por último consideró criterios históricos y/o arqueológicos que demostraban un uso antiguo de esas especies (Meyer et al., 2012). En años posteriores se demostró que la realidad no es tan sencilla y estos criterios sufrieron modificaciones, por ejemplo Harlan propuso que no todos los cultivos presentan alta variabilidad de tipos en su centro de origen (Harlan, 1975), o Fuller que consideró dividir el centro de origen situado en la India en cinco subcentros (Fuller, 2009). Con el tiempo también diversos autores propusieron nuevas áreas donde se domesticaron cultivos, incluyendo la isla de Nueva Guinea (Allaby, 2007, Denham et al., 2003), la Amazonia (Clement, 1999a; Clement, 1999b; Clement et al., 2010), la zona oriental de Norte América (Smith, 2006) y los deltas de ríos del África Occidental (Harlan, 1971; Portères, 1976). Así pues, visto lo difícil que supone definir los centros de origen, los científicos prefieren la denominación de centros de domesticación, incluyendo a todas aquellas zonas donde han tenido lugar procesos de domesticación (Meyer et al., 2012).

Vavilov fue un visionario, pionero en tomar conciencia de la importancia que tenía esa diversidad y de lo importante que era conservarla y ponerla a disposición de la comunidad científica. Fue consciente antes que nadie que distintos motivos como la industrialización, la globalización de la agricultura o el incremento desmesurado de la población, estaba provocando que el número de especies cultivadas se estuviese reduciendo de una manera drástica, siendo un hecho todavía más evidente en las últimas décadas (Khoury et al., 2014). Ejemplos de esto es que según la FAO solamente tres especies, arroz, maíz y trigo, suponen más de un 60 % del total de calorías consumidas por la humanidad, o que solamente 30 especies suponen más de un 95 % de las necesidades de energía alimentaria.

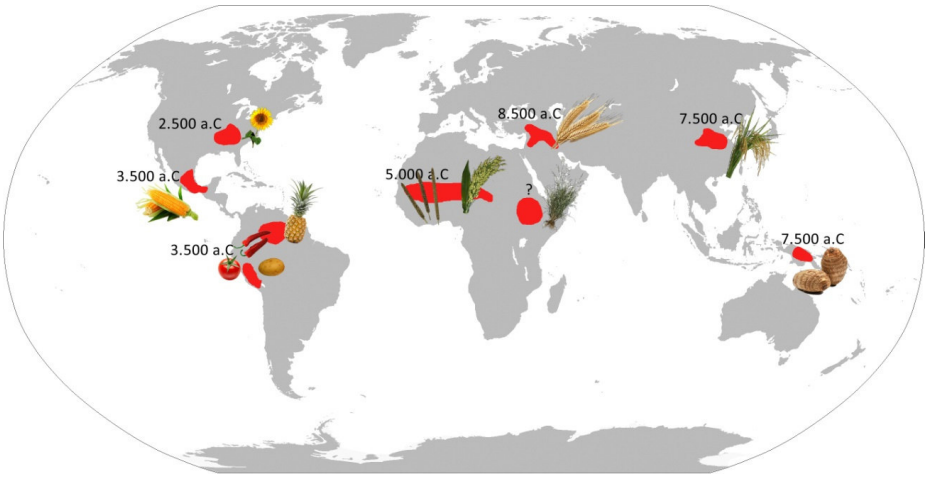

Figura 1. Fecha aproximada del origen de la agricultura en distintas regiones del planeta basado en la descripción de Meyer y Purugganan (2013).

A esta reducción del número de especies cultivadas, hay que añadir la progresiva pérdida de variabilidad dentro de ellas, incluso dentro de estos centros de origen descritos por Vavilov. Un patrimonio perdido para las generaciones futuras, que es irreversible en algunos casos y muy grave en el resto.

Son varias las iniciativas que pueden tomarse para paliar esta pérdida de variabilidad en los recursos fitogenéticos. Los bancos de germoplasma, los jardines de introducción y otros métodos “ex-situ” son algunas de ellas. Obviamente son métodos indiscutibles en la mejora

genética tradicional y en la investigación en biología vegetal, ya que facilita un acceso rápido y eficiente a estos recursos, pero presenta el problema de que se "congela" la diversidad conservada. Es por ello que distintas organizaciones como la FAO o el Convenio sobre Diversidad Biológica (CDB), recomiendan realizar una conservación "in-situ" de estos materiales, promoviendo el cultivo de variedades tradicionales en sus centros de origen, conviviendo con sus silvestres relacionadas y permitiendo su evolución generación tras generación.

Frente al reducido número de cultivos en la actualidad, existen centenares de especies que sólo son cultivadas a nivel local, pero presentan características que las podrían convertir en cultivos de importancia. Esta ampliación del espectro de cultivos empleados presenta numerosas ventajas que contribuyen a mejorar la calidad alimentaria (Mayes et al., 2012), centrando la atención en zonas subdesarrolladas, como forma de conservar la diversidad cultural y alimenticia. Por otro lado disminuyen los efectos de plagas y enfermedades, aumentan la sostenibilidad de la agricultura al disminuir los inputs, como combustibles, plaguicidas y fertilizantes, contribuyendo también a la conservación del suelo. Por último, en países desarrollados son cultivos que proporcionan a los productores y mercados un producto de alto valor económico dirigido a consumidores con alto poder adquisitivo o "gourmets", son productos que además no suelen ser excedentarios.

Cabe mencionar que históricamente los humanos siempre han estado interesado en la introducción de nuevo cultivos en sus territorios. Bien siguiendo rutas comerciales, bien a través de pueblos nómadas o mediante rutas de descubrimientos, son muchos los cultivos que se han dispersado, alejándose de su centro de origen. Muchos de estos intentos de introducción fracasaron, pero algunos concluyeron con éxito y en la actualidad poca gente conoce que por ejemplo, los cítricos en la región mediterránea proceden realmente del sudeste asiático, los melones y sandías proceden del África subsahariana o las Solanáceas de mayor importancia proceden de Sudamérica. De hecho, de los cultivos más importantes, solamente la lechuga es originaria de la región mediterránea.

En términos de dispersión de cultivos, el descubrimiento de América supuso un choque en múltiples ámbitos; uno de ellos fue el descubrir pobladores que manejaban técnicas avanzadas de agricultura, que en el caso de la región andina suponía vencer las adversidades que les ofrecía un territorio muy accidentado y una climatología muy extrema.

Pero sobre todo supuso el descubrimiento de nuevas especies cultivadas y completamente domesticadas por estos pueblos. Quizá el ejemplo más importante de esto es el tomate, que previamente a la llegada de los españoles sufrió una difusión desde la región andina a la región de Mesoamérica donde fue domesticado y donde sufrió un cuello de botella reduciendo su variabilidad, problema que aún afecta a la mejora de esta especie. Aún así, es en México donde se encuentra la mayor variabilidad dentro de los tipos domesticados y es en la región andina donde se encuentran especies silvestres relacionadas con él. Fue desde México donde se distribuyó a distintas regiones del viejo mundo, con distintos niveles de aceptación dependiendo del país. En Italia y España se aceptó de manera relativamente rápida, pero en el resto de Europa se consideró tóxica durante siglos, todo debido a la mala fama de las Solanáceas europeas, tóxicas y/o alucinógenas la mayoría de ellas. La historia del tomate en Europa demuestra que no siempre es fácil la introducción de estos nuevos cultivos y que muchas veces toca luchar por superar supersticiones y creencias infundadas.

El tomate es un ejemplo importante de una especie cuyo centro origen se encuentra en América, pero son otras muchas las que proceden de este continente. Por ejemplo, plantas cultivadas por sus raíces o tubérculos, como multitud de tipos de patatas, el boniato y la mandioca; por sus frutos como algunas calabazas y varias especies de pimientos y por sus semillas como el cacao, el cacahuete o maní, y la judía común. Como representante de los cereales destaca el maíz, y como frutas de postre exóticas destacan la piña, el aguacate, la papaya, la fruta de la pasión o la chirimoya.

En el caso de la región andina, un territorio inmenso donde su gran diversidad climática ha favorecido el desarrollo de una gran variedad de especies vegetales, destaca un grupo de cultivos domesticados desde hace milenios y cultivados por sus frutos. Entre las más importantes están diversas bayas andinas como la mora andina o de Castilla (*Rubus glaucus* Benth.) o la mora gigante colombiana (*Rubus macrocarpus* Benth.), la papaya de montaña o papayuelo (*Carica pubescens* Lenne & Koch), la lúcuma (*Pouteria lucuma* (Ruiz & Pav.) Kuntze), el lulo o naranjilla (*Solanum quitoense* Lam.), el tamarillo o tomate de árbol (*Cyphomandra betacea* (Cav.) Sendtn.), el alquequenje (*Physalis peruviana* L.), y diversas especies de fruta de la pasión (*Passiflora* spp.). La mayoría de estos cultivos han permanecido marginados desde la llegada de los

conquistadores españoles, por lo que el desarrollo de variedades, conocimientos sobre requerimientos de suelo y nutrientes, las técnicas de propagación, etc..., están poco avanzados. Otro cultivo procedente de esta región es el pepino dulce (*Solanum muricatum* Aiton), especie de la que se ocupa esta tesis doctoral.

1.2.- La importancia del estudio de la variabilidad genética en la colecciones de germoplasma

1.2.1.- Los recursos fitogenéticos (RRFF):

Se considera recurso fitogenético, cualquier material de origen vegetal, con capacidad de reproducción, ya sean semillas o propágulos, con un valor real o potencial para la alimentación y la agricultura.

Como ya se ha comentado, en la actualidad, las necesidades de alimentos recaen en unas pocas especies, dejando en desuso otras muchas. Además de esto, dentro de las especies más cultivadas se ha producido una progresiva pérdida de diversidad en la mayor parte de los cultivos, según la FAO principalmente motivado por el uso de variedades modernas frente a las variedades locales o tradicionales, es lo que se denomina erosión genética. Esta erosión genética compromete el futuro de la agricultura, limitando las opciones de los cultivos a adaptarse a cambios, como por ejemplo el tan comentado cambio climático, la aparición de nuevas plagas y enfermedades o satisfacer nuevas necesidades o exigencias comerciales.

El abandono por parte de los agricultores de las variedades que cultivaban tradicionalmente, ha supuesto que muchas de ellas se pierdan irremediablemente. Sin embargo, cuando la comunidad científica fue consciente de la problemática se comenzó a desarrollar distintas iniciativas de conservación de estos recursos. Se elaboraron unas normas de recolección y transferencia de estos materiales, se definió el papel que juegan los distintos agentes implicados en las actuaciones a realizar, así como las normas de funcionamiento de las entidades responsables de conservar estos recursos a largo plazo. Todo esto quedó plasmado en el Tratado Internacional sobre los Recursos Fitogenéticos para la Alimentación y la Agricultura (FAO, 2004), un tratado que la mayoría de los países ya han firmado, comprometiéndose a su cumplimiento.

1.2.2.- Los bancos de germoplasma:

Son los bancos de germoplasma o bancos de semillas las instituciones encargadas de recoger y conservar estos RRFF. Conservando la variabilidad existente en las especies cultivadas, ya sean variedades comerciales o tradicionales, así como sus especies silvestres relacionadas u otros materiales de mejora. Si bien el trabajo de recolectar, documentar y mantener estas entradas requiere un esfuerzo considerable, este trabajo se hace pequeño cuando esta ingente cantidad de semillas conservadas requieren describirse. En esta descripción se distinguen dos aspectos; por un lado la caracterización, que pretende identificar los atributos invariables de estos materiales, como pueden ser el color de las flores, forma del fruto, etc…, normalmente caracteres cualitativos; y por otro lado la evaluación que permite determinar caracteres de interés agronómico como precocidad, resistencia a plagas y enfermedades o contenido de determinados compuestos. El conocer estos atributos de interés son los que dan sentido a este tipo de conservación en los bancos de germoplasma (conservación *ex situ*), que lleva implícito un uso tanto presente como futuro de estos materiales. Así pues toda esta información generada en los trabajos de descripción es de máxima utilidad para los mejoradores, ya que manteniendo esta diversidad a su alcance les permite una respuesta rápida a las nuevas necesidades.

Para que toda esta cantidad de información recogida por los distintos bancos de germoplasma sea utilizable, se requiere el empleo de claves estandarizadas, que faciliten el intercambio de información. Desde la recogida de las muestras (los llamados datos de pasaporte), los datos de gestión o los datos de descripción, se emplean los denominados descriptores. En este sentido organismos internacionales como el IPGRI o la FAO en colaboración con organismos locales se han encargado de desarrollar estos descriptores adaptados a las distintas especies conservadas en los bancos de germoplasma.

En el caso del pepino dulce, en el año 2004 se desarrolló un descriptor morfológico desarrollado por el COMAV en colaboración con la FAO (Prohens et al., 2004). Desde su edición este descriptor ha sido usado en varios ensayos de caracterización del pepino dulce y de sus especies relacionadas (Rodríguez-Burruezo et al., 2011; Blanca et al., 2007), contribuyendo a un mejor conocimiento de estas especies. En esta tesis se ha empleado este descriptor para un estudio de la diversidad de

una colección de entradas de pepino dulce conservadas en el banco de germoplasma del COMAV junto especies silvestres relacionadas.

Si bien es importante disponer de descriptores morfológicos que nos permitan conocer los materiales al alcance de los mejoradores, también es importante disponer de descriptores fenológicos que nos permitan definir cada uno de los estadios de desarrollo de las plantas. Esto es útil, por ejemplo, para delimitar los estadios donde es más eficaz un tratamiento fitosanitario, cuando es apreciable un determinado carácter, cuando se ha alcanzado la madurez fisiológica o para extraer conclusiones de un determinado microclima. En este contexto, la escala BBCH (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) es una clave que permite describir y delimitar los distintos estados de desarrollo fenológico en plantas. Inicialmente fue desarrollada una clave general (Hack et al., 1992), para posteriormente desarrollarse claves específicas adaptadas a cada cultivo. Hasta la fecha el pepino dulce no disponía de una escala BBCH de caracterización fenológica por lo que los investigadores debían recurrir a una clave general de Solanáceas (Feller et al., 1995) o de alguna especie cercana como patata (Hack et al., 1993) o tomate (Feller et al., 1995).

1.2.3.- Los marcadores moleculares para el estudio de la variabilidad genética:

1.2.3.1.- ¿Qué es un marcador molecular? Aplicaciones en la conservación de RRFF.

La diversidad o variabilidad entre organismos se debe al efecto combinado del medioambiente y a diferencias en la secuencia de ADN. Estas diferencias suelen ser pequeños cambios que pretenden identificar los marcadores moleculares. Así pues un marcador molecular o marcador genético se define como un gen o una secuencia de ADN que puede ser usada para identificar un organismo, una especie o una característica fenotípica asociada a él.

En el caso de la conservación de los RRFF tiene aplicaciones directas, como puede ser la búsqueda de genes de interés, y sobre todo en estudios filogenéticos. Se emplean pues, ampliamente complementando los trabajos de caracterización morfológica.

1.2.3.2.- Marcadores más usados para evaluar la diversidad genética:

Existen distintas formas de clasificar a los marcadores moleculares; por ejemplo pueden clasificarse en dominantes (cuando no se pueden diferenciar los individuos homocigotos de los heterocigotos) o codominantes (sí se pueden diferenciar homocigotos y heterocigotos). También pueden clasificarse en si precisan de amplificación por PCR, u otra técnica como hibridación de sondas o digestión con enzimas. Otra clasificación importante es si necesitan o no de un conocimiento previo de la secuencia a estudiar.

Los marcadores AFLP (Amplified Fragment Length Polymorphism) son unos marcadores ampliamente usados en los estudios de la variabilidad. Combinan una digestión del ADN con dos enzimas de restricción con una amplificación por PCR de estos fragmentos previamente digeridos. Son marcadores que presentan muchas ventajas: por un lado, a pesar de ser dominantes, se obtiene una gran cantidad de fragmento polimórficos. Son marcadores que no requieren de grandes infraestructuras para su utilización y sobre todo que no se necesita tener conocimiento de la secuencia. En el caso del pepino dulce se han utilizado en varios trabajos. Por ejemplo Blanca y colaboradores en 2007 (Blanca et al., 2007) emplearon los AFLP para estudiar la variabilidad en una colección de entradas de pepino dulce y especies relacionadas, determinando cuáles eran las zonas de origen de mayor variabilidad y qué especies silvestres se encuentran más próximas a la cultivada. En otro trabajo de Prohens y colaboradores en 2006 (Prohens et al., 2006), se llevó a cabo con estos marcadores, la caracterización de prácticamente la totalidad de especies incluidas en la sección *Basarthrum* del género *Solanum*, donde está incluida el pepino dulce.

Otros marcadores ampliamente usados son los microsatélites o SSR. Los microsatélites son secuencias cortas de ADN que se repiten de manera consecutiva y el polimorfismo viene dado por diferencias en el número de esas repeticiones. Para ello es necesario amplificar estas secuencias vía PCR usando cebadores que flanquean los microsatélites. Son marcadores con un gran interés y un gran potencial, ya que presentan un gran polimorfismo, son codominantes y son sencillos de emplear. Como desventajas podemos comentar que, al ser fragmentos que difieren en pocas pares de bases, requieren sistemas electroforéticos de alta resolución para detectar los polimorfismos, usándose normalmente

analizadores de fragmentos de tipo capilar. Otra desventaja es que es necesario conocer la secuencia de las regiones flanqueantes para diseñar los cebadores. En ocasiones, los microsatélites se pueden transferir entre especies, de esta manera especies poco estudiadas a nivel genómico pueden verse favorecidas si existe alguna especie cercana más ampliamente estudiada y de la que se dispongan de estos marcadores. Así, en esta tesis, se han transferido con éxito marcadores microsatélites desarrollados en tomate para estudiar la variabilidad de una colección de entradas de pepino dulce y especies relacionadas.

Finalmente, otros marcadores de desarrollo más reciente son los SNV (Single Nucleotide Variant), que consisten en variaciones en la secuencia del ADN que afectan a un solo nucleótido. Pueden ser SNPs, cuando una base se ve sustituida por otra, o INDELs, cuando se producen pequeñas inserciones y deleciones. Son con diferencia los marcadores más abundantes en la naturaleza y los que más ampliamente se están utilizando en distintas disciplinas. Existen varios métodos de detección, pero los más eficaces están basados en la secuenciación del ADN por lo que hasta ahora su utilización estaba restringida a especies con un alto conocimiento a nivel genómico.

1.3.- Métodos de secuenciación masiva. Aplicaciones en especies menores

1.3.1.- Introducción a las técnicas de secuenciación:

Desde el descubrimiento de que la molécula del ADN era la portadora de la información genética (Avery et al., 1944) y de su estructura (Watson y Crick, 1953), el conocer la secuencia de bases que la componen, denominado secuenciación, supuso un gran reto para los científicos. Esta motivación fue dirigida principalmente a la secuenciación del ADN humano, objetivo cumplido a principios del siglo XXI gracias al trabajo de un consorcio formado por numerosos grupos de investigación (Venter et al., 2001). Al mismo tiempo se secuenciaron otras especies modelo como el ratón (Chinwalla et al., 2002), la mosca del vinagre (Adams et al., 2000) o *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000) la primera planta en ver su genoma completamente secuenciado.

La secuenciación de estas primeras especies supuso un gran esfuerzo, implicando numerosos grupos de investigación y grandes recursos económicos. La tecnología usada fue la secuenciación automática basada en el método de Sanger (Sanger et al., 1977), un método muy fiable,

pero a la vez lento y sobre todo muy caro. Como ejemplo se estima que la secuenciación del genoma humano costó aproximadamente unos 3.000 millones de dólares, lo que supone aproximadamente un dólar por cada base secuenciada.

El fundamento de esta secuenciación Sanger es la polimerización de copias del ADN con el uso de 4 dideoxinucleótidos distintos (uno para cada base) que sirven como terminadores de esa reacción de polimerización. Inicialmente se usaban geles de acrilamida para determinar el tamaño exacto de cada fragmento y con ello la posición de las distintas bases, pero posteriormente este proceso se automatizó utilizando dideoxinucleótidos marcados con fluoróforos y resolviendo su tamaño mediante electroforesis capilar. La automatización de este proceso fue desarrollada por la empresa Applied Biosystems y fue a partir de este hecho cuando se empezó a contemplar la posibilidad real de secuenciar organismos completos, ya sean genomas o transcriptomas.

1.3.2.- Tecnologías de secuenciación masiva de nueva generación (NGS):

Progresivamente el coste de la secuenciación automática basada en el método Sanger fue bajando, pero resultaba evidente que por mucho que se redujera no nos permitiría conocer de forma rápida y económica los genomas de otras especies menos importantes. Es entonces cuando varias compañías inician el desarrollo de nuevas técnicas, basándose en el desarrollo reciente de la nanotecnología, de esta manera surgen los primeros secuenciadores de ADN en paralelo de alto rendimiento, es lo que se denomina secuenciación de segunda generación. Un ejemplo de estos equipos son 454 (Roche), Solexa (Illumina), o SOLiD (Applied Biosystems) entre otros. Son tecnologías que inicialmente sólo eran capaces de leer secuencias cortas, por lo que su uso se restringía a la resecuenciación de genomas ya conocidos de una manera muy económica. Pero en la actualidad son sistemas capaces de leer secuencias lo suficientemente largas para permitir su empleo en la secuenciación de genomas o transcriptomas *de novo*.

Hoy en día se está hablando de la secuenciación de tercera generación, con el desarrollo de nuevos equipos que pretenden aumentar el rendimiento, disminuyendo el tiempo y el coste. La más conocida es PacBio (Eid et al., 2009) y básicamente consiste en un conjunto de técnicas que permiten la secuenciación molécula a molécula a tiempo real (SMRT® – Single Molecule Real Time Sequencing).

1.3.3.- La genómica en las especies menores:

De lo expuesto, se puede concluir que el avance en los últimos años en las técnicas de secuenciación masiva de nueva generación (NGS), ha permitido llevar a cabo proyectos de secuenciación, ya sea de transcriptomas o de genomas completos en organismos no modelos y con pocos o nulos conocimientos a nivel genómico. En la presente tesis, se presenta el análisis de manera exhaustiva del primer transcriptoma de pepino dulce. Se ha realizado un ensamblado *de novo*, una anotación estructural y funcional y una comparación con los genomas de tomate y patata. Además de ello, se han evaluado diferentes genes candidatos de caracteres de interés agronómico, así como un estudio filogenético comparando variantes en las secuencias de pepino dulce con los de otras Solanáceas.

1.4.- El pepino dulce es un cultivo prometedor

El pepino dulce (*Solanum muricatum* Aiton.) presenta un consumo bastante elevado en su zona de origen, donde es frecuente encontrarlo en los mercados locales, donde se considera un fruto muy apreciado. Su fruto, una baya, es refrescante, aromática y de sabor agradable, existiendo una gran variación de tamaño, forma y color entre distintos cultivares. Además es un fruto que puede tener varios usos en función de su momento de recolección; inmaduro puede consumirse como ensalada, mientras que maduro se considera fruta de postre, siempre que supere un mínimo de contenido en azúcar. También puede usarse para la elaboración de zumos y batidos, así como mermeladas, confituras y compotas.

1.4.1.- Taxonomía:

Como se ha comentado, el pepino dulce pertenece a la familia de las Solanáceas, y al género *Solanum*. Dentro de este género se clasifican otras especies como la patata, la berenjena y más recientemente el tomate, antes incluido en el género *Lycopersicon*. Dentro del género *Solanum*, el pepino dulce pertenece al subgénero *Potatoe*, a la sección *Basarthrum* y a la serie *Muricata*, siendo su único miembro.

El nombre científico por el que se conoce el pepino dulce, *Solanum muricatum*, fue dado en el siglo XVIII por William Aiton, del Royal Botanic Garden de Kew, en Londres (Aiton, 1814). Previamente había recibido otros nombres como *Melongena laurifolia* (Schultes y Romero-Castañeda, 1962), debido a que los frutos de algunos cultivares pueden

parecerse a la berenjena (*Solanum melongena*). Anteriormente a la denominación dada por Aiton, los españoles Ruiz y Pavón, a raíz de una expedición botánica en Perú y Chile, dieron al pepino dulce el nombre de *Solanum variegatum*, debido al característico veteado de los frutos (Ruiz y Pavón, 1957). Otro término por el que se ha conocido esta especie fue *Solanum guatemalense*, sobre todo en Norteamérica, debido a que la primera introducción de la planta en Estados Unidos a finales del siglo XIX fue realizada a partir de material procedente de Guatemala (Wickson, 1889).

El termino específico dado por Aiton, *muricatum*, significa con protuberancias cortas y duras (Aiton, 1814), y ha ocasionado alguna confusión, tal y como sugieren Schultes y Romero-Castañeda (Schultes y Romero-Castañeda, 1962). Según estos autores algunas fuentes indican que la planta podría tener espinas. Sin embargo, se piensa que el término "muricatum" hace referencia al aspecto que suelen presentar los tallos de la planta cuando crecen en condiciones de alta humedad, con el crecimiento de raíces adventicias (Figura 2).

1.4.2.- Origen y domesticación. Especies relacionadas:

Dentro de la sección *Basarthrum*, encontramos 22 especies, siendo el pepino dulce la única cultivada, aunque los frutos de alguna especie de esta sección se consumen de manera esporádica. Son varios los estudios que se han realizado para determinar cuál es el ancestro del pepino dulce y es aún a día de hoy una cuestión indeterminada (Blanca et al., 2007; Prohens et al., 2006). Cabe indicar que el pepino dulce no se conoce en estado silvestre; es posible que aún no se haya descubierto este ancestro (Brücher, 1970), pero es más probable que, o bien este ancestro se haya extinguido, o bien el cultivo se ha diferenciado mucho de la especie a partir de la cual se domesticó. En cualquier caso tanto para un proceso como otro se requiere un periodo largo tiempo.

Figura 2. Raíces adventicias en los tallos de la variedad Sweet Round de pepino dulce.

Dentro de la sección *Basarthrum* es el único miembro de la serie *Muricata*, pero se encuentra estrechamente relacionado con un grupo de especies silvestres, pertenecientes a la serie *Caripensa*. Es dentro de esta serie donde encontramos a las especies más probablemente involucradas en el origen del pepino dulce. Estas especies son las siguientes:

- *S. caripense* Humb. & Bonpl. *ex* Dunal: Es una especie que presenta una distribución muy amplia, con varios morfotipos, alguno de los cuales son similares a *S. muricatum* (Rodríguez-Burruezo et al., 2011). Es una planta que crece cerca de asentamientos humanos, y en ciertos países sus frutos se recolectan para su consumo ya que son muy dulces (Nuez y Ruiz, 1996). Es la especie que presenta menos diferencias de cariotipo con *S. muricatum* (Bernardello y Anderson, 1990) y se cruza con facilidad con ella, dando lugar a híbridos fértiles (Rodríguez-Burruezo et al., 2011), es por tanto una especie candidata a ser el ancestro del pepino dulce (Figura 3).

Figura 3. Frutos de la entrada E-7 de *S. caripense*.

- *S. tabanoense* Correll: Algún estudio propone a esta especie como el ancestro de *S. muricatum* (Brücher, 1970; Brücher, 1966; Brücher, 1968). Su fruto es ligeramente más grande que el de *S. caripense* y de una forma más parecida a la del pepino dulce (Figura 4). Sin embargo a nivel genético, a pesar de que cruza con el pepino dulce, estos híbridos son de menor fertilidad. Por otro lado su hábito de crecimiento también es diferente, siendo *S. tabanoense* de tipo rastrero y trepador (Nuez y Ruiz, 1996).
- *S. basendopogon* Bitter: Es otra especie que cruza con el pepino dulce, pero las semillas híbridas no son viables (Anderson, 1977). Sin embargo *S. basendopogon* presenta similitudes morfológicas con el pepino dulce. También se caracteriza por cruzar bien con *S. caripense* dando híbridos con alta fertilidad (Anderson, 1977).
- *S. cochoae* G.J. Anderson & Bernardello: Esta especie fue descrita más recientemente y viene a sumarse a la lista de posibles candidatos a ser el o los ancestros del pepino dulce (Anderson y Bernardello, 1991). Cuando se cruza con pepino dulce, solamente un 10 % de los cruzamientos tiene éxito, y de estos se recuperan muy pocas semillas con baja tasa de germinación. Aun así, los híbridos obtenidos demostraron una alta fertilidad (Nuez y Ruiz, 1996).

Figura 4. Frutos de la entrada E-257 de *S. tabanoense*.

En definitiva el origen del pepino dulce es un asunto aún bajo discusión, y en el caso de que sus ancestros todavía existan y según los últimos estudios (Blanca et al., 2007), *S. caripense* aparece como su ancestro más probable, sin descartar que otras especies como *S. tabanoense*, *S. basendopogon*, *S. cochaae* o incluso cualquier otra todavía desconocida hayan contribuido a la formación de *S. muricatum* a través de procesos de introgresión.

El lugar donde se llevó a cabo esta domesticación se desconoce, aunque parece que está claro que fue en la región andina (desde el sur de Colombia al sur de Perú), incluyendo en la actualidad los países de Colombia, Perú y Ecuador (Anderson, 1979). Uno de los métodos para determinar esta área de domesticación se basa en la distribución del ancestro silvestre del que procede. En este sentido, si el ancestro fuese *S. tabanoense* o *S. basendopogon* esa área se podría delimitar muy estrechamente. Por el contrario, si el ancestro fuese *S. caripense,* esta delimitación sería imposible, ya que esta especie presenta una distribución muy extensa (Anderson, 1977; Anderson, 1975; Heiser, 1992).

Más acuerdo hay entre los investigadores para indicar que el pepino dulce se domesticó hace muchos años (Prohens et al., 1996), y es que son varios los hechos que parecen apoyar esta idea:

- El hecho de que no se encuentre en estado silvestre hace pensar que o bien la especie silvestre se ha extinguido, o bien la especie cultivada se ha diferenciado mucho. Tanto un proceso como el otro requiere cierto tiempo.
- Ya existía cuando los españoles llegaron a esos territorios, con una enorme diversidad de cultivares. Esta diversidad precisaba de largo tiempo de cultivo.
- Aparece en representaciones ornamentales precolombinas (Figura 5) de culturas como la Paracas, Moche y Chimú.

Figura 5. Representación del pepino dulce en una vasija de la cultura Moche

1.4.3.- Historia y difusión del pepino dulce:

Previamente a la llegada de los españoles a los territorios del imperio Inca en el siglo XVI, el pepino dulce ya se cultivaba y se consumía, constancia de ello son los numerosos restos arqueológicos de diferentes culturas como Paracas, Moche y Chimú (Prohens et al., 1996). Además de ello, todos los cronistas que describieron los territorios del reino de Perú mencionaron la importancia del pepino dulce y la amplia presencia de su cultivo. Era una planta que sorprendió a estos cronistas por su agradable sabor, su variabilidad de formas y colores y lo saludable que parecía ser. Un ejemplo de este tipo de crónicas es la que le dedica el cronista Pizarro en el capítulo "*De las fructas que ay en el Reino del Pirú*" de su obra "*Relación del descubrimiento y conquista de los reinos del Perú*" (1572).

Todo parece indicar que el pepino dulce se cultivaba en mayor medida en los territorios bajo el dominio incaico, pero es muy probable que también se cultivara en la actual Bolivia, y en la región del Tucumán (norte de Argentina) y en norte de Chile.

Al ser una planta de importancia en estos territorios, y dada la aparente buena impresión que causó entre los españoles, cabe pensar que estos intentaron llevársela a la península. Sin embargo, no existen referencias de ello, y en caso de haberse introducido, probablemente no se hubiese adaptado y se hubiese abandonado la intención de cultivarlo.

Sí que existe constancia de una difusión temprana dentro de América. Por ejemplo, en la primera mitad del siglo XVII fue llevada a México (Cobo, 1964) así como al resto de Mesoamérica.

La primera referencia a la difusión del pepino dulce hacia Europa fue a raíz de la expedición botánica a los reino de Perú y Chile realizada por Ruiz y Pavón, a finales del siglo XVIII (Prohens et al., 1996). Durante esta expedición, que duró 12 años, se realizaron varios envíos tanto de plantas, como de semillas a España. Muchos de estos envíos fracasaron, pero alguno debió de llegar ya que esta especie ya figura en los catálogos del Jardín Botánico de Madrid de 1785. También tuvo éxito un envío de material vegetal al Jardín Botánico de Tenerife. En las Canarias este cultivo se adaptó con facilidad, y aún hoy se sigue cultivando.

Es probable que en esta misma expedición de Ruiz y Pavón se enviaran plantas a la corte del rey de Francia, y de ahí pasaran a los Jardines de Kew, donde Aiton le dio el nombre científico por el que se conoce actualmente (Aiton, 1789).

Posteriores referencias, ya en el siglo XIX, indican que el pepino dulce sufrió lo que podría considerarse un "redescubrimiento", por ejemplo se cita su adaptación al cultivo al aire libre en las proximidades de París (Tioutine, 1937), o que fue introducida a finales del siglo XIX como planta ornamental en Rusia donde tuvo mucha aceptación (Bukasov, 1930). También hay constancia de su presencia en los mercados de Inglaterra probablemente cultivados por agricultores locales (Anónimo, 1903) o en Italia donde se realizaron algunas experiencias sobre su cultivo la primera mitad del siglo XX (Baccarini, 1908; Nanetti, 1912; Casella, 1955).

A finales del siglo XIX fue introducido en los Estados Unidos, desde Guatemala (Anónimo, 1892) adaptándose, en Florida y California, donde fue bastante visible en los mercados.

En 1918, a partir de plantas procedentes de Canarias fue introducido en la isla de Cuba (Esquivel y Hammer, 1991), donde se le denomina melón pera o huevo de gato.

En 1952 se introdujo en Marruecos, donde se llevó a cabo una plantación comercial, que pretendía abastecer los mercados de Agadir, Francia e Inglaterra (Chapot, 1955).

En 1906 el pepino dulce fue introducido en Nueva Zelanda (Cossio, 1988), y en los años treinta era cultivado por el famoso viverista Hayward R. Wright, apareciendo en algunos catálogos comerciales (Morley-Bunker, 1983). En la actualidad, en Nueva Zelanda se han desarrollado numerosas variedades adaptadas a sus condiciones de cultivo, y junto con Chile, son los países que presentan una mayor exportación de este fruto.

1.4.4.- Composición del fruto e importancia nutracéutica:

El pepino dulce es una fruta que destaca por su alto contenido en agua (>92 %) y con un bajo contenido calórico (250 kcal/kg) (Rodríguez-Burruezo et al., 2011). Otras características importantes son su alto contenido en potasio y vitamina C, así como en carotenoides que le otorgan ese color amarillo (Hsu et al., 2011; Di Scala et al., 2011). La tabla 1 muestra el contenido en otros compuestos menos importantes cuantitativamente (Redgwell y Turner, 1986).

El fruto de pepino dulce destaca por su aroma agradable que se asemeja al del melón. Este aroma es el resultado de una compleja y específica combinación de compuestos volátiles, muy variable además entre las distintas variedades. A pesar de ser una característica tan apreciada en esta especie, son pocos los estudios dirigidos a determinar y evaluar esta fracción volátil responsable del aroma. Shiota *et al.* (Shiota et al., 1988) identificaron más de 30 compuestos en tres variedades de pepino dulce. Los compuestos encontrados más importantes fueron los acetatos de los alcoholes 3-metil-2-buten-1-ol y 3-metil-3-buten-1-ol, junto con los acetatos de hexilo, butilo y propilo. Encontraron además diferencias entre variedades que permiten explicar las diferencias más importantes de aroma entre ellas. Posteriormente Rodriguez-Burruezo y colaboradores (Rodríguez-Burruezo et al., 2004) realizaron un análisis de

los constituyentes volátiles del aroma de pepino dulce, determinando la existencia de tres grupos de aromas, que permitían diferenciar y caracterizar diferentes grupos varietales. Estos aromas son a fruta madura, constituido por acetatos y prenol, a vegetal verde, constituidos por aldehídos C6 y C9 y a fruta exótica formado por lactonas, mesifurano y β-damascenona.

Son varios los estudios que le otorgan al pepino dulce propiedades nutraceúticas, como propiedad antioxidante, antidiabética, antiinflamatoria y antitumoral (Hsu et al., 2011; Sudha et al., 2011; Shathish y Guruvayoorappan, 2014). Estas propiedades se han convertido en un objetivo prioritario en la mejora del pepino dulce, cosa que puede estimular su demanda.

1.4.5.- Manejo del cultivo. Estreses:

1.4.5.1.- Propagación y establecimiento del cultivo:

El método de propagación habitual del pepino dulce es mediante esquejes más o menos lignificados. Su semilla es viable y germina con relativa facilidad, pero no es un método común de propagación de este cultivo ya que los cultivares son altamente heterocigotos, por lo que se produce una elevada segregación en la descendencia (Prohens, 1997).

Esta propagación por esquejes resulta muy sencilla; se eligen plantas madre vigorosas y con buen estado fitosanitario y se cortan trozos de las ramas de uso 25-35 cm (Nuez y Ruiz, 1996). Es posible realizar la plantación directa de estas estacas, pero se recomienda en vías de una mayor supervivencia y una mayor homogeneidad del cultivo realizar una plantación previa en vermiculita con una solución fungicida de amplio espectro o un producto comercial enraizante (Nuez y Ruiz, 1996). El realizar una plantación previa también nos permite reducir el ciclo de cultivo, realizando el trasplante tras los últimos fríos y permitiendo el cuajado de los frutos antes de que lleguen las altas temperaturas del verano (Prohens, 1997).

1.4.5.2.- Sistemas de conducción de la planta y marcos de plantación:

En las zonas de producción de su centro de origen el pepino dulce se cultiva sin ningún tipo de poda o entutorado, desarrollándose de forma rastrera sobre el suelo, en campos que desde la distancia se asemejan a los de patatas. Los marcos de plantación son muy variables en función de las zonas y del grado de tecnificación del cultivo. Así por ejemplo en Chile

recomiendan plantaciones a marco cuadrado de no más de 10.000 plantas por hectárea (Carriel et al., 1982). En otros países suelen usar marcos más amplios que permitan el paso cómodo entre las plantas. Esta forma de cultivo es de manejo sencillo y de bajo coste, sin embargo, presenta el problema de que los frutos están en contacto con el suelo, por lo que pueden verse afectados por podredumbres o daños en su epidermis que reducen su valor comercial (Carriel et al., 1982).

Tabla 1.- Rango de concentraciones de componentes en el pericarpio de frutos maduros de pepino dulce (Redgwell y Turner, 1986).

Compuesto	Valores por 100 g
Peso seco (g)	6.8-8.2
Proteína (g)	0.10-0.13
Lípidos y pigmentos (mg)	24.6-44.4
Azúcares solubles (g)	4.9-6.4
Almidón (mg)	20.0-90.0
Celulosa (mg)	154-220
Hemicelulosa (mg)	40.1-53.6
Pectina (mg)	26.7-34.5
Vitamina C	46.0-68.8
Ácidos orgánicos no volátiles (mg)	119-153
Aminoácidos libres (mg)	52-70
Nitrógeno (mg)	23-30
Fósforo (mg)	10.7-12.3
Potasio (mg)	115-123
Azúfre (mg)	3.0-4.0
Calcio (mg)	2.3-3.0
Magnesio (mg)	5.3-6.1
Sodio (mg)	0.76-2.30
Hierro (mg)	0.20-0.31
Manganeso (mg)	0.06-0.07
Cinc (mg)	0.02-0.05
Cobre (mg)	0.02-0.03
Boro (mg)	0.03-0.05

Es por lo que se aconseja el empleo de sistemas de cultivo que impliquen algún sistema de entutorado que mantenga la planta y los frutos alejados del suelo. Este sistema favorece también una mayor aireación de la planta que minimiza la acción de las plagas al favorecer su tratamiento. Este sistema de cultivo también permite una mayor iluminación de los frutos que repercute en una mejora del color de epidermis, un factor de calidad muy valorado en el pepino dulce (Martínez et al., 1995).

Cabe indicar que es una especie que se adapta perfectamente a cultivo bajo invernadero. En el caso de Nueva Zelanda, se suele realizar una poda a tres brazos, eliminando los brotes laterales de manera similar al tomate, con la diferencia que el pepino dulce desarrolla varios brotes laterales desde la misma axila foliar, por lo que se debe realizar varias pasadas de poda, incrementándose los costes de cultivo (Nuez y Ruiz, 1996). Se suelen dejar entre 3 y 5 racimos por brazo, despuntando estos unas pocas hojas por encima del último racimo.

1.4.5.3.- Riego y abonado:

El pepino dulce es una planta que tiene un sistema radical poco profundo, por lo que requiere un aporte frecuente de agua durante todo el periodo de cultivo, sobre todo hasta que los frutos hayan alcanzado su tamaño definitivo. La dosis y frecuencia dependerá mayormente del tipo de suelo, si este es ligero, precisará de aportes más frecuentes que si es arcilloso (Prohens, 1997).

En general la planta se adapta muy bien al riego por goteo y no es muy sensible al exceso de humedad o encharcamiento. De igual manera tolera muy bien periodos de estrés hídrico prolongados, recuperándose rápidamente una vez superadas las condiciones de estrés (Nuez y Ruiz, 1996).

En cuanto al abonado depende principalmente de la fertilidad del suelo, pero se puede decir que no es una planta excesivamente exigente en cuando a fertilización, y se le supone que existen diferencias entre cultivares en este sentido (Prohens, 1997). Incluso se considera que en suelos muy fértiles la planta presenta un gran crecimiento vegetativo, en detrimento de la producción de frutos y haciendo más difícil el manejo del cultivo al exigir una poda mayor (Morley-Bunker, 1983).

1.4.6.- Plagas y enfermedades:

En general se puede decir que existen muchas plagas y pocas enfermedades que pueden atacar al pepino dulce. Como plagas más importantes podemos destacar:

- La araña roja (*Tetranychus urticae*), difícil de controlar en invernaderos en temporada cálida.
- Las moscas blancas (*Trialeurodes vaporarium*, *Bemisia tabaci*), que afectan principalmente a cultivos bajo invernadero.
- Pulgones (varias especies).
- El escarabajo de la patata (*Leptinotarsa decemlineata*).
- Las moscas minadoras (*Liriomyza trifolii*, *Tuta absoluta*).

Existen otras muchas plagas que pueden afectarlo, prácticamente todas las que afectan a cultivos hortícolas, pero debido a que es una planta muy vigorosa, se recupera rápidamente de ataques severos (Nuez y Ruiz, 1996; Prohens, 1997)

En cuanto a enfermedades, pueden afectarle sobre todo en climas húmedos y lluviosos la alternaria y el mildiu (Morley-Bunker, 1983), aunque quizá tengan mayor incidencia las virosis (Prohens, 1997). Las más comunes son las siguientes:

- Virus del bronceado o TSWV (*Tomato spotted wilt virus*), es un virus que se transmite principalmente por trips. En pepino dulce produce síntomas parecidos a los que produce en tomate, aunque no se produce una disminución aparente de la producción.
- Virus del mosaico del tomate o ToMV (*Tomato mosaic virus*). Es un virus que se transmite mecánicamente con una alta eficiencia. En tomate produce daños graves y en el caso del pepino dulce algunos cultivares sí se ven afectados de manera severa.
- Virus del mosaico del pepino dulce o PepMV (*Pepino mosaic virus*). También se transmite mecánicamente y produce un amarilleamiento en hojas jóvenes, aunque como el resto de los virus no produce una pérdida apreciable en la producción.

En definitiva, de manera aislada los virus no son un problema grave para el cultivo del pepino dulce, quizá el mayor problema es que al ser una especie de propagación vegetativa, pueden llegar a acumularse varias virosis a través de los ciclos de cultivo, que pueden requerir el empleo de técnicas como el cultivo de meristemos, la termoterapia o la quimioterapia para su saneamiento (Andrade y del Carmen, 1984; Jones et al., 1986). En este aspecto la conservación in vitro de estos materiales, evitando el contacto con el exterior es una medida a tener en cuenta.

1.4.7.- Estreses abióticos:

A continuación se expone el comportamiento del pepino dulce a los distintos estreses abióticos.

Sequía: como se ha comentado en el apartado de abonado, las raíces de pepino dulce son bastante superficiales por lo que se hace importante una alta frecuencia de riegos, recomendándose el riego localizado por goteo. A pesar de esto, es una planta que tolera muy bien el déficit hídrico, recuperándose muy rápidamente cuando cesan las condiciones de estrés (Morley-Bunker, 1983).

Salinidad: La salinidad está relacionada con el estrés hídrico, ya que el exceso de sal, al reducir el potencial hídrico, dificulta la absorción de agua por la plantas, por lo tanto el pepino dulce es una planta que la tolera bastante bien. En algún ensayo realizado en Israel (Pluda et al., 1993a; Pluda et al., 1993b) y España (Ruiz y Nuez, 1994a; Ruiz y Nuez, 1994b) se observó que una salinidad moderada mejoraba la calidad organoléptica de los frutos, es por tanto un cultivo adecuado para terrenos afectados por un ligero exceso de sal.

Altas temperaturas: Hay dos fases del desarrollo de la planta, floración y cuajado de frutos, que pueden verse seriamente afectadas por temperaturas elevadas, convirtiéndose en un factor limitante en muchas áreas de cultivo. Es por ello que en zonas cálidas se recomienda adelantar todo lo posible el cultivo para que cuando lleguen las altas temperaturas los frutos ya están cuajados (Nuez y Ruiz, 1996).

Bajas temperaturas: Aunque no está bien estudiado el efecto de las bajas temperaturas, se puede considerar que el pepino dulce es bastante sensible a las mismas (MacRae et al., 1986). Los daños producidos dependerán de la magnitud de esas bajas temperaturas.

1.4.8.- Recolección y post-cosecha:

La maduración de los frutos de pepino dulce no tiene lugar de forma agrupada, por lo que la cosecha no se agrupa en el tiempo y tiende a extenderse durante varios meses, con unos rendimientos de cultivo que depende de, además de la variedad y el modo de cultivo, del periodo durante el que se extienda esta recolección.

Existe una controversia en cuanto a si el pepino dulce es un fruto climatérico o no (Nuez y Ruiz, 1996). Algunos autores afirman que basándose en sus características respiratorias, es un fruto climatérico (El-Zeftawi et al., 1988), otros autores (Heyes et al., 1994; Ahumada y Cantwell, 1996) en cambio afirman que no lo es, al no obtener una síntesis endógena de etileno cuando se aplicaba a los frutos propileno. En este trabajo de Heyes se consiguió un cambio en el color del fruto y ablandamiento de la carne, pero no se modificó el contenido en azúcares. Según el trabajo de Martí y Valero (Martı´ y Valero, 2003) todo parece apuntar que existen diferencias entre cultivares en este asunto, además de que sería necesario redefinir o matizar el concepto de fruto climatérico.

Debido a que la máxima calidad organoléptica del fruto se alcanza cuando este está completamente maduro, hace difícil su manipulación y transporte. Aun así es un fruto que puede conservarse en buen estado durante bastantes días tras su recolección, hasta 60 días en cámaras de almacenaje refrigerado en 5 y 10ºC (Goubran, 1985), incluso algunos autores aconsejan mantener los frutos a 10ºC durante 4 semanas para mejorar la calidad organoléptica (Morley-Bunker, 1983).

1.4.9.- Tipos varietales más comunes:

El centro de origen del pepino dulce se encuentra en territorios que en la actualidad corresponden con Perú, Ecuador, Colombia y Bolivia. A pesar de su larga historia, sigue siendo en esas zonas un cultivo marginal, sobre el que no se han realizado programas de mejora, por lo que no existen nombres diferenciados para los distintos cultivares, o bien estos se han perdido. Lo contrario ocurre en Nueva Zelanda y España donde sí se han desarrollado nuevos cultivares con trabajos de mejora, que implican selección a partir de semillas y cruzamientos entre líneas y posterior selección de clones mejor adaptados a sus condiciones de cultivo.

Tipos varietales cultivados en Ecuador:

Ecuador es posible que sea el país donde más diversidad de tipos se pueden encontrar (Schultes y Romero-Castañeda, 1962; Prohens, 1997; Heiser, 1964), sin embargo predominan dos clases principales de cultivares:

- Cultivares con frutos de tamaño grande y formas globosas, donde el color de fondo del fruto inmaduro es verde y con un veteado escaso con bandas púrpuras bien definidas.
- Cultivares más pequeños y formas más alargadas, en ocasiones casi cilíndricas. El color de los frutos inmaduros es casi blanco y el veteado morado es más abundante, ocupando en ocasiones un alto porcentaje del fruto, y sus bandas menos definidas.

De las dos formas predomina la primera ya que presenta mejores características para su transporte y manipulación, con una carne de mayor consistencia que resulta más apreciada.

Tipos varietales cultivados en Perú:

Según Delgado de la Flor (Delgado de la Flor et al., 1988) en Perú predominan frutos con formas acorazonadas.

Siguiendo la clasificación hecha por Correll (Correll, 1962), Sánchez-Vega (Sánchez-Vega, 1992) considera dos variedades botánicas, por un lado la variedad "Protegenum" de hojas compuestas y la variedad "Typica" de hojas simples. Según este autor en la Sierra de Cajamarca predomina la forma típica, con frutos subesféricos, de ápice hendido y color verde amarillento con algún jaspe violeta. En cambio en zonas de costa se encuentra la forma *glaberrimum*, también de la variedad "Typica", que presenta hojas sin vellosidad. De esta forma se distinguen dos variedades:

- Morado listado: Con hojas verde oscuro, ramas suberectas y frutos ovoide-cónicos de tamaño variable. Con pulpa amarilla muy dulce, muy apreciados.
- Oreja de burro: Con hojas de color verde claro, ramas largas y semipostradas. Con frutos de color blanquecino con pocas manchas y de tamaño grande-mediano. Pulpa también blanquecina y menos dulce.

Tipos varietales cultivados en Chile:

En Chile tampoco existen variedades como tal, pero también se diferencian tipos característicos de cada zona de cultivo (Prohens, 1997). Así en el norte del país (Coquimbo, La Serena), se cultivan tipos de forma ovalada o acorazonada, con los extremos redondeados, con escaso veteado. En la zona central (Quillota, Valparaíso), se cultiva un tipo alargado, con el extremo apical puntiagudo, de color cremoso con bastantes vetas moradas. Este tipo presenta un ciclo de cultivo más corto adaptándose mejor a condiciones templadas, y debido a su forma alargada presenta más problemas para el transporte y la manipulación.

Como curiosidad, una variedad de pepino dulce registrada en Estados Unidos (Gomberoff, 1991), la Cascade Gold, procede de una mutación de unas plantas cultivadas en la provincia de La Serena, que se caracteriza por ser muy dulce y no tener el retrogusto típico de algunos frutos de pepino dulce. Es además una variedad que se adapta bien al cultivo en zonas más frías.

Tipos varietales cultivados en Nueva Zelanda:

Es el país donde existen más variedades registradas de pepino dulce. Algunas variedades han sido introducidas directamente procedentes de la región andina, mientras que otras se han obtenido en la propia Nueva Zelanda mediante selección a partir de semillas (Nuez y Ruiz, 1996). Algunas de estas variedades más importantes son:

- Asca: muy productivo. Fruto grande, ovoide, de color amarillo claro, vetas verdes y contenido en sólidos solubles medio-bajo.
- Kawi: de producción media. Fruto de tamaño medio, alargado, verde pálido, vetas púrpuras y contenido en sólidos solubles medio (Figura 6).
- El Camino: el más cultivado y de buena producción. Fruto de tamaño medio, forma ovoide-acorazonada, amarillo, vetas púrpuras y contenido en sólidos solubles alto. Quizá sea la variedad de pepino dulce más famosa del mundo y en la que más estudios se han realizado (Figura 6).

Otras variedades neozelandesas son: Miski, Suma, Schmidt, Toma, Lincold Long, Lincold Gold, Golden Litesripe.

Figura 6. Frutos de El Camino (izquierda) y Kawi (derecha).

Tipos varietales cultivados en Australia:

Australia es un país en el que también se han desarrollado variedades propia. Existen variedades que básicamente se clasifican por la forma de sus frutos en: alargados, ovoides y ovoides grandes (la mayoría). Algunas de estas variedades son: Pepino Gold, Naragold, Golden Spendour, Wayfarer Special, Temptation y Colossal. También se cultivan ciertas variedades de origen neozelandés.

Tipo varietales cultivados en España:

En España, tras un programa de selección y mejora de varios años a partir de semillas procedentes del centro de origen del pepino dulce, se han ido liberando cultivares en los últimos años. Todos ellos han sido desarrollados por el COMAV en la Universitat Politècnica de València y se encuentran adaptados a la climatología mediterránea. Las más importantes son:

- Sweet Long (Ruiz et al., 1997): de producción media. Fruto alargado, amarillo dorado, vetas moradas y contenido en sólidos solubles elevado (Figura 7).
- Sweet Round (Ruiz et al., 1997): producción media (inferior a Sweet Long). Fruto redondo, amarillo dorado, vetas moradas y contenido en sólidos solubles elevado.
- Puzol (Prohens et al., 2002): de producción alta. Frutos grandes y elongados, amarillo dorado, abundante veteado púrpura y contenido en sólidos solubles medio (Figura 7).

- Turia (Rodríguez-Burruezo et al., 2004): de producción alta. Fruto de tamaño medio, ovalado, color dorado, vetas moradas definidas y contenido en sólidos solubles medio (Figura 7).
- Valencia (Rodríguez-Burruezo et al., 2004): de producción media-alta. Frutos de tamaño medio, elongados, color dorado, vetas estrechas moradas y contenido en sólidos solubles muy alto (Figura 7).

1.4.10.- Objetivos de mejora en el pepino dulce:

El pepino dulce, pese a ser un cultivo con gran potencial en muchas regiones, es una especie poco estudiada en la mayor parte de los aspectos. Para que esta especie se adapte a unas condiciones de cultivo más amplias y a un mayor espectro de consumidores se requiere trabajar principalmente en los aspectos que limitan su cultivo, que dificultan su transporte, manipulación y conservación post-cosecha y en la calidad del fruto, tanto en calidad organoléptica como calidad nutricional.

Figura 7. Frutos de las principales variedades de pepino dulce desarrolladas en España.

1.4.10.1.- Aspectos que limitan su cultivo:

- Duración del ciclo de cultivo y agrupación de la producción:

Para permitir que el cultivo del pepino dulce sea viable en zonas templadas y frías se requiere trabajar en la selección de materiales con un ciclo de cultivo más corto. En el caso de nuestras condiciones de cultivo,

lo interesante sería obtener frutos maduros antes de que llegara el calor del verano, y en el caso de zonas más frías, el objetivo sería recoger la cosecha antes de que llegasen las primeras heladas (Nuez y Ruiz, 1996). Un ejemplo de esto sería la variedad desarrollada por Gomberoff en 1991 (Gomberoff, 1991) a partir de una mutación de un cultivar chileno, con un precocidad tal que permitía su cultivo en el estado de Washington, al norte de los Estados Unidos.

Por otro lado uno de los problemas que presenta el cultivo es que el periodo de recolección puede alargarse varios meses, al no madurar los frutos de manera agrupada, aumentándose los costes de cultivo. Para agrupar esta producción tradicionalmente se ha empleado ethephon (ácido 2-cloroetilfosfonico, precursor del etileno) (Araya y Juan, 1987) así como prácticas culturales como el entutorado y la poda (Prohens et al., 1997), pero también se ha encontrado variabilidad genética, encontrándose cultivares más precoces (Prohens et al., 1997; Ruiz y Nuez, 1997). Por lo que, pese a ser un carácter que todavía no se ha estudiado, es susceptible llevar a cabo programas de mejora genética con este objetivo.

- Cuajado:

A pesar de tener una floración abundante en un amplio rango de condiciones climáticas el cuajado de los frutos suele presentar ciertas dificultades (Prohens, 1997), originando irregularidad en la producción. Estos fallos en el cuajado pueden ser debidos a varios factores como las temperaturas desfavorables (Ruiz y Nuez, 2000), la falta de liberación del polen, la competencia de la fructificación con el desarrollo vegetativo y la baja intensidad luminosa. Otro factor importante son las características propias del cultivar, habiendo alguno de ellos que presenta mayor facilidad para el cuajado de frutos, bien sea por la características florales o del polen, bien porque son partenocárpicos. Seleccionar estos cultivares y evaluar la genética de este carácter es un paso importante para desarrollar variedades adaptadas a diferentes zonas de cultivo.

- Solución de problemas post-cosecha:

Quizá uno de los aspectos más estudiados en pepino dulce sean los relacionados con la conservación post-cosecha y el procesado (Di Scala et al., 2011; Prono-Widayat et al., 2003). Es un fruto que para que tenga una elevada calidad organoléptica debe recogerse en estado de madurez avanzada, por este motivo, soporta mal el transporte y la manipulación. A

pesar de esto, frutos limpios, sin golpes ni daños en la piel pueden conservarse en buenas condiciones durante bastante tiempo, tanto en cámaras como a temperatura ambiente. Existe una gran variabilidad de comportamiento en la forma de madurar en los distintos cultivares, es por ello que un correcto estudio de estos caracteres y su comportamiento se hacen imprescindibles para iniciar programas de selección y mejora.

1.4.10.2.- La calidad del fruto:

- Calidad organoléptica o sensorial:

Uno de los problemas que aparece cuando se pretende cultivar el pepino dulce en condiciones agroclimáticas diferentes a las de su origen, es que se produce una merma en su calidad organoléptica (Prohens, 1997). Principalmente se produce una reducción en el contenido en sólidos solubles, mayormente azúcar, lo que dificulta su introducción en nuevos mercados como fruta de postre. Otro problema que aparece en estas condiciones es que se acentúa el retrogusto típico que presenta el pepino dulce, que consiste en la persistencia de un ligero sabor amargo o picante y una sensación de aspereza en la lengua (Prohens et al., 2002; Levy et al., 2006). Es importante entonces la selección de clones que mejor se adapten a cada condición de cultivo seleccionando por contenido en sólidos solubles y realizando evaluaciones sensoriales de la calidad organoléptica global haciendo especial énfasis para minimizar este retrogusto antes mencionado.

- Calidad nutracéutica:

La nutracéutica es un término relativamente reciente que se usa para definir aquellos productos o compuestos naturales que presentan una acción terapéutica al ser ingeridos. La palabra en sí, deriva de la fusión de las palabras nutrición y terapéutica. Esta acción terapéutica no hay que entenderla como que los alimentos curen *per se*, sino que son alimentos que ayudan en el tratamiento de determinadas enfermedades o previenen el desarrollo de las mismas. Quizá entonces la denominación más correcta de estos productos sea la de “alimento funcional”.

Tradicionalmente, tanto la calidad organoléptica como la nutracéutica no se ha considerado en los programas de mejora, priorizando otros objetivos como la mejora de la productividad, uniformidad, calidad externa y resistencia a estreses (Harlan, 1975), pero esta tendencia está cambiando. Numerosos expertos en nutrición afirman que una dieta que sustituya las grasas animales por frutas y verduras se

considera un factor positivo en la prevención de enfermedades cardiovasculares, diabetes, cáncer, y otras enfermedades degenerativas (Cámara, 2006). Es por este motivo que en los últimos años, para satisfacer la demanda de los consumidores de una dieta más saludable, los investigadores y mejoradores han comenzado a trabajar para modificar la composición de frutas y verduras, mejorando el contenido en determinadas sustancias beneficiosas, y a día de hoy puede considerarse este, un objetivo de mejora prioritario para muchos cultivos.

Dentro de las frutas y verduras existen muchos grupos de compuestos con interés nutracéutico (Cámara, 2006; Shashirekha et al., 2015), como por ejemplo:

- Vitaminas y sus precursores como el ácido ascórbico.
- Minerales incluyendo microelementos como el cobre, el hierro y el cinc.
- Fibra alimentaria.
- Polifenoles.
- Carotenoides.
- Glucosinolatos.
- Folatos.
- Fitoesteroles y otros.

Normalmente, para cada especie, los objetivos mejora se centran en la mejora de aquellos compuestos o grupos de compuestos que presentan propiedades más relevantes.

Cabe indicar que en la actualidad ya se han liberado variedades cuya característica principal es una mayor calidad nutracéutica, ejemplos de esto son la sandía "Fashion", que presenta un elevado contenido en licopeno y citrulina (Tarazona-Díaz et al., 2011), la variedad de tomate "Lycomate" de alto contenido en licopeno, o el famoso arroz dorado (Potrykus, 2001; Paine et al., 2005), capaz de sintetizar en su grano elevadas cantidades de β-caroteno, pero que debido a su origen transgénico ha visto entorpecido su cultivo.

En el caso del pepino dulce se han descrito numerosas propiedades beneficiosas para la salud. Destaca por su elevado contenido en potasio (>1g/kg) y vitamina C (>200mg/kg) (Rodríguez-Burruezo et al., 2011;

Redgwell y Turner, 1986; Pluda et al., 1993b; Sanchez et al., 2000), así como un elevado contenido en polifenoles y flavonoides (Sudha et al., 2011). Se ha descrito que tiene propiedades hipotensivas (Redgwell y Turner, 1986), diuréticas (Sánchez-Vega, 1992), y gracias a su elevado contenido en polifenoles antioxidantes su consumo puede prevenir la diabetes (Hsu et al., 2011; Orhan et al., 2014) y por último también presenta actividad antitumoral (Ren y Tang, 1999). En este sentido las especies silvestres relacionadas con el pepino dulce suponen un reservorio de propiedades nutricionales y nutracéuticas de interés para la mejora del pepino dulce. *S. caripense* y *S. tabanoense* son consumidas en su zona de origen por la extremada dulzor de sus frutos (Rodríguez-Burruezo et al., 2011), y son varios los trabajos que describen un elevado contenido en compuestos bioactivos (Prohens y Nuez, 2001; Rodríguez-Burruezo et al., 2003; Prohens et al., 2005).

Explotar estas características tan importantes del pepino dulce puede favorecer la aceptación de esta especie por los consumidores, para ello es imprescindible mantener una elevada calidad organoléptica del fruto. En esta tesis se ha realizado un primer trabajo de caracterización de la composición nutricional de las entradas de pepino dulce y relativas conservadas en el banco de germoplasma del COMAV. En cinco entradas de esta colección se evaluó además el perfil de polifenoles presentes en el fruto, la actividad antioxidante y se realizó un ensayo con líneas celulares que pretendió evaluar la actividad biológica de extractos de esas entradas frente a un estrés inducido.

OBJETIVOS

El objetivo principal de esta tesis es el desarrollo de herramientas para la caracterización morfológica, molecular, genómica y nutracéutica del pepino dulce. Con estas herramientas se pretende la correcta caracterización, ya sea de los materiales estudiados en este trabajo, como de otros cultivares u otras entradas conservadas en los bancos de germoplasma, con vistas al desarrollo de variedades comerciales adaptadas a diferentes zonas de cultivo, ofreciendo materiales demandados por los consumidores, interesados en productos novedosos, pero sobre todo en productos saludables.

Para cumplir este objetivo principal, la estructura del trabajo se ha dividido en cuatro partes que engloban los cinco artículos presentados:

1.- Desarrollo de una clave fenológica BBCH específica para pepino dulce que permita la identificación precisa de los distintos estados fenológicos en este cultivo.

2.- Estudio de la diversidad morfológica y molecular mediante marcadores microsatélites transferidos de tomate, de una colección de entradas de pepino dulce y especies silvestres relacionadas.

3.- Secuenciación del primer transcriptoma en pepino dulce, ensamblaje *de novo*, análisis comparativo con los genomas de tomate y patata, estudio filogenético, estudio de genes relacionados con la domesticación y desarrollo masivo de marcadores moleculares.

4.- Caracterización de la calidad nutracéutica en la colección de entradas de pepino dulce y especies silvestres relacionadas:

> 4.1.- Evaluación del contenido en materia seca, proteínas, antioxidantes, pigmentos y minerales.
>
> 4.2.- Estudio del perfil de polifenoles, capacidad antioxidante y actividad biológica de cuatro entradas de pepino dulce y una de la especie silvestre *S. caripense*.

RESULTADOS

3.1.- Phenological growth stages of pepino (*Solanum muricatum*) according to the BBCH scale

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Scientia Horticulturae, 2015, 183, 1–7.

ABSTRACT

The pepino (*Solanum muricatum*) is a solanaceous vegetatively propagated fruit crop of Andean origin. We provide a detailed description of phenological stages because it is of interest for pepino crop management and research. Given the increasing prominence of this crop, and the fact that it morphologically and developmentally variable, and different from other major solanaceous crops, we have developed a pepino specific BBCH (Biologische Bundesanstalt, Bundessortenamt, CHemische Industrie) numerical scale. Nine principal stages are described for germination/rooting, leaf development, formation of side shoots, main shoot elongation, inflorescence emergence, flowering, development of fruit, ripening of fruit and seed, and senescence. Secondary stages (two-digit scale) have been identified for all principal stages. Complementary descriptions using mesostages (three-digit scale) have been developed for leaf development, formation of side shoots, inflorescence emergence, and flowering phenological stages. A description of all phenological stages combined with illustrations is provided. The utility of the BBCH scale has been validated by comparing several traits of agronomic interest at specific developmental stages in a collection of pepino local varieties, modern cultivars and wild relatives. The BBCH scale developed provides uniform criteria for the description, identification and selection of phenological stages of the pepino and will facilitate the management, breeding and conservation of genetic resources of this crop.

Keywords: characterization, development stages, phenological scale, Solanaceae, varietal differences

1. Introduction

The pepino (*Solanum muricatum* Aiton) is an herbaceous crop domesticated in the northern Andes, where its closest wild relatives, from *Solanum* section *Basarthrum* also thrive (Anderson et al., 1996; Blanca et al., 2007). The pepino can be very variable in shape and colour, and is mostly consumed when fully ripe as a fresh fruit. At maturity, it has a characteristic mild sweet flavour and intense fruity aroma, which has some resemblance to that of melon (Prohens et al., 2005). In the last few decades demand for pepinos in commercial exotic fruit markets has grown, which has increased the interest and production of this crop not only in its region of origin but also in other temperate regions of the world (Rodríguez-Burruezo et al., 2011). As for other emerging crops, there is

little information on production statistics, but the production in Ecuador is estimated at around 400 ha (Hidalgo, 2006).

The pepino has a number of specific features that distinguish it from major solanaceaous fruit crops such as tomato (*S. lycopersicum* L.), pepper (*Capsicum annuum* L.) or eggplant (*S. melongena* L.) (Rodríguez-Burruezo et al., 2011). These include vegetative propagation; in agricultural practice, the pepino is usually propagated by cuttings which root easily when placed in a wet substrate. An alternative way of clonal propagation is the use of *in vitro* micropropagation, which allows the production of disease free plants (Cavusoglu and Sulusoglu, 2013). Also, the pepino grows luxuriantly, and such vegetative growth may compete with fruit set, so the highest yields are obtained when the lateral side-shoots are removed, nitrogen fertilization is controlled to avoid excessive vegetative growth, and the plants are trained with vertical strings using a one or two main shoot system (Kowalczyk and Kobryn, 2003). Another difference with major solanaceous fruit crops is that many pepino cultivars display a strong tendency to parthenocarpy, with some cultivars obligately parthenocarpic (Prohens et al., 2005). In addition, the pepino fruit needs a long time (up to 70 days) to fully ripen since. Finally, fruit quality, especially sugar concentration, may be influenced by temperature during ripening; high temperatures result in a lower sugar content and in the development of an off-flavour (Rodriguez-Burruezo et al., 2011).

The development of characterization tools for the precise and standardized description of the pepino plants and fruits is essential for an increased efficiency and effectiveness of research experiments, breeding programmes, conservation of germplasm and for the comparison of experimental data (Gotor et al., 2008; Meier et al., 2009). As a result, we produced a list of standarized descriptors (IPGRI and COMAV, 2004). Although this list is useful for the description of characteristics of pepino varieties and wild relatives and for the study of the morphological variation in collections and segregating generations, no standardized scales to precisely describe the phenological stage of pepino plants, which would be of great utility for agronomic and botanical research (Meier, 2001), are available. The BBCH (Biologische Bundesantalt, Bundessortenamt und Chemische Industrie) scale for the phenological identification of the growth stages of all species of mono- and dicotyledonous plants (Lancashire et al., 1991). This scale uses 10 principal stages (0-9), each of which is further divided into 10 secondary

(0-9) growth stages. An extended BBCH-scale, using mesostages (0-9), was proposed for some crops using a third-digit scale (Meier, 2001). Both scales (simple and extended) have been developed and are widely accepted for many crops (Meier et al., 2009). The development stages of pepino have not yet been defined and described. Given the increasing interest in pepino cultivation and breeding (Rodríguez-Burruezo et al., 2011), we suggest that the development and validation of a phenological BBCH scale for pepino might be of interest for the efficient development of this emerging crop.

2. Material and methods

2.1. Plant material

Phenological observations were made by the authors through a period of more than half a century (initiated C.B. Heiser in the 1960s (Heiser, 1964) and followed up by G.J. Anderson) of pepino research, cultivation, evaluation and breeding of pepinos. These proposals are based on research that has included examination of pepino plants growing in different environments and cultivation conditions in its native home in the Andean region, as well under cultivation outside and in glasshouses in the USA, Spain, and a number of other countries. In addition, the phenological cycle of pepino was specifically studied for the development of the BBCH scale in a characterization trial performed from February to July 2014 in an experimental greenhouse on the campus of the Universitat Politècnica de València (Valencia, Spain). This latter area has a typical Mediterranean climate, with mild winters and long warm and dry summers. Materials used in this trial included 14 clonal pepino varieties consisting of six local varieties from the Andean region and eight commercial cultivars. In addition eight accessions of the wild species most closely related to the domesticate pepino were studied, including: *S. caripense* Humb. and Bonpl. ex Dun. (four accessions), *S. catilliflorum* G.J. Anderson, Martine, Prohens and Nuez (one accession), *S. perlongistylum* G.J. Anderson, Martine, Prohens and Nuez (one accession), *S. tabanoense* Correll (one accession) and *S. trachycarpum* Bitter and Sodiro (one accession) (Anderson, 1979; Anderson et al., 2006). Pepino materials were vegetatively propagated *in vitro* and after acclimatization were transplanted in the greenhouse in 1 m deep benches filled with silica sand as substrate. Wild relatives were germinated from seed and one individual was clonally propagated *in vitro* for the trial. For each of the pepino varieties and wild relatives, five plants were cultivated

and arranged in a completely randomized design. Watering and fertilizers were applied with the drip irrigation system. Plants were cultivated in the winter-spring cycle and trained using vertical strings. For self-incompatible wild relatives, manual pollinations with compatible pollen were carried out to ensure fruit set. In order to validate the BBCH scale for comparison of varieties, several traits of agronomic importance (stem length, fruit length, fruit width, fruit length/width ratio, time from transplant to beginning of ripening, and soluble solids content) were taken at specific BBCH stages. Univariate analyses of variance (ANOVA) were performed for the traits considered. Significance of differences among clones was studied using the Student-Newman-Keuls multiple range test at a significance level of P=0.05.

2.2. Pepino BBCH scale characteristics

Based on the existing extended BBCH-scale (Meier, 2001), the completed growth cycle of pepino was divided into nine principal growth states, including germination (for seed propagation) / rooting (for vegetative propagation) (stage 0), leaf development (stage 1), formation of side shoots (stage 2), main shoot elongation (stage 3), inflorescence emergence (stage 5), flowering (stage 6), development of fruit (stage 7), ripening of fruit and seed (stage 8), and senescence (stage 9). BBCH-scale stage 4 (development of harvestable vegetative plant part or vegetatively propagated organs / booting) is not applicable to pepino. Each principal growth stage was classified into secondary stages, ordered from 0 to 9, which can represent an ordinal number or a percentage (1=10%, 2=20%, etc.) that are used to describe precise time points or shorts intervals of development within each principal stage. The combination of the principal stage number with the secondary stage number results in a two-digit code. For situations in which the growth stages are not defined with sufficient precision with the two-digit code, the inclusion of a mesostage (with a 0 to 9 code) between the principal and secondary stages provides a further subdivision and results in a three-digit scale (Meier, 2001). This results in a three-digit scale, that can be used as an alternative to the regular two-digit scale. For main stages where the mesostage is not applicable, then a 0 is used for the mesostage when the three-digit scale is used. The principal growth stages do not need to proceed in the strict sequence defined, but may occasionally proceed in parallel (Meier, 2001). In this case, if two or more principal stages proceed in parallel, they can be indicated using a diagonal stroke (e.g., 33/61 or 303/601).

3. Results and discussion

Unlike major solanaceous fruit crops, like tomato, pepper, or eggplant, that are propagated by seeds, the pepino is mostly propagated vegetatively in the agricultural practice (Prohens et al., 2005; Cavusoglu and Sulusoglu, 2013). Although potato (*Solanum tuberosum* L.) is also vegetatively propagated, the fact that potato is cultivated for its tubers and pepino for its fruits results in many differences in the phenology of both crops. There are also important morphological and developmental differences in pepino vs. other major solanaceous crops (Prohens et al., 1998; IPGRI and COMAV, 2004; Prohens et al., 2005; Rodríguez-Burruezo et al., 2011); these differences strongly argue for the development of a BBCH scale specifically for the pepino.

The two-digit scale provides a precise definition of most of the phenological growth stages in most crops (Meier, 2001). As a result of our observations we have developed a two-digit BBCH scale for pepino. However, for stages 1 (leaf development), 2 (formation of side shoots), 5 (main shoot elongation), and 6 (flowering) we consider that mesostages appropriate for more precise description in certain circumstances, and therefore, we have also developed the three-digit scale. In Solanaceous crops for which the BBCH scale is available, the use of mesostages is common in particular for stages involving the development of vegetative aerial parts, and flowering, and fruiting (Hack et al., 1993, Feller et al., 1995; Ramírez et al., 2013). Below, we provide a description of the phenological cycle stages for pepino based on our studies. The pepino BBCH phenological stages scale provides a complement to the descriptors for pepino (IPGRI and COMAV, 2004), that we developed for describing the morphological variation of the crop. Furthermore, we have validated the utility of the BBCH scale for the comparison among pepino varieties and wild relatives of several agronomically relevant traits (Prohens et al., 2005; Rodríguez-Burruezo et al., 2011) measured at specific developmental stages.

3.1 Principal growth stage 0: germination / rooting

This stage describes the germination of seed when plants are produced from seed and the rooting of explants when plants are vegetatively propagated (Table 1). Seed propagation in pepino is used in breeding programmes (Rodríguez-Burruezo et al., 2011) and is also the natural reproductive system of pepino wild relatives (Anderson et al.,

1979, 2006). Vegetative propagation, either using herbaceous cuttings for rooting in a substrate like peat, perlite, or vermiculite, or through *in vitro* micropropagation, is used in the commercial production of pepino (Cavusoglu and Sulusoglu, 2013). When propagated by seed, this stage begins with the dry seeds (stage 00 or 000), that after being sown in a substrate, in a Petri dish, or in an *in vitro* culture medium, typically take a few days to get fully imbibed (stage 03 or 003). In a period between three and 30 days, the radicle emerges from the seed (stage 05 or 005) and after three days to one week after this stage, the emergence of cotyledons takes place (stage 09 or 009). When vegetatively propagated, the stage begins with the cuttings or explants (stage 00 or 000). After being placed in a wet substrate or in *in vitro* growing medium there is a swelling of the explant part in contact with the substrate or medium (stage 00 or 001) and in a few days root protuberances are evident (stage 03 or 003). On occasions the cuttings already have hardy root protuberances, however we consider that the stage 03 or 003 is reached when these protuberances are swollen and in the process of breaking for developing actively growing adventitious roots (stage 05 or 005). The subsequent stage is when axillary buds begin breaking (stage 07 or 007), which is followed after two to six days by the buds showing green tips (stage 09 or 009).

3.2 Principal growth stage 1: Leaf development

The development of the young plant mostly involves the growth and appearance of new leaves. The number of leaves in the most developed (main) shoot determines the phenological stage code (Table 2). Pepino leaves are alternate and can be simple or pinnate, depending on the variety and on the stage of development of the plant and leaf (IPGRI and COMAV, 2004). For seed propagated plants, this main stage begins with the cotyledons being completely unfolded and, in the case of vegetatively propagated plants, with the dominant axillary bud leaf emerging (stage 10 or 100). The following stages continue with the unfolding of subsequent leaves in the main shoot so that when the first leaf is unfolded the plant is at stage 11 or 101 and end when at least the 9 (two-digit scale) or 19 leaves (three-digit scale) of the main shoot are unfolded (stages 19 and 119, respectively).

3.3. Principal growth stage 2: Formation of side shoots

Pepino plants form side shoots derived from axillary buds of the main shoot, or in the case of vegetatively propagated plants, from buds

other than the dominant bud in the cutting or explant. This main stage begins with the first primary apical side shoot being visible (stage 21 or 201) and ends when at least nine or more apical side shoots are visible (stage 29 or 209) (Table 3). Depending on the training system of the plant, side shoots are left to grow (in the case of untrained plants or plants trained in a hedgerow system) or removed in the case of plants trained to one or two main shoots. The appearance and development of side shoots are stimulated by conditions that are favourable for vegetative growth (i.e., high humidity and high soil nitrogen). The production of many side shoots competes directly with fruit set and development of fruits. Because of this, the highest fruit production is obtained in pruned plants where the side shoots have been removed (Kowalczyk and Kobryn, 2003). If side shoots are removed, then this growth stage is not applicable to the pepino crop.

3.3. Principal growth stage 3: Main shoot elongation

The shoots of the pepino plant are indeterminate and the maximum length of the main shoot depends on the training system. The main shoot length may reach more than 200 cm when the plant is trained in greenhouse-cultivated plants. Reciprocally, the main shoots of un-trimmed and untrained plants are much shorter, due to competition (Prohens et al., 1996; Kowalczyk and Kobryn, 2003). The scale begins with the length of the main shoot up to 10 cm long (stage 31 or 301) and ends up when the elongation of the main shoot has ceased (stage 39 or 309) (Table 4). The time required for passing from one stage to the next depends on the cultivation techniques as well as on the environmental conditions. That is, the main shoot grows faster when plants are trained and pruned and it grows slower when plants are not trained nor pruned.

3.4. Principal growth stage 4: Development of harvestable vegetative plant parts or vegetatively propagated organs / booting (main shoot)

The pepino is almost always cultivated for its harvestable fruits, though rarely plants may be grown as an ornamental (Prohens et al., 1996). In consequence, this principal growth stage, which is included in the BBCH scale (Meier, 2001), is not applicable to the pepino.

3.5. Principal growth stage 5: Inflorescence emergence

The number of inflorescences in the most developed (main) shoot determines the phenological stage code (Table 5). And, given that the pepino has indeterminate growth, the number of inflorescences is a

matter of time and age; i.e., inflorescences continue to be produced as long as the main shoot continues to grow. Typically, the first inflorescence is visible (stage 51 or 501) after 10-20 leaves (e.g., stage 19 in the two-digit scale, and between stages 110 and 119 in the three-digit scale) have been matured along the main shoot (this is usually when the main shoot has reached between 20 and 70 cm (e.g., between stages 31 and 34). Subsequently, new inflorescences appear each two to four nodes. Depending of the rate of growth, it may take 3 to 8 days to pass from one stage to the subsequent stage (e.g., from stage 51 or 501 to stage 52 or 502).

3.6. Principal growth stage 6: Flowering

The pepino inflorescence is an indeterminate pseudoterminal cymose raceme with one or two axis and 5 - 20 hermaphrodite flowers (Anderson, 1979), which open acropetaly, i.e., from the base towards the tip of an inflorescence. Pepino flowers are white, purple or white marked with purple and have inserted or slighty exserted stigma. This phenological stage is determined by the opening of the first (basal) flower of each of the inflorescence (Table 6). Opening of all flowers of the inflorescence usually takes 3 to 10 days, depending on growth conditions and number of flower buds in the inflorescence. The pepino is self-compatible and mostly autogamous (Mione and Anderson, 1992), although when pollinators are present, a frequent situation in open field cultivation or in greenhouses where bumblebees are used for stimulating pollination, a high degree of outcrossing may occur (Murray et al., 1992). When no pollination occurs, the flower may set parthenocarpic fruits (Prohens et al., 1998). In any case, infructescences usually include 1 to 3 mature fruits.

3.7. Principal growth stage 7: Development of fruit

The pepino fruit is a fleshy berry with two to three locules that follows a sigmoidal growth pattern (Schaffer et al., 1989). The fruit usually weighs between 100 and 400 g, the weight depending both on genetics (the cultivar) and the environment (growth conditions). The shape of the fruit, as well as the colour patterning, also depend on the cultivar (Rodríguez-Burruezo et al., 2011). The fruit usually takes between 30 to 50 days to grow to full size (Prohens and Nuez, 2001). At this time the fruit is physiologically unripe and has a green colour and may be harvested for use in salads in the same way as cucumbers (Prohens et al., 1996).

Phenological stage 7 begins when the first fruit of the oldest (lowest) infructescence bears the first mature (in size and colour) fruit (Table 7). Given that it is quite unusual that more than six clusters bearing fruit appear on individual shoots, the scale for this phenological stage begins with the first fruit of the first cluster (stage 71 or 701) and ends with nine or more clusters in the main shoot having the first fruit having reached typical size and shape (stage 79 or 709).

3.8. Principal growth stage 8: Ripening of fruit and seed

The pepino fruit takes between 7 to 25 days after reaching full size until, until it is fully ripe (Prohens and Nuez, 2001). When fully ripe, the fruit has a pale green to golden yellow colour, which may be covered by purple stripes or not. The fruit normally is very aromatic and has a mild flavour, with sugar content ranging between 6% to 10%, and with a low acidity (Rodríguez-Burruezo et al., 2011). The fruit may be parthenocarpic or seeded, and in the latter case it may contain up to 200 small seeds (Anderson, 1979). Seeds are physiologically mature when the fruit evinces the typical fully ripe colour. Phenological stage 8 is determined by the percentage of fruits produced by the plant that have reached the typically fully ripe colour (Table 8). The scale begins with 10% of the fruits showing the typical fully ripe colour (stage 81 or 801) and ends with all fruits having the typical fully ripe colour (stage 89 or 809).

3.9. Principal growth stage 9: Senescence

Like tomato, pepper, eggplant and other solanaceous fruit crops, the plant of pepino is perennial, although it is usually grown as an annual (Prohens et al., 1996; Rodríguez-Burruezo et al., 2011). This is because after six to nine months of production, plants begin to develop symptoms of senescence, especially under intensive cultivation conditions, with oldest leaves getting yellowish and subsequently brownish. This situation may be aggravated when plants are affected by pests or diseases, problems that accelerate senescence. This phenological stage begins with the initiation of leaf yellowing (stage 91 or 901) and ends when all fruits have been harvested (stage 99 or 909) (Table 9).

3.10. Validation of the utility of the BBCH scale

Measurement at a specific developmental stage is of great relevance for comparison of different varieties in characterization and phenomics studies (Fiorani and Schurr, 2013). In our case, measuring traits of agronomic interest at specific BBCH developmental stages in a

collection of cultivated pepino accessions and wild relatives has allowed a precise characterization that has resulted in the detection of significant ($P<0.05$) differences among accessions for all traits (Table 10). We have found that wild relatives have, with the exception of *S. trachycarpum* (E-34), a longer stem when the first inflorescence in the main shoot is visible (stage of 51/501). This is probably caused by the fact that selection during the domestication process of the pepino has favoured more compact plants that are better adapted to cultivated environments (Anderson et al., 1996; Prohens et al., 1996; Meyer and Purugannan, 2013). Many differences have been found in fruit length and width, measured in the first fruit of the first cluster that reaches the typical form and colour (stage 71/701), in the materials evaluated (Table 10). This is in agreement with the high variation and heritability of this trait (Prohens et al., 2005). Also, as expected, cultivated materials have had a fruit size generally larger than those of wild relatives. There are, as well, many differences in fruit shape, measured as fruit length/width ratio at this same stage (71/701),. Differences in cultivated pepino have been much larger than in the wild relatives, which is something expected as artificial selection has yielded materials highly variable for fruit shape (Prohens et al., 1996; Rodríguez-Burruezo et al., 2011). For earliness, measured as time from transplant to beginning of fruit ripening (stage 81/801), few significant differences have been found, the only significant ones being between Col-1 and, Puzol, and E-7 accessions (earlier) and Sweet Long (later) (Table 10). Finally, for soluble solids content, an important trait for fruit quality (Rodríguez-Burruezo et al., 2011), many differences have been found in the materials measured at stage 81/801. Wild relatives have generally had significantly higher levels of soluble solids content than cultivated materials, with several accessions having contents above 10% (Table 10), confirming that wild relatives are sources of variation of interest for pepino quality breeding (Prohens et al., 2005). In pepino, as in other crops (like tomato) in which there is a sequential fruit set, (Aurand et al., 2012), differences may exist among fruit characteristics harvested at different developmental stages and therefore it is important to measure the traits at the same developmental stage in order to have comparable and relevant measures. In summary, the BBCH scale has proved as very useful to compare different pepino varieties at the same developmental stage, which results in information of relevance for horticulturists and breeders.

4. Conclusions

The specific BBCH scale developed for pepino, with its two-digit (simple) and three-digit (extended) versions, allows the precise identification of the phenological stages of this crop. We have shown that the measurement of traits of agronomic interest at specific BBCH developmental stages is important because it allows the proper comparison of varieties, given that there is no bias due to differences in developmental stages. The BBCH scale offers a standardized tool that will help pepino researchers, agronomists, breeders, and germplasm curators in an efficient management, breeding, and conservation of genetic resources of this emerging crop.

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Table 1
Description of the phenological stages of pepino growth stage 0 (germination / rooting) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description	
		Germination (seed propagation)	Rooting (vegetative propagation)
00	000	Dry seeds	Cuttings
00	001	Beginning of seed imbibition	Swelling of the cutting/explant
03	003	Seed imbibition complete	Root protuberances evident
05	005	Radicle emerges from seed	Adventitious roots developing
07	007	Hypocotyl with cotyledons breaking through seed coat	Beginning of axillary bud breaking
09	009	Emergence: cotyledons break through soil surface	Axillary buds showing green tips

Table 2
Description of the phenological stages of pepino growth stage 1 (leaf development) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
10	100	Cotyledons completely unfolded (seed propagation) or emerging leaf in dominant axillary bud having grown to 1 cm (vegetative propagation)
11	101	First true leaf on main shoot fully unfolded
12	102	2nd leaf on main shoot unfolded
13	103	3rd leaf on main shoot unfolded
1.	10.	Stages continuous until...
19	109	9 or more leaves on main shoot unfolded (2-digit scale) 9^{th} leaf on main shoot unfolded
-	110	10^{th} leaf on the main shoot unfolded
-	11.	Stages continuous till...
-	119	19 or more leaves on main shoot unfolded

Table 3
Description of the phenological stages of pepino growth stage 2 (formation of side shoots) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
21	201	First primary apical side shoot visible
22	202	2nd primary apical side shoot visible
2.	20.	Stages continuous till . . .
29	209	9 or more primary apical side shoots visible (two-digit scale) 9th primary apical side shoot visible (three-digit scale)
-	210	10th primary apical side shoot visible
-	21.	Stages continuous till...
-	219	19 or more primary apical side shoots visible

Table 4
Description of the phenological stages of pepino growth stage 3 (main shoot elongation) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
31	301	Main shoot up to 10 cm long
32	302	Main shoot up to 20 cm long
33	303	Main shoot up to 40 cm long
34	304	Main shoot up to 70 cm long
35	305	Main shoot up to 100 cm long
36	306	Main shoot up to 130 cm long
37	307	Main shoot up to 160 cm long
38	308	Main shoot up to 200 cm long
39	309	Elongation growth of the main shoot is ceased

Table 5
Description of the phenological stages of pepino growth stage 5 (inflorescence emergence) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
51	501	First inflorescence in the main shoot visible
52	502	2nd inflorescence in the main shoot visible
53	503	3th inflorescence in the main shoot visible
5.	50.	Stages continuous until...
59	509	9 or more inflorescences in the main shoot visible (two-digit scale) 9th inflorescence in the main shoot visible (three-digit scale)
-	510	10th inflorescence in the main shoot visible
-	51.	Stages continuous until...
-	519	19 or more inflorescences in the main shoots visible

Table 6
Description of the phenological stages of pepino growth stage 6 (flowering) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
61	601	First inflorescence in the main shoot: first flower open
62	602	2nd inflorescence in the main shoot: first flower open
63	603	3th inflorescence in the main shoot: first flower open
6.	60.	Stages continuous until...
69	609	9 or more inflorescence in the main shoot with open flowers (two-digit scale) 9th inflorescence in the main shoot: first flower open (three-digit scale)
-	610	10th inflorescence in the main shoot: first flower open
-	61.	Stages continuous until...
-	619	19th inflorescence in the main shoot: first flower open

Table 7
Description of the phenological stages of pepino growth stage 7 (development of fruit) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
71	701	First fruit cluster in the main shoot: First fruit reaches typical size and form and colour
72	702	2nd fruit cluster in the main shoot: First fruit reaches typical size and form
73	703	3th fruit cluster in the main shoot: First fruit reaches typical size and form
7.	70.	Stages continuous until...
79	709	9 or more fruit clusters in the main shoot with the first fruit having reached typical size and form

Table 8

Description of the phenological stages of pepino growth stage 8 (ripening of fruit and seed) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
81	801	10% of fruits show typical fully ripe colour
82	802	20% of fruits show typical fully ripe colour
83	803	30% of fruits show typical fully ripe colour
84	804	40% of fruits show typical fully ripe colour
85	805	50% of fruits show typical fully ripe colour
86	806	60% of fruits show typical fully ripe colour
87	807	70% of fruits show typical fully ripe colour
88	808	80% of fruits show typical fully ripe colour
89	809	Fully ripe: all fruits have typical fully ripe colour

Table 9
Description of the phenological stages of pepino growth stage 9 (senescence) using a 2-digit and a 3-digit scale BBCH scale.

2-digit code	3-digit code	Description
91	901	Beginning of leaf yellowing
95	905	50% of leaves brownish
99	909	All fruits harvested

Table 10

Differences among cultivated pepino (*Solanum muricatum*) varieties and accessions of wild relatives for traits of agronomic interest at specific phenological stages as defined by the BBCH scale. The phenological stage at which each trait was measured in both the two-digit and three-digit scales is indicated within square brackets.

Variety/accession	Stem length (cm) [51/501][a]	Fruit length (cm) [71/701]	Fruit width (cm) [71/701]	Fruit length/width ratio [71/701]	Time from transplant to beginning of ripening (d) [81/801]	Soluble solids content (%) [81/801]
Local varieties of cultivated pepino (*S.muricatum*)						
37-A	64.4 a	7.34 de	4.10 ab	1.79 def	147 ab	5.58 ab
Col-1	53.4 a	7.34 de	7.90 cdef	0.93 ab	143 a	7.22 bcde
CH2-22	50.6 a	7.86 e	7.74 cdef	1.03 ab	153 ab	7.52 cde
OV-8	70.6 a	5.70 cde	5.60 bcd	1.03 ab	153 ab	6.80 abcd
PT-154	46.4 a	7.87 e	11.10 g	0.72 a	150 ab	7.27 bcde
RP-1	52.0 a	5.64 cde	8.14 def	0.71 a	145 ab	5.90 abc
Modern varieties of cultivated pepino (*S.muricatum*)						
El Camino	53.4 a	7.61 e	6.04 bcde	1.27 bc	145 ab	6.92 bcd
Kawi	46.8 a	14.47 g	9.37 f	1.55 cd	150 ab	5.60 ab
Puzol	43.0 a	10.90 f	6.84 cde	1.60 de	143 a	7.56 cde
Quito	60.4 a	6.57 de	6.10 bcde	1.08 ab	149 ab	5.13 a
Sweet Long	44.0 a	10.77 f	5.73 bcde	1.89 ef	161 b	6.40 abcd
Sweet Round	50.2 a	7.17 de	8.30 ef	0.87 ab	145 ab	7.87 def
Turia	47.6 a	15.50 g	7.43 cdef	2.08 fg	150 ab	6.70 abcd
Valencia	44.0 a	11.62 f	5.34 bc	2.21 g	147 ab	7.58 cde
Wild relatives[b]						
BIRM/S 1034 (*S. ca.*)	115.0 b	2.77 ab	2.73 a	1.01 ab	153 ab	9.27 fg
E-7 (*S. ca.*)	143.8 c	3.52 abc	3.62 ab	0.97 ab	144 a	10.28 gh
EC-40 (*S. ca.*)	99.0 b	2.82 ab	2.78 a	1.01 ab	151 ab	8.02 def
QL-013 (*S. ca.*)	104.2 b	3.24 abc	2.94 a	1.10 ab	152 ab	10.38 gh
P-80 (*S. ct.*)	93.7 b	1.16 a	1.80 a	0.94 ab	152 ab	10.30 gh
P-62 (*S. pe.*)	99.2 b	2.15 ab	2.25 a	0.96 ab	147 ab	10.50 gh
E-257 (*S. ta.*)	96.2 b	4.60 bcd	3.67 ab	1.26 bc	154 ab	8.90 efg
E-34 (*S. tr.*)	63.2 a	2.50 ab	2.12 a	1.18 b	150 ab	11.60 h

[a]Means separated by different letters within each column are significantly different at P<0.05, according to the Student-Newman-Keuls multiple range test.

[b]The species corresponding to each of the wild accessions is indicated in brackets according to the following code: *S. c.=S. caripense*; *S. ct.=S. catilliflorum*; *S. pe.=S. perlongistylum*; *S. ta.=S. tabanoense*; *S. tr.=S.trachycarpum*).

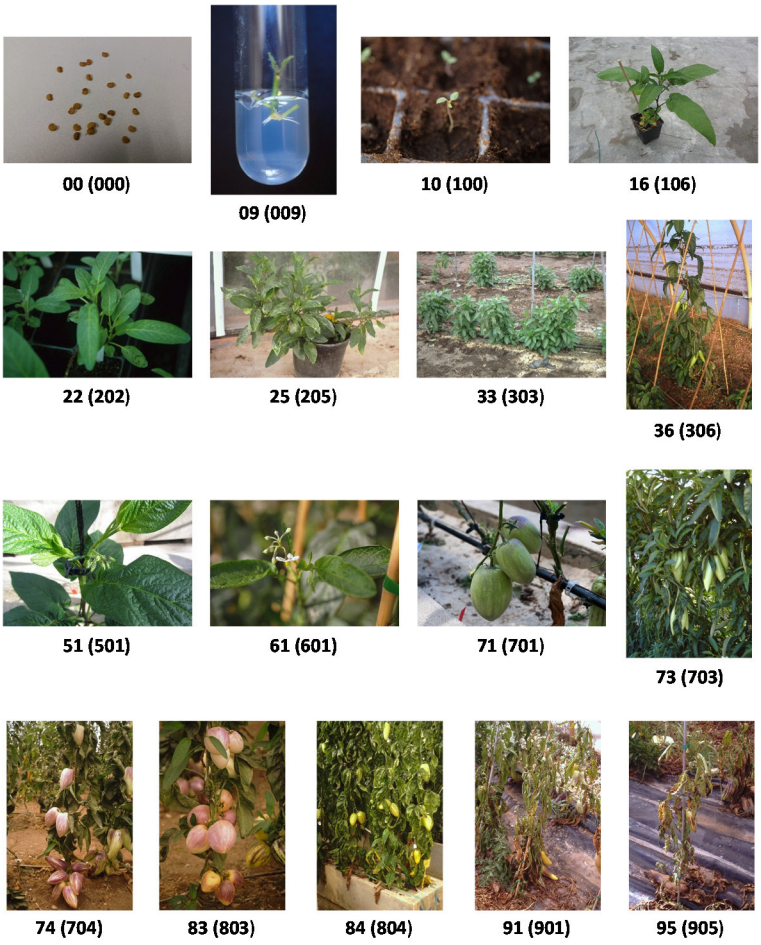

Fig. 1. Illustrations of some of the phenological stages of pepino (*Solanum muricatum*) according to the BBCH scale. Two-digit and three-digit (between brackets) scale codes are indicated. See Tables 1-9 for the description of each of the phenological stage codes

3.2.- Morphological and molecular characterization of local varieties, modern cultivars and wild relatives of an emerging vegetable crop, the pepino (*Solanum muricatum*), provides insight into its diversity, relationships and breeding history

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Euphytica, 2015, 206, 2, 301-318.

Abstract

Availability of standardized morphological and molecular characterization data is essential for the efficient development of breeding programmes in emerging crops. Pepino (*Solanum muricatum*) is an increasingly important vegetatively propagated vegetable crop for which concurrent data on morphological descriptors and molecular markers are not available. We evaluated 58 morphological traits, using a collection of 14 accessions of pepinos (including local Andean varieties and modern cultivars) and 8 of wild relatives, using the IPGRI and COMAV descriptors lists coupled with 20 EST-SSRs from tomato. High morphological diversity was found in both cultivated and wild accessions; all morphological traits except three were variable. Cultivated pepino and wild relatives were significantly different for 26 traits. Also, local varieties and modern cultivars of pepino were different from each other for 13 morphological traits and were clearly separated in a principal components analysis (PCA). Fourteen of the 20 tomato EST-SSRs were polymorphic, with an average number of alleles per locus of 4.07 and a polymorphic information content (PIC) value of 0.4132. This revealed a high degree of transferability from tomato to pepino and wide molecular diversity in the collection. Cultivated materials manifest high levels of observed heterozygosity, suggesting that it is related to heterosis for yield associated with heterozygosis. SSR data clearly differentiated cultivated and wild materials. Furthermore, for pepinos, the modern varieties were genetically much less diverse than the traditional local varieties. However, both groups of cultivated material expressed a low degree of genetic differentiation. A strong correlation (r=0.673) between morphological and molecular distances was found. Our results provide foundational information for programmes of germplasm conservation, and that can be used to enhance breeding for this emerging crop.

Keywords: Breeding · Descriptors · Germplasm · Heterozygosity · *Solanum muricatum* · SSRs

Introduction

Modern breeding programmes in emerging crops are often limited by scanty or non-existent phenotypic and genetic information, and by small germplasm collections (FAO 2010; Mayes et al. 2012). Complementary studies of morphological and molecular diversity provide relevant information for identifying sources of variation in breeding

programmes, for establishing relationships among plant materials, as well as a foundation for promoting breeding and for germplasm conservation (Rao and Hodgkin 2002; Khoury et al. 2010).

The pepino (*Solanum muricatum* Aiton) is an emerging usually vegetatively propagated vegetable crop native to the Andean region (Anderson et al. 1996). This crop is phylogenetically close to tomato (*S. lycopersicum* L.) and potato (*S. tuberosum* L.) (Spooner et al. 1993; Särkinen et al. 2013). The pepino is cultivated for its juicy and aromatic fruits. Although the pepino is locally important in the Andean region since long ago (Prohens et al. 1996), in recent decades the increasing interest in exotic fruit markets has promoted increasing interest in pepino cultivation in several countries including New Zealand, Australia, Spain, Turkey, Israel and China (Levy et al. 2006; Yalçin 2010; Rodríguez-Burruezo et al. 2011; Abouelnasr et al. 2014). Nutritionally, pepino fruits contain high levels of potassium and vitamin C, and it is low in calories. Furthermore, it offers some properties of medicinal interest, such as antidiabetic, antidiuretic and antihypotensive activities (Hsu et al. 2011; Rodríguez-Burruezo et al. 2011; Sudha et al. 2012).

Most of the plant material cultivated in the Andean region consists of local varieties that have not been subjected to formal breeding and are adapted to local climatic conditions and preferences for flavour, size and fruit shape and colour (Anderson et al. 1996; Prohens et al. 1996). Local varieties of the pepino are commonly cultivated outdoors in their native range, and they usually have a poor performance when introduced in other regions (where the pepino is cultivated either outdoors or in greenhouses: Prohens et al. 1996; Rodríguez-Burruezo et al. 2011). As a consequence of the usually poor performance, several improved cultivars adapted to non-Andean climates and to protected cultivation have been developed in New Zealand, Spain, and Israel (Dawes and Pringle 1984; Simms et al. 1996; Ruiz et al. 1997; Prohens et al. 2002; Rodríguez-Burruezo et al. 2004a, 2004b; Levy et al. 2006). These materials have been developed using conventional approaches including generating genetically variable populations by means of seed propagation of collections from the Andean region or by hybridization between different vegetatively propagated clones in order to exploit heterosis (Rodríguez-Burruezo et al. 2011).

Wild pepino relatives which, like the domesticated pepino, are included in the section *Basarthrum* of genus *Solanum* (Anderson 1975,

1979) represent a genetic resource of interest for pepino breeding (Rodríguez-Burruezo et al., 2003a). Among the wild relatives, the highly variable *S. caripense* Humb. and Bonpl. ex Dun., as well as *S. tabanoense* Correll, form part of the primary genepool of pepino. Fully fertile interspecific hybrids and backcross generations to pepino have been obtained among these species (Anderson 1979; Rodríguez-Burruezo et al. 2003a, 2011). Other species of interest for pepino breeding include *S. trachycarpum* Bitter and Sodiro, which grows in dry areas (Anderson 1979), and *S. catilliflorum* G.J. Anderson, Martine, Prohens and Nuez and *S. perlongistylum* G.J. Anderson, Martine, Prohens and Nuez, which are among the most recent species discovered and described for this section (Anderson et al. 2006) and that remain to be studied as potential genetic resources for pepino breeding.

Given the interests in crop diversity and enhancement, the precise and standardized morphological and molecular characterization of the pepino would be of great utility for breeding programmes, for germplasm conservation and for comparison of experimental data of different trials and plant materials (Rao and Hodgkin 2002; Khoury et al. 2010). Fortunately, an internationally accepted list of morphological descriptors for the extensive characterization of vegetative, inflorescence and flower, fruit and seed traits of pepino is available (IPGRI and COMAV 2004). However, no reports are known to us on the utilization of this list of descriptors for the morphological characterization of pepino collections. Although several studies have been made on phenotypic diversity of pepino, including wild relatives of interest for breeding, they have mostly dealt with specific traits of agronomic interest (Rodríguez-Burruezo et al. 2003a, 2011; Muñoz et al. 2014)

Similarly, few studies have been done on the molecular diversity of collections of cultivated pepino and wild relatives (Anderson et al. 1996; Blanca et al. 2007). The evaluation of the cpDNA-RFLPs polymorphism in the pepino and wild relatives of *Solanum* section *Basarthrum* revealed that the cultivated pepino was closely related to *S. caripense* and *S. tabanoense* (Anderson et al. 1996). A subsequent study using AFLP markers and the sequence variation in the DNA sequence of the nuclear gene 3-methylcrotonyl-CoA carboxylase revealed that cultivated pepino is highly diverse and showed that this cultigen was genetically differentiated from wild relatives (Blanca et al. 2007). AFLP markers have also been used to evaluate the genetic distances among four pepino cultivars as a

predictor for heterosis for yield traits (Rodríguez-Burruezo et al. 2003b). However, no studies have been performed with other molecular markers in pepino. Unlike AFLPs, which are dominant (Meudt and Clarke 2007), SSRs are co-dominant and particularly valuable because they allow the precise assignment of allelic states and evaluation of the level of heterozygosity of individual pepino clones. Furthermore, SSRs (1) have a high reproducibility and therefore are ideal for comparison among different experiments and laboratories, (2) are multiallelic, (3) have locus specificity, (4) are abundant and (5) are randomly distributed throughout the genome (Kalia et al. 2011). For species like the pepino in which no genomic libraries or expressed sequence tags (EST) sequences are available, SSRs may be transferred from close relatives, like tomato, in which there has been an abundance of SSRs developed (Frary et al. 2005; Suresh et al. 2014). In this respect, EST-SSRs usually offer a greater degree of transferability among species, as transcribed regions have a greater degree of conservation than non-transcribed regions (Kalia et al. 2011).

The simultaneous study of morphological and molecular diversity of the pepino and wild relatives also provides information on the morphological and molecular variation and relationships of the crop to wild relatives, as well as on the association between morphological and molecular variation. Here, we evaluate the morphological and molecular diversity using standardized descriptors and highly repeatable SSR markers in a collection of local varieties and modern cultivars of pepino, as well as in a set of accessions from wild relatives of interest for breeding. The information obtained will be of interest for breeders and germplasm managers, as well as for understanding the evolution of the crop.

Material and methods

Plant material

We studied a total of 22 accessions, of which six corresponded to local pepino varieties from the Andean region, eight to improved pepino cultivars, and eight to wild relatives (different species) (Table 1). Local varieties originated in Colombia (1), Chile (2), Ecuador (2) and Peru (1). Modern varieties were developed in New Zealand (2), Spain (5) and the United Kingdom (1) as a result of selection and breeding programmes (Dawes and Pringle 1984; Simms et al. 1996; Ruiz et al. 1997; Prohens et

al. 2002; Rodríguez-Burruezo et al. 2004a, 2004b). Wild relatives were represented by accessions of *S. caripense* (4), *S. catilliflorum* (1), *S. perlongistylum* (1), *S. tabanoense* (1) and *S. trachycarpum* (1). The material is part of the germplasm collection of the Instituto de Conservación y Mejora de la Agrodiversidad valenciana (Valencia, Spain).

Five clonal replicates obtained by *in vitro* micropropagation (Cavusoglu and Sulusoglu 2013) were used for each of the 22 accessions. Clonal replicates were grown in a glasshouse in Valencia (GPS coordinates: lat. 39º 29' 01" N, long. 0º 20' 27" W) using a completely randomized design. Rooted plantlets were transplanted to benches filled with quartz sand in January 2014. Plants were spaced 55 cm in the bench, with 115 cm between bench centers. Plants were drip irrigated every 4 h for 5 min. Fertilization was applied through the drip irrigation system during the growing cycle. A combination of different fertilizers was used to achieve a final concentration of main ions and cations in the irrigation solution of 11.47 mM NO_3^-, 1.00 mM NH_4^+, 1.50 mM $H_2PO_4^-$, 6.75 mM K^+, 3.25 mM Ca^{2+}, 2.50 mM Mg^{2+} and 2.82 mM SO_4^{2-}. Microminerals were supplied by adding the following salts to the irrigation water: 50 µM H_3BO_3, 10 µM FeEDTA, 4.5 µM $MnCl_2$, 3.8 µM $ZnSO_4$, 0.3 µM $CuSO_4$ and 0.1 µM $(NH_4)_6Mo_7O_{24}$. Flowers were vibrated mechanically (to approximate the natural bee pollination syndrome of vibratile pollination; Anderson and Symon 1988) twice a week to stimulate fruit set. For the self-incompatible wild species *S. caripense*, *S. perlongistylum* and *S. tabanoense* (Mione and Anderson 1992; Anderson et al. 1996), manual pollination using pollen from other plants from each of the species was used in order to ensure fruit set. Phytosanitary treatments against spider mites (*Tetranychus urticae* Koch.) and whiteflies (*Bemisia tabaci* Gennadius) were performed when necessary.

Morphological and agronomic characterization

Individual plants were characterized using 58 primary descriptors (IPGRI and COMAV 2004). These descriptors include two plant (P code), seven stem (St code), 12 leaf (L code), three inflorescence (I code), six flower (Fl code), 24 fruit (Fr code), and four seed (Se code) traits. Eighteen traits corresponding to these primary descriptors are quantitative, seven are meristic (traits in which the parts or components are counted) and the other 33 traits are measured in a scale with predetermined values (Table 2).

Molecular characterization

Genomic DNA was extracted from young leaves of each clone according to the CTAB procedure (Doyle and Doyle, 1987). DNA quality was evaluated on 0.8% agarose gels, dyed with GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA) and the DNA concentrations estimated using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA) spectrophotometer. Extracted DNA was diluted to a concentration of 20 ng/µL.

We used 20 simple sequence repeat (SSR) markers that proved to be polymorphic in tomato (Table 3) and that are distributed throughout the tomato genome (Frary et al. 2005). SSRs were amplified following the M13-tail method described by Schuelke (2000) to facilitate the incorporation of a dye label during PCR. Amplifications were performed in a total volume of 10 ng DNA, 1 mM $MgCl_2$, 0.05 µM of forward primer, 0.25 µM of reverse primer, 0.2 µM of fluorescent-labelled M-13 primer, 0.2 mM of dNTPs and 1 unit of *Taq* polymerase in 1x PCR buffer. PCR amplifications were performed in a Mastercycler ep gradient S thermocycler (Eppendorf, Hamburg, Germany) using the following programme: 1 cycle for 2 min at 94 ºC, 35 cycles of 15 s at 94ºC, 30 s at annealing temperature (Table 3), 45 s at 72 ºC, followed by 10 min extensive at 72 ºC. SSR alleles were resolved on an ABI PRISM 3100 DNA (Applied Biosystems, Carlsbad, California, USA) genetic analyzer using GeneScan 3.7 (Applied Biosystems) software and precisely sized using GeneScan 500 LIZ molecular size standards with genotyper 3.7 (Applied Biosystems) software.

Data analysis

Range and mean values for the morphological descriptors for the 14 accessions of cultivated pepino and for the eight accessions of its wild relatives, as well as for the six local varieties and eight modern cultivars of cultivated pepino, were calculated using average values for each accession. Significance of differences among groups (cultivated pepino vs. wild species, and local varieties vs. modern cultivars) was tested using Student's *t* tests. A principal components analysis (PCA) was performed for standardized morphological data using pairwise Euclidean distances among accessions. Monomorphic traits were excluded from the PCA analysis.

For the molecular (SSR) data, the number of alleles and of private alleles for each of the groups considered (all accessions, all cultivated accessions, local varieties, modern cultivars, and wild relatives) were calculated. The polymorphism information content (*PIC*) for each SSR marker was calculated as indicated Botstein et al. (1980). Observed heterozygosity (H_o) was calculated for each accession. Pairwise genetic similarities among accessions were calculated using the codominant genetic distance (Smouse and Peakall 1999). In this context, for a single-locus with four different alleles (*i*, *j*, *k* and *l*) a set of squared distances are defined as $d^2(ii, ii)=0$, $d^2(ij, ij)=0$, $d^2(ii, ij)=1$, $d^2(ij, ik)=1$, $d^2(ij, kl)=2$, $d^2(ii, jk)=3$, and $d^2(ii, jj)=4$. In order to obtain the genetic distance between two accessions, genetic distances are summed across loci under the assumption of independence (Smouse and Peakall 1999). A principal coordinates analysis (PCoA) was performed using pairwise genetic similarities. Total genetic diversity (H_T), among groups genetic diversity (D_{ST}), within groups genetic diversity (H_S), relative magnitude of genetic differentiation (G_{ST}) and standardized G_{ST} (G'_{ST}) were calculated according to Nei (1973). Correlations between morphological and molecular distances were investigated with a Mantel (1967) test.

Results

Morphological characterization

A wide morphological diversity was found in the collection (Figure 1). Fifty-five out of the 58 morphological descriptors evaluated were variable in the collections studied. The three morphological traits which were not variable were Fr-Stripes (all clones bore fruits with stripes), Fr-Locules (all clones bore fruits with two locules), and Se-Type (all clones had seeds with no wings). Furthermore, when considering only the cultivated materials, Fl-CorollaShape was also monomorphic (all clones had rotate a corolla).

Differences between cultivated and wild clones

Significant differences were found between the cultivated pepino and wild relatives for 26 traits (Table 4). On average, the cultivated pepino is less tall than the wild relatives, with significantly lower values for traits related to plant size (P-Size, St-LengthInfl1, St-InternLength or I-LeavesInfl1). The cultivated pepino plants are characterized by: more root protuberances at the stem nodes (St-Protuberances), less pubescence (St-

Pubescence), fewer divided leaves (L-Type) (i.e., fewer compound, and more simple leaves) and more bifurcated (I-Type) inflorescences than the wild relatives (Table 4). Regarding sexual reproduction traits, the cultivated pepino has less style exsertion (Fl-StyleExsertion), lower pollen production (Fl-PollenProd) and fewer seeds per fruit (Se-SeedsFruit) than wild relatives. Many differences are found for fruit traits; in particular cultivated pepinos are not surprisingly larger (Fr-Length, Fr-Width, Fr-PlacentLength, Fr-PlacentBreadth), have more luminous (Fr-L*), yellow (Fr-b*) and glossy (Fr-Glossiness) skin, and more yellow (Fr-FleshColour), and better tasting (Fr-Flavour and Fr-OffFlavour) flesh, although with less soluble solids content (Fr-Soluble Solids), than the wild relatives (Table 4). However, the range of variation within cultivated pepinos and related wild species was generally large and overlapped for all but six traits, of which three were related to fruit size (Fr-Length, Fr-Width, Fr-PlacentLength), two to fruit taste (Fr-Flavour and Fr-SolubleSolids), and the remaining one to the number of seeds per fruit (Se-SeedsFruit) (Table 4).

Differences between local varieties and modern cultivars

Local pepino varieties differed significantly from modern cultivars for 13 traits (Table 5). However, despite the significance of differences in the averages of the two categories of cultivated pepinos for these traits, the range of variation for all traits of local cultivars and modern varieties overlapped. Local varieties, on average, had more pigmented stem and leaves (St-Colour and L-AnthVeins) and shorter internode length (St-InternLength) than modern varieties. Most modern varieties had simple leaves, while local varieties mostly had compound and flat leaves, which resulted in differences among both groups for several leaf shape and type traits (L-LaminaWidth, L.LWRatio, L-Type, L-Leaflets, L-Surface) (Table 5). Modern varieties had, on average, greater pollen production (Fl-PollenProd) and a larger number of seeds (Se-SeedsFruit) than local varieties. Also, fruits of modern varieties were, on average larger and more elongated (Fr-Length and Fr-LW Ratio), and had a higher intensity of green colour (Fr-a*) than local varieties.

Principal components analysis

The first and second components of the PCA performed with all accessions accounted, respectively, for 29.7% and 11.8%, of the total variation among accession means. The first component was positively

correlated with plant size vigour and growth traits (P-Size, St-LengthInfl1, St-InternLength, I-LeavesInfl1), high pollen and seed production (Fl-PollenProd and Se-SeedsFruit), and with fruits having off-flavour (Fr-OffFlavour) and high soluble solids content (Fr-SolubleSolids), and negatively with the density of root protuberances in the stem nodes (St-Protuberances), convex leaf surface (L-Surface), multiparous inflorescences (I-Type), fruit size traits (Fr-Length, Fr-Width, Fr-PlacentLength, and Fr-PlacentBreadth), fruit glossiness (Fr-Glosiness), fruit flesh with no chlorophyll (Fr-FleshColour), and sweet flavour (Fr-Flavour) (Table 6). The second principal component was positively correlated with anthocyanin pigmentation of plant parts (St-Anthocyanins, St-Colour, L-PetioleColour, and L-AnthVeins), compound leaves (L-LaminaWidth, L-Type and L-Leaflets), greater number of flowers per inflorescence (I-NFlowers), more luminous (Fr-L*), less green (Fr-a*), mottled (Fr-Mottling), and fasciated (Fr-Fasciation) fruits, and negatively with dropping (L-Attitude), elongated (L-LWRatio) and convex (L-Surface) leaves, pigmented flowers (Fl-CorollaColour) and obovoid fruits (Fr-WidestPart) (Table 6).

The projection of the accessions on a two-dimensional PCA plot showed that the first component clearly separates wild accessions in the right part (i.e., positive values) and cultivated pepino in the left part (i.e., negative values) of the graph (Figure 2). No overlap was found for the first component values between cultivated pepino and wild relatives. The second component clearly separates local varieties and modern cultivars of cultivated pepino, so that the former plot in the upper part (i.e., positive values) of the graph, while the latter plot in the lower part (i.e., negative values) (Figure 2). This second component also separates the different wild species from each other. The highest values belong to *S. caripense*, followed by the group of the morphologically similar *S. perlongistylum* and *S. catilliflorum*, then by *S. tabanoense*, and finally by *S. trachycarpum* (Figure 2). The PCA plot also shows that the groups of local varieties of pepino and modern varieties show a considerable degree of dispersion in the PCA graph. Although the four accessions of the wild *S. caripense* plot in the same section of the PCA graph, they are distinct for the second component (Figure 2). Interestingly, the local varieties originating in Chile (CH and OV) and Colombia (Co) plot close to most of the modern varieties developed in Spain (SL, SR, Tu and Va) (Figure 2).

Molecular characterization

Out of the 20 tomato SSRs tested, 14 were found to be polymorphic. The six other SSRs either did not amplify (SSR13, SSR51 and SSR136) or were monomorphic (SSR38, SSR150 and SSR248).

SSR characterization

The 14 polymorphic SSRs amplified 57 alleles, with an average of 4.07 alleles/locus and a range between 2 and 8 in the collection (Table 7). When considering cultivated accessions only, two of the SSRs (SSR14 and SSR66) were monomorphic, and the average number of alleles per locus was 2.5, with a range between 1 and 6. The number of alleles for each SSR locus for the local varieties of cultivated pepino was identical to that found for all pepino accessions, except for locus SSR20, in which five alleles were found instead of six (Table 7). As a result, the average number of alleles per locus was very similar to that obtained for all the cultivated accessions. Modern varieties have many fewer alleles per locus, with an average of 1.29, and polymorphism was only found for four SSR loci, in which only two alleles were detected (Table 7). For wild relatives, all SSR loci were polymorphic, except locus SSR578. The average number of alleles per locus was 3.0, with up to 5 alleles being detected for loci SSR45 and SSR306 (Table 7). No SSR was found to be specific and universal to cultivated or wild accessions. The average value for the *PIC* parameter of the 14 polymorphic SSRs was of 0.4132, with a range for individual SSR loci between 0.0499 (SSR66) and 0.7021 (SSR306) (Table 7).

The mean value for observed heterozygosity (H_o) was 0.149, with a range between 0 and 0.333 (Table 8). All the alleles were homozygous for the accessions of the modern pepino cultivar, Sweet Round. Similarly, the wild accessions P-80 (*S. catilliflorum*), P-62 (*S. perlongistylum*) and E-257 (*S. tabanoense*) were homozygous. When considering average values, local varieties of cultivated pepino had the highest H_o value (0.193), while the wild relatives had the lowest (0.117).

Principal coordinates analysis

The first and second principal coordinates of the PCoA analysis performed with SSR data account for 26.0% and 10.6% of the total variation, respectively. The first principal coordinate clearly separated cultivated (right part of the graph) and wild (left part of the graph)

accessions (Figure 3). As occurred with the PCA for morphological data, no overlap was found for the first coordinate values between cultivated pepino and wild relatives. With the exception of accession 37A, which showed highly negative values for the second principal coordinate, all cultivated pepino accessions had positive or moderately negative values for the second component (Figure 3). Regarding wild relatives, the second principal coordinate clearly separated two groups of wild relatives, one formed by *S. caripense* and *S. tabanoense*, with positive values for the second coordinate, and another one formed by *S. catilliflorum*, *S. perlongistylum* and *S. trachycarpum*, with negative values. All modern varieties clustered together in the same area of the PCoA plot, while local varieties were more dispersed (Figure 3).

Genetic differentiation

Total diversity (H_T) of the collection had a value of H_T=0.458, with the cultivated pepino having a H_T=0.237 and wild relatives a H_T=0.458 (Table 9). The among-groups diversity (D_{ST}) between cultivated pepino and wild relatives had a value of D_{ST}=0.107, resulting in a relative magnitude of genetic differentiation (G_{ST}) value of G_{ST}=0.274 and a standardized G_{ST} value (G'_{ST}) of G'_{ST}=0.430 (Table 9). When comparing the local varieties and modern cultivars of pepino, the total diversity of local varieties was much higher (H_T=0.336) than that of modern varieties (H_T=0.096), with the among groups diversity being relatively very low (D_{ST}=0.021), resulting in low values of G_{ST} (0.047) and G'_{ST} (0.089) (Table 9).

Correlation between morphological and genetic distances

Correlations obtained from the Mantel test between the matrices of morphological and genetic distances were high (r=0.673). The graphical representation of the relationships between morphological and genetic distances shows that for both distances the values between local varieties are generally higher than those of modern varieties (Figure 4). For the wild species, there was a wide range of morphological and genetic distances, with the lowest values for both distances being between *S. caripense* accessions. When comparing accessions of local varieties and modern cultivars of the pepino, it became evident that some local accessions (Chilean accessions) are morphologically and molecularly similar to most of the modern varieties, while others are as different as local varieties among themselves (Figure 4). Values for both

morphological and genetic distances between cultivated (local varieties and modern cultivars) and wild accessions were high (Figure 4).

Discussion

A combination of morphological and molecular data provides relevant complementary and synergistic information of great interest for plant breeders and for germplasm curators, in particular for those working with emerging crops (Rao and Hodgkin 2002; Khoury et al. 2010; Rodríguez-Burruezo et al. 2011; Yildiz 2014). In the case of the pepino, a standardized morphological descriptors list is available (IPGRI and COMAV 2004), but the descriptors previously have not been validated or used for the characterization of a diverse germplasm collection of pepino. We have demonstrated that most of the IPGRI and COMAV (2004) descriptors used are variable (95% for the whole collection and 93% for cultivated pepino). This allows the acquisition of multiple characterization (i.e., phenomics) data of agronomic interest in the pepino and wild relatives for a precise morphological description. Among the few non-variable traits, some are of relevance for the taxonomic discrimination, like the type of seed (Se-Type), which is specific for discrimination between the species used here and other wild relatives of *Solanum* section *Basarhtrum* (Anderson 1979), or in the case of the cultivated pepino, the corolla shape (Fl-CorollaShape) which is rotate, while in the wild *S. tabanoense* is stellate (Anderson 1975).

Regarding molecular data, SSR markers are preferred to other molecular markers for the standardized characterization of germplasm (Ghislain et al. 2009; Vilanova et al., 2014) as, among other properties, they are highly repeatable, co-dominant, and allow an adequate discrimination among closely related materials (Kalia 2011). Because there are no SSR markers available for the pepino, we tested tomato EST-SSRs for transferability, given that the pepino and tomato are phylogenetically close relatives (Spooner et al. 1993; Särkinen et al. 2013), indicated conclusion supported as well by the viable somatic hybrids between the two species that have produced flowers and fruits (Sakomoto and Taguchi 1991). Our results show that a large proportion (70%) of tomato EST-SSRs are transferrable and polymorphic in the pepino collection studied. Furthermore, considerable SSR variation has been detected in the collections of pepino and wild relatives studied, with an

average number of alleles and *PIC* values almost as high as the values obtained for a highly variable tomato germplasm collection that included wild relatives (Frary et al. 2005). This indicates that the large set of SSRs available in tomato (Frary et al. 2005; Suresh et al. 2014) represents a genomic tool of interest for pepino characterization and breeding, as well as for mapping and synteny studies.

The morphological characterization results reveal that the pepino and its close wild relatives are notably variable but clearly distinct, with significant differences for average values for almost one half of the descriptors evaluated and a clear separation in the PCA analysis. The domestication syndrome in the case of the pepino includes larger fruits and very variable for fruit shape (i.e., the organ for which it is cultivated – illustrating one of Darwin's conclusions about domesticates: the greatest variation in cultivated plants will be in that feature for which they are cultivated) that are more luminous, glossy and yellow and more compact plants (Anderson et al. 1996; Prohens et al. 1996). However, we have also found important changes in reproductive traits, like an increased number of root protuberances at the nodes (that facilitate vegetative reproduction), shorter styles (that facilitate selfing), a reduction in pollen production (that may accompany the selfing syndrome, or vegetative reproduction) and fewer seeds per fruit. The fact that pepino is vegetatively propagated probably favoured the selection of parthenocarpic materials (Prohens et al. 1998), which means that traits that promote effective sexual reproduction are released from selection. Cultivated pepinos also offer a better perceived flavour, probably resulting for a selection for lower acidity and lack of off-flavour (Prohens et al. 2005). But, pepino cultigens also have a lower content in soluble solids content (Rodríguez-Burruezo et al. 2003a), which is undesirable for producing sweet tasting fruits, obviously highly desirable in the marketplace (Rodríguez-Burruezo et al. 2011). As in other crops, selection for yield may have brought a reduction in the concentration of sugars due to the "dilution effect" associated to high yields (Davis 2009). However, it has been demonstrated that it is possible to obtain backcrosses resembling the cultivated pepino with interspecific hybrids derived from *S. caripense* and *S. tabanoense*. Such hybrids have high yield and soluble solids content levels higher than those of the cultivated recurrent parent, suggesting that these wild species contain genes not present in the cultivated species that can be useful for improving the soluble solids content of pepino (Rodríguez-Burruezo et al. 2003a, 2011).

The local varieties and modern cultivars of pepinos also differ by a number of significant morphological differences, and, as a consequence, theycluster in different areas of the PCA diagram. Breeding for higher yield and fruit typologies adapted to markets has resulted in modern varieties with larger and more elongated fruits. The elongated fruits may be constitute a selection for shipping: they pack better in layers in boxes, which may result in fewer bruises than in round fruits. Also, modern varieties have a higher production of pollen and higher number of seeds per fruit, probably as a result of selection for higher yield under conditions that may not favour expression of parthenocarpy. Oddly, and surprisingly, although markets favor golden yellow fruits (Rodríguez-Burruezo et al. 2011), modern varieties have a greener (a* parameter) skin colouration than local varieties. In tomato, enhancing chloroplast development in the fruit increases sugar contents in fruit (Cocaliadis et al. 2014), and if the same occurs in pepino this might be the underlying reason for which breeders have unconsciously selected for fruits with a greener skin. However, this hypothesis remains to be tested.

The high morphological diversity observed in the collections studied is matched by high levels of molecular diversity. A high level of molecular diversity was already observed for AFLP and DNA sequence of a nuclear gene (Blanca et al. 2007). The EST-SSR markers evaluated are scattered over the genome of tomato and may constitute a good representation of different regions of the genome of pepinos as well, if the high degree of synteny exists between the two closely related crops (Peters et al. 2012). The results reveal that cultivated pepino clones manifest a considerable heterozygosis, which is expected as a high degree of heterozygosis is associated with heterosis for yield (Rodríguez-Burruezo et al. 2003b). Heterozygosis for DNA sequence data had already been observed by Blanca et al. (2007) in some pepino clones and wild relatives. In the case of modern varieties, despite the lower heterozygosity compared to local varieties, the level of observed heterozygosis has been similar to that of local varieties. This may be taken as evidence that breeders have selected for highly heterozygous individuals in the modern breeding programs. The Sweet Round variety, which has been the only modern cultivar homozygous for the 14 loci scored must be heterozygous for other loci as it does not breed true (Ruiz et al. 1997). With the exception of *S. caripense*, wild relatives present low levels of observed heterozygosity. This is probably caused by the fact that many populations of wild species of *Basarthrum* other than the widespread *S. caripense* are

composed of few individuals (Anderson 1975, 1979), which favours fixation of alleles, even despite the self-incompatibility of some of these species, like *S. perlongistylum* and *S. tabanoense* (Mione and Anderson 1992; Anderson et al. 1996).

Wild relatives show greater molecular diversity than the cultivated pepinos (Blanca et al. 2007). In addition the genetic differentiation between the cultivated and wild materials was quite high (G_{ST}=0.274 and G'_{ST}=0.430), indicating that wild relatives contain a large diversity that is not represented in the genetic background of the cultivated pepino. This suggests that wild relatives constitute an important source of variation for pepino breeding (Rodríguez-Burruezo et al. 2003a; Blanca et al. 2007). Local varieties of pepino show much greater genetic diversity than modern varieties, but their differentiation was very low (G_{ST}=0.047 and G'_{ST}=0.089), indicating that the genetic diversity of the modern varieties is mostly present in the local varieties. This is expected as modern varieties have been derived by selection of segregating generations derived from local varieties (Dawes and Pringle 1984; Simms et al. 1996; Ruiz et al. 1997; Prohens et al. 2002; Rodríguez-Burruezo et al. 2004a, 2004b; Levy et al. 2006). Also, in contrast to tomato (Lin et al. 2014), no modern pepino cultivars have been released incorporating artificially introgressed traits from wild relatives, which increases genetic diversity of modern cultivars. The low diversity present in the modern varieties indicates that, as occurred in many crops (Cooper et al. 2001), a genetic bottleneck has taken place during the selection and hybridization programmes performed by breeders. Our data confirm the information provided by breeders (Dawes and Pringle 1984; Simms et al. 1996; Ruiz et al. 1997; Prohens et al. 2002; Rodríguez-Burruezo et al. 2004a, 2004b; Levy et al. 2006) indicating that they have mostly used local varieties from the peripheral southern (Chile) range of distribution of pepino, where the diversity is much lower than in the center of diversity of the crop in Ecuador, southern Colombia and northern Peru (Anderson et al. 1996; Blanca et al. 2007). In fact in the PCoA analysis, the local varieties closest to the modern varieties cluster are those from Chile. Thus, different results might be expected with different selections of pepino cultivars and (particularly) with different *S. caripense* wild collections.

Conclusions

The characterization using the IPGRI and COMAV (2004) morphological descriptors list and tomato SSRs molecular markers (Frary et al. 2005) has revealed a large variation in the collection studied. These characterization tools will allow the identification of new sources of morphological and genetic variation in pepino and wild relatives, the study of diversity and establishment of the relationships in pepino and wild relatives. Cultivated pepino and wild relatives display high morphological and molecular diversity, but the two groups are clearly differentiated from each other. Modern cultivars are notably morphological different from local varieties, and are much less variable at the molecular level indicating the existence of a genetic bottleneck during the modern breeding history of this crop. All of these data are of relevance for modern and efficient pepino breeding based on phenotypic and molecular marker selection as well as for the management and conservation of pepino germplasm collections.

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Table 1 Plant materials used for the study of morphological and molecular (SSR) variation in a germplasm collection of local varieties and modern cultivars of cultivated pepino (*S. muricatum*) and wild relatives (other species of *Solanum* section *Basarthrum*).

Accession	Code	Species	Origin[a]
Pepino local varieties			
37-A	37	*S. muricatum*	Ecuador (Azuay)
Col-1	Co	*S. muricatum*	Colombia
CH2-22	CH	*S. muricatum*	Chile
OV-8	OV	*S. muricatum*	Chile (Limarí)
PT-154	PT	*S. muricatum*	Peru
RP-1	RP	*S. muricatum*	Ecuador
Pepino modern cultivars			
El Camino	EC	*S. muricatum*	New Zealand
Kawi	Ka	*S. muricatum*	New Zealand
Puzol	Pu	*S. muricatum*	Spain
Quito	Qu	*S. muricatum*	United Kingdom
Sweet Long	SL	*S. muricatum*	Spain
Sweet Round	SR	*S. muricatum*	Spain
Turia	Tu	*S. muricatum*	Spain
Valencia	Va	*S. muricatum*	Spain
Wild relatives			
BIRM/S 1034	c1	*S. caripense*	Ecuador
E-7	c2	*S. caripense*	Ecuador
EC-40	c3	*S. caripense*	Ecuador (Loja)
QL-013	c4	*S. caripense*	Ecuador
P-80	ct	*S. catilliflorum*	Peru (Abancay)
P-62	pe	*S. perlongistylum*	Peru (La Mar)
E-257	ta	*S. tabanoense*	Ecuador (Loja)
E-34	tr	*S. trachycarpum*	Ecuador

[a]Origin refers to the country and province (when known) of the collection in the case of wild relatives and local varieties of pepinos, and to the country where the modern cultivar of the pepino was developed.

Table 2 Morphological and agronomic descriptors used for the characterization of cultivated pepino (*S. muricatum*) and wild relatives. Full details on each descriptor can be consulted elsewhere (IPGRI and COMAV 2004).

Descriptor	Code	Range (scale) / units
Plant descriptors (P)		
Plant size	P-Size	1-9 (3=small; 7=large)
Vigour of the plant	P-Vigour	1-9 (3=weak; 7=strong)
Stem descriptors (St)		
Stem length at first inflorescence	St-LengthInfl1	cm
Degree of ramification	St-Ramification	1-9 (3=low; 7=high)
Intensity of anthocyanin of shoot tip	St-Anthocyanin	0-9 (0=absent; 7=strong)
Root protuberances at the node	St-Protuberances	0-9 (0=absent; 7=many)
Stem pubescence density	St-Pubescence	0-9 (0=glabrous; 7=dense)
Stem colour	St-Colour	1-5 (1=green; 5=dark purple)
Internode length	St-InternLength	cm
Leaf descriptors (L)		
Petiole length	L-PetioleLength	mm
Petiole colour	L-PetioleColour	1-5 (1=green; 5=dark purple)
Foliage density	L-Density	1-9 (3=sparse; 7=dense)
Leaf attitude	L-Attitude	1-3 (1=semi-erect; 3=dropping)
Leaf lamina length	L-LaminaLength	cm
Leaf lamina width	L-LaminaWidth	cm
Leaf blade length/width ratio	L-LWRatio	---
Type of leaves	L-Type	1-2 (1=simple; 2=compound)

Table 2 Cont.

Descriptor	Code	Range (scale) / units
Number of leaflets	L-Leaflets	---
Leaf colour	L-Colour	1-5 (1=light green; 5=purple)
Anthocyanin coloration of leaf veins	L-AnthVeins	1-9 (3=green; 7=purple)
Leaf surface attitude	L-Surface	1-9 (3=flat; 7=very convex)
Inflorescence descriptors (I)		
Number of leaves from ground to first inflorescence	I-LeavesInfl1	---
Inflorescence type	I-Type	1-3 (1=generally uniparous; 3=generally multiparous)
Number of flowers per inflorescence	I-NFlowers	---
Flower descriptors (Fl)		
Corolla shape	Fl-CorollaShape	1-3 (1=stellate, 3=rotate)
Corolla colour	Fl-CorollaColour	1-6 (1=white; 6=purple)
Sepal length	Fl-SepalLength	mm
Stamen length	Fl-StamenLength	mm
Style exsertion beyond anther cone	Fl-StyleExsertion	mm
Pollen production	Fl-PollenProd	0-9 (0=none; 7=high)
Fruit descriptors (Fr)		
Number of fruits per infructescence	Fr-FruitInfruct	---
Number of fruits per plant	Fr-FruitPlant	---
Fruit size uniformity	Fr-Uniformity	1-9 (3=low; 7=high)
Fruit length	Fr-Length	cm

Table 2 Cont.

Descriptor	Code	Range (scale) / units
Fruit width	Fr-Width	cm
Position of the widest part of the fruit	Fr-WidestPart	1-9 (3=less than ¼ way from base to tip; 7=more than ½ way from base to tip)
Fruit length/width ratio	Fr-LWRatio	---
Fruit primary colour L* parameter	Fr-L*	---
Fruit primary colour a* parameter	Fr-a*	---
Fruit primary colour b* parameter	Fr-b*	---
Fruit stripes	Fr-Stripes	0-1 (0=absent; 1=present)
Fruit mottling	Fr-Mottling	0-1 (0=absent; 1=present)
Fruit surface covered by additional colour	Fr-AddColour	1-3 (1=less than 10%; 3=between 30 and 50%)
Fruit epidermis glossiness	Fr-Glossiness	3-7 (3=dull; 7=bright)
Number of locules per fruit	Fr-Locules	---
Inner placental area length	Fr-PlacentLength	cm
Inner placental area breadth	Fr-PlacentBreadth	cm
Inner placental length/breadth ratio	Fr-PlacentLBRatio	---
Fruit flesh colour	Fr-FleshColour	1-8 (1=dark green; 8=salmon)
Fruit flavour	Fr-Flavour	1-9 (3=acidic; 9=sweet)
Fruit flesh colour	Fr-FleshColour	1-8 (1=dark green; 8=salmon)

Table 2 Cont.

Descriptor	Code	Range (scale) / units
Fruit cracking	Fr-Cracking	0-9 (0=absent; 9=severe)
Fruit fasciation	Fr-Fasciation	0-9 (0=absent; 9=severe)
Fruit soluble solids content	Fr-SolubleSolids	%
Seed descriptors (Se)		
Number of seeds per fruit	Se-SeedsFruit	---
Seed colour	Se-Colour	1-7 (1=white; 7=black)
Seed diameter	Se-Diameter	1-3 (1=small (<1.5 mm); 3=large (>2.5 mm))
Type of seed	Se-Type	1-3 (1=not winged; 3=winged)

Table 3 EST-SSR tomato markers used in the present study along with their repeat motif, annealing temperature, expected size, and linkage group in which they map in the tomato genetic map (Frary et al. 2005).

SSR locus	Repeat motif	Annealing temperature	Expected size	Linkage group
SSR13	$(AAG)_6$	50	102	5
SSR14	$(ATA)_9$	55	166	3
SSR20	$(GAA)_8$	50	157	12
SSR38	$(TCT)_8$	55	237	8
SSR43	$(TAC)_7$	55	237	4
SSR45	$(AAT)_{14}$	50	246	7
SSR51	$(ACAA)_6$	50	148	1
SSR52	$(AAC)_9$	50	202	7
SSR66	$(ATA)_8$	50	185	2
SSR80	$(TTTCAA)_2(GTACAA)_2(CAA)_7$	50	186	11
SSR111	$(TC)_6(TCTG)_6$	50	188	3
SSR128	$(CAG)_6(CAA)_3(CAG)_7$	50	123	6
SSR136	$(CAG)_7$	50	149	11
SSR150	$(CTT)_7$	50	217	1
SSR248	$(TA)_{21}$	55	251	10
SSR285	$(TTAT)_2(AT)_6$	55	276	7
SSR306	$(ATT)_7$	55	258	4
SSR578	$(AAC)_6(ATC)_5$	55	294	6
SSR590	$(TC)_6(AC)_4$	55	161	5
SSR593	$(TAC)_7$	55	295	4

Table 4 Mean and range for the morphological descriptors for which significant differences were found between accessions of the cultivated pepino (*S. muricatum*) and its wild relatives.

	Cultivated species		Wild relatives		
Descriptor[a]	Mean	Range	Mean	Range	Prob. t
N	14		8		
P-Size	4.9	3.4-6.6	6.5	5.0-7.0	0.0014
St-LengthInfl1	51.9	43-71	101.8	63-144	<0.0001
St-Protuberances	4.6	3.0-7.0	2.3	0.0-3.0	0.0002
St-Pubescence	2.7	0.0-3.0	4.7	0.0-7.0	0.0067
St-InternLength	5.3	4.2-6.0	7.5	4.3-9.3	0.0001
L-LaminaLength	31.7	25-37	26.9	20-34	0.0180
L-LWRatio	1.8	1.0-3.0	1.2	0.8-2.2	0.0469
L-Type	1.4	1.0-2.0	1.9	1.0-2.0	0.0077
L-Surface	4.7	3.0-7.0	3.4	3.0-5.0	0.0026
I-LeavesInfl1	11.6	8-17	16.8	13-19	0.0001
I-Type	2.6	1.0-3.0	1.4	1.0-3.0	0.0008
Fl-StyleExsertion	2.8	1.4-3.9	3.9	1.3-5.2	0.0223
Fl-PollenProd	3.4	0.0-5.4	5.7	5.0-7.0	0.0007
Fr-Uniformity	5.0	3.0-6.2	5.9	5.0-7.0	0.0249
Fr-Length	9.1	4.8-15.4	2.9	1.7-4.6	<0.0001
Fr-Width	7.2	4.1-11.1	2.7	1.8-3.6	<0.0001
Fr-L*	60.3	51-65	54.7	40-63	0.0495
Fr-b*	23.6	17-29	18.7	8-24	0.0241
Fr-Glossiness	4.5	3.0-5.7	3.3	3.0-5.0	0.0039
Fr-PlacentLength	5.1	2.2-9.7	1.4	0.6-2.1	0.0012
Fr-PlacentBreadth	0.68	0.2-1.8	0.15	0.1-0.2	0.0020
Fr-FleshColour	5.2	3.0-6.7	2.8	2.0-4.0	0.0001
Fr-Flavour	5.8	5.0-7.0	2.0	1.0-3.0	<0.0001
Fr-OffFlavour	0.66	0.0-3.0	2.75	0.0-5.0	0.0037
Fr-SolubleSolids	6.6	4.9-7.7	9.7	7.8-11.4	<0.0001
Se-SeedsFruit	0.26	0.0-0.7	3.08	1.0-4.0	<0.0001

[a]See Table 2 for a full definition of the descriptors.

Table 5 Mean and range for the morphological descriptors for which significant differences were found between local varieties and modern cultivars of cultivated pepino (*S. muricatum*).

	Local varieties		Modern cultivars		
Descriptor[a]	Mean	Range	Mean	Range	Prob. t
N	6		8		
St-Colour	3.1	2.0-4.0	2.2	2.0-3.4	0.0195
St-InternLength	4.8	4.2-5.4	5.6	5.1-6.0	0.0024
L-LaminaWidth	24.6	16-34	16.5	11-31	0.0354
L-LWRatio	1.3	1.0-1.7	2.3	1.1-3.0	0.0082
L-Type	1.7	1.3-2.0	1.2	1.0-1.5	0.0057
L-Leaflets	3.0	1.0-5.0	1.6	1.0-3.0	0.0269
L-AnthVeins	4.3	3.0-5.0	3.3	3.0-3.8	0.0196
L-Surface	4.1	3.0-5.4	5.2	4.6-7.0	0.0478
Fl-PollenProd	2.5	0.0-4.6	4.1	3.0-5.4	0.0402
Fr-Length	6.8	4.8-7.9	10.8	6.6-15.5	0.0185
Fr-LWRatio	1.0	0.7-1.8	1.6	0.9-2.2	0.0382
Fr-a*	-3.3	-6.1--1.3	-6.4	-11.7--3.1	0.0393
Se-SeedsFruit	0.10	0.0-0.4	0.38	0.0-0.7	0.0223

[a]See Table 2 for a full definition of the descriptors.

Table 6 Correlation coefficients between morphological descriptors and the two first components (29.7% and 11.8% of the total variance explained by the first and second principal components, respectively) for accessions evaluated of the cultivated pepino and wild relatives. Only those correlations with absolute values ≥0.15 have been listed.

Descriptor[a]	First principal component	Second principal component
P-Size	0.172	
St-LengthInfl1	0.225	
St-Anthocyanins		0.181
St-Protuberances	-0.178	
St-Colour		0.227
St-InternLength	0.188	
L-PetioleColour		0.280
L-Attitude		-0.190
L-LaminaWidth		0.270
L-LWRatio		-0.235
L-Type		0.201
L-Leaflets		0.249
L-AnthVeins		0.155
L-Surface	-0.178	-0.180
I-LeavesInfl1	0.199	
I-Type	-0.159	
I-NFlowers		0.217
Fl-CorollaShape	0.198	
Fl-CorollaColour		-0.185
Fl-PollenProd	0.163	
Fr-Length	-0.196	
Fr-Width	-0.220	
Fr-WidestPart		-0.160
Fr-L*		0.152
Fr-a*		0.259
Fr-Mottling		0.164
Fr-Glossiness	-0.183	
Fr-PlacentLength	-0.178	
Fr-PlacentBreadth	-0.172	
Fr-FleshColour	-0.186	
Fr-Flavour	-0.224	
Fr-OffFlavour	0.159	
Fr-Fasciation		0.247
Fr-SolubleSolids	0.184	
Se-SeedsFruit	0.211	

[a]See Table 2 for a full definition of the descriptors.

Table 7 SSR markers successfully amplified and polymorphic in the collection of cultivated pepino and wild relatives evaluated, number of alleles per SSR locus of each of the groups considered and PIC value.

SSR locus	Number of alleles					PIC
	All accessions (n=22)	All cultivated accessions (n=14)	Cultivated local varieties (n=6)	Cultivated modern cultivars (n=8)	Wild relatives (n=8)	
SSR14	3	1	1	1	3	0.3360
SSR20	8	6	5	2	3	0.6134
SSR43	4	3	3	1	2	0.2604
SSR45	6	2	2	1	5	0.3665
SSR52	3	2	2	1	2	0.4156
SSR66	2	1	1	1	2	0.0499
SSR80	2	2	2	2	2	0.3715
SSR111	4	2	2	1	4	0.4297
SSR128	5	2	2	1	4	0.3079
SSR285	4	3	3	1	3	0.5188
SSR306	6	4	4	1	5	0.7021
SSR578	2	2	2	2	1	0.3693
SSR590	4	3	3	2	2	0.5774
SSR593	4	2	2	1	4	0.4669
Mean	4.07	2.50	2.43	1.29	3.00	0.4132

Table 8 Observed heterozygosity (H_o) for the polymorphic SSR loci in each of the accessions of cultivated pepino (*S. muricatum*) and wild relatives evaluated, and mean values (±SE) for the cultivated pepino local varieties, modern cultivars and for wild relatives.

Accession	H_o
Pepino local varieties	
37-A	0.154
Col-1	0.231
CH2-22	0.214
OV-8	0.167
PT-154	0.091
RP-1	0.300
Mean local varieties	0.193±0.029
Pepino improved cultivars	
El Camino	0.154
Kawi	0.333
Puzol	0.154
Quito	0.143
Sweet Long	0.154
Sweet Round	0
Turia	0.077
Valencia	0.167
Mean improved cultivars	0.148±0.033
Wild relatives	
BIRM/S 1034 (*S. caripense*)	0.154
E-7 (*S. caripense*)	0.250
EC-40 (*S. caripense*)	0.154
QL-013 (*S. caripense*)	0.286
P-80 (*S. catilliflorum*)	0
P-62 (*S. perlongistylum*)	0
E-257 (*S. tabanoense*)	0
E-34 (*S. trachycarpum*)	0.091
Mean wild relatives	0.117±0.040

Table 9 Total genetic diversity (H_T), among groups genetic diversity (D_{ST}), within groups genetic diversity (H_S), relative magnitude of genetic differentiation (G_{ST}) and standardized G_{ST} (G'_{ST}) (Nei, 1973) estimated from data for the cultivated pepino (*S. muricatum*) and wild relatives accessions.

Group	Sample size	H_T	D_{ST}	H_S	G_{ST}	G'_{ST}
All	22	0.458	0.107	0.350	0.274	0.430
Cultivated pepino	14	0.237				
Wild relatives	8	0.401				
Cultivated pepino	14	0.237	0.021	0.216	0.047	0.089
Local varieties	6	0.336				
Modern cultivars	8	0.096				

Figures

Fig. 1 Diversity in fruit size, shape and colour in the cultivated pepino and wild relatives collection studied. Fruits of wild species are indicated by white arrows.

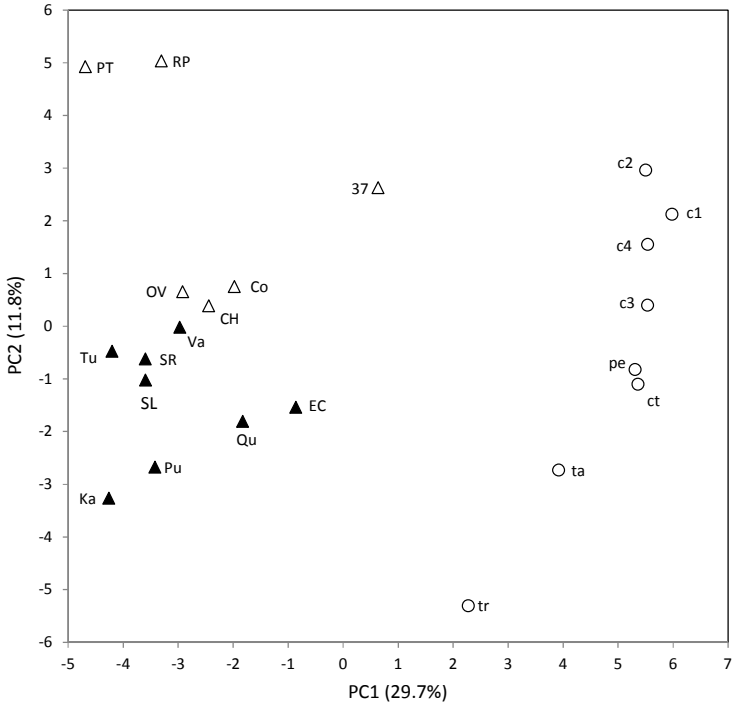

Fig. 2 Principal components analysis (PCA) similarities based on 55 variable morphological descriptors among 22 accessions of local varieties (open triangle), modern cultivars (solid triangle) of cultivated pepino and wild relatives (open circle). First (PC1) and second (PC2) principal components account for 29.7% and 11.8% of the total variation, respectively.

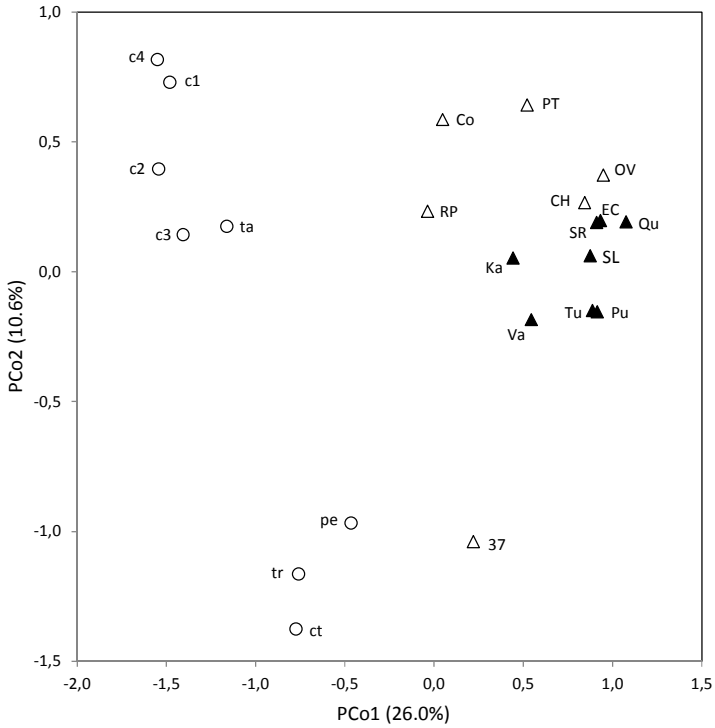

Fig. 3 Principal coordinates analysis (PCoA) similarities based on 14 polymorphic EST-SSRs among 22 accessions of local varieties (open triangle) and modern cultivars (solid triangle) of cultivated pepino and wild relatives (open circle). First (PC1) and second (PC2) principal coordinates account for 26.0% and 10.6% of the total variation, respectively.

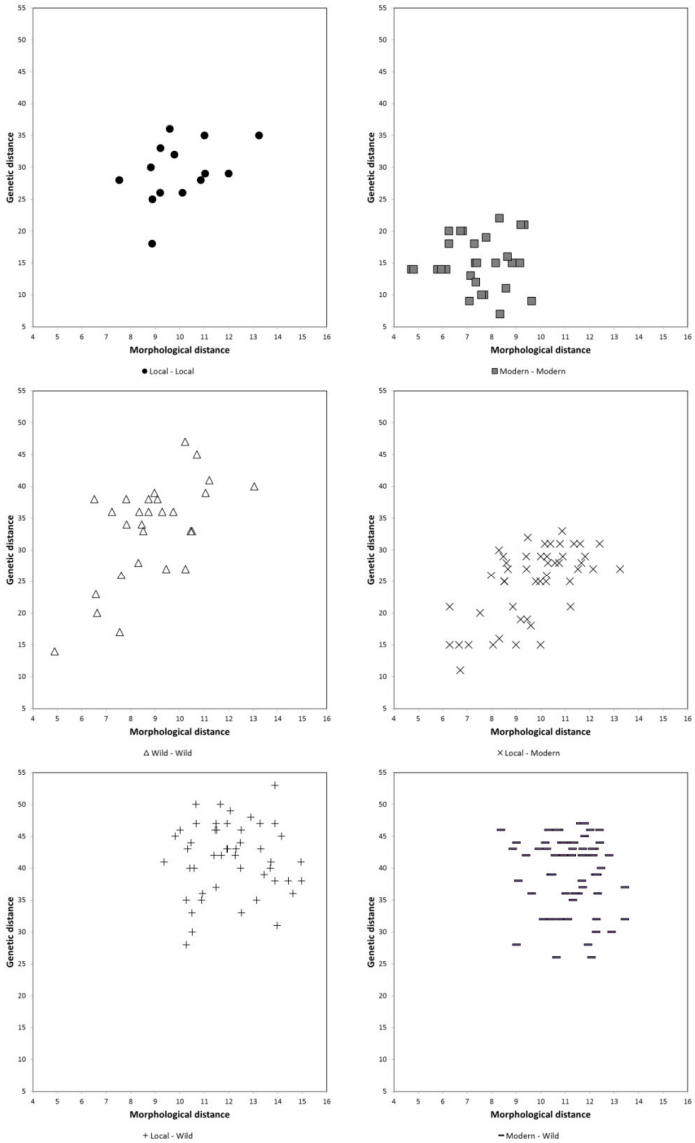

Fig. 4 Relationships between morphological and molecular distances among pairs of accessions of pepino and wild relatives. Distances between pairs of accessions are represented for each combination of groups: Local and local (solid circle; above left); modern and modern (grey square; above right); wild and wild (white triangle; center left); local and modern (× cross; center right); local and wild (+ cross; below left); and, modern and wild (horizontal dash; below right).

3.3.- The first *de novo* transcriptome assembly of pepino (*Solanum muricatum*) and its wild relative *S. caripense*: Comprehensive analysis and comparison with closely related potato and tomato genomes

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Submitted to BMC Genomics.

Abstract

Background: *Solanum* sect. *Basarthrum* is phylogenetically very close to potatoes (*Solanum* sect. *Petota*) and tomatoes (*Solanum* sect. *Lycopersicon*), two groups with great economic importance, and for which *Solanum* sect. *Basarthrum* represents a tertiary gene pool for breeding. Within section *Basarthrum* there is a cultigen, the pepino (*Solanum muricatum*) and several wild species, among which *S. caripense* is prominent due to its major involvement in the origin of pepino and its wide geographical distribution. Despite its interest as emerging crop and for potato and tomato breeding, the pepino has been barely studied at genomic level.

Results: Using Illumina HiSeq-2000, RNA-Seq was performed from a pool of three tissues (young leaf, flowers in pre-anthesis and mature fruits) from *S. muricatum* and *S. caripense*, generating almost one hundred eleven millions reads among the two species. A high quality de novo transcriptome was assembled from *S. muricatum* clean reads resulting in 75,832 unigenes with an average length of 704 bp. These unigenes were functionally annotated based on similarity of public databases. We used Blast2GO, to conduct an exhaustive study of the gene ontology, including GO terms, EC numbers and KEGG pathways. Pepino unigenes were compared to both potato and tomato genomes in order to determine their estimated relative position, and to infer genes prediction models. Candidate genes related to traits of interest in other Solanaceae were evaluated by presence or absence and compared with *S. caripense* transcripts. In addition, by studying five genes, the phylogeny of pepino and five other Solanaceae were studied. The comparison of *S. caripense* reads against *S. muricatum* assembled transcripts result in thousands of intra and interspecific SNVs. In addition, more than 1,000 SSRs were identified in the pepino transcriptome.

Conclusions: This study represents the first genomic resource for pepino. We suggest that, the data we generated will be useful not only for work with the pepino, its relatives and improvement, but also for potato and tomato breeding. The high quality of the transcriptome presented here allows application in comparative studies in the genus *Solanum*. The accurate transcript annotation will enable us to figure out the gene function in particular traits of interest. The high number of markers (SSR and SNV) obtained will be useful for several applications with great interest for breeding, diversity, synteny, evolution, and phylogenetic studies.

Keywords: *Solanum muricatum*, transcriptome, *S. caripense*, pepino, potato, tomato, Solanaceae, functional annotation, phylogeny, candidate genes, molecular markers.

Introduction

Pepino (*Solanum muricatum* Aiton) is a neglected herbaceous domesticate native to the Andean region, where wild relatives (*Solanum* section *Basarthrum*) are naturally distributed [1, 2]. The pepino is vegetatively propagated cultigen grown for its fruit, which is a juicy berry, of variable shape depending on the cultivar, and which typically weighs between 100 and 400 g. The fruit has an attractive appearance, with most successful cultivars producing fruits with a golden yellow skin covered with purple stripes. From the nutritional point of view it has outstanding levels of potassium and vitamin C, and a low content in calories [3]. Apart from being cultivated in its region of origin, the pepino has been introduced in other countries like New Zealand, China and Turkey as a potential new horticultural crop [4, 5].

One of the most interesting features of pepino is its phylogenetically close relationship with potato and tomato [6, 7]. In fact, pepino and its wild relatives in *Solanum* section *Basarthrum* are part of the tertiary gene pool of both potato and tomato [8, 9]. Cultivated potato, tomato and pepino share the same basic number of chromosomes (n=12) [10, 11], although tomato and pepino are diploid and most cultivated potato cultivars are polyploid [12]. The phylogenetic proximity between these species has important implications, as this allows the use of genomic resources from tomato and potato for pepino breeding, as has been demonstrated with the high transferability of tomato SSRs to the pepino [13]. Reciprocally, this close relationship may facilitate also the use of the pepino as a source of variation for tomato and potato breeding, including resistance to several diseases in both crops, and parthenocarpy and improvement of flavour in the case of tomato [3, 8, 14]. In this respect, somatic hybrids between tomato and pepino, as a first step for introgression of pepino traits into tomato, have been obtained [9].

Wild relatives of domesticates are a source of variation of interest for improving cultivated species [15] and for studying the domestication process [16]. In this context, *S. caripense* Dunal, locally known as "mamoncillo" or "tzimbalo", is easily hybridized with the pepino and

hybrids are highly fertile [3]. AFLP and genic DNA sequence studies indicated that *S. caripense* is one of the species that has been involved in the origin and evolution of the cultivated pepino [17]. In the Andean region, the pepino frequently grows in close vicinity of the widely distributed *S. caripense* and other cross-compatible wild relatives and introgression and gene flow evidence has been found [17]. *Solanum caripense* is of particular interest as it presents traits of interest for pepino breeding such as high levels of soluble solids content [18], or a high content of bioactive phenolic acids (unpublished results). In addition, some accessions of *S. caripense* have displayed resistance to Tomato Mosaic Virus (ToMV) [19] and to *Phytophthora infestans* [20], the most important disease of potato [21], and could represent alternative sources of variation for breeding for resistance to these diseases.

Despite being an important crop in the Andean region during pre-Columbian times [22] and its potential as a new crop for many areas with mild climates, few molecular studies and tools have been developed for *S. muricatum* – the pepino. The pepino and its wild relatives have not been thoroughly studied at a genome-wide level in the context of molecular studies and tools. As of July 2015, only 126 nucleotide sequences had been deposited in the NCBI's GenBank database, all of them resulting from a single study [17]. In addition, there are few studies of molecular markers and their applications in pepinos. Some of the previous studies used cp-DNA restriction fragments length polymorphism (RFLP) [1], AFLP and gene sequence haplotypes [17], RAPDs [23] and EST-SSRs derived from tomato [13] to study diversity in the pepino and its wild relatives. Apart from these studies, an intra-specific low-density genetic map with SNPs taken from the sequencing of a set of COSII was produced in the pepino wild relative *S. caripense* with the aim of mapping the resistance to *Phytophthora infestans* [20].

High throughput sequencing of transcriptomes (RNA-Seq) has opened the way to study the genetic and functional information in neglected crops and species. RNA-Seq is genome-independent and is especially useful for analysing the transcriptome of species without complete genome information or a reference genome [24, 25], as is the case of the pepino and wild relatives. In this context, RNA-Seq can be helpful to: (1) listing the transcripts and other RNAs from one or several tissues; (2) investigating the transcriptional structure of genes, splicing patterns, and gene isoforms; (3) studying post-transcriptional

modification and mutations; and (4) quantifying gene expression [26]. These transcriptomics studied have provided a basis for scanning the evolution of polyploidy in plants [27, 28], for study of phylogenies in some families including the Solanaceae [29], to compare patterns associated with domestication [30], and to develop markers *en masse* [31-33].

In the present work, we used the Illumina pair-end sequencing technology to perform RNA-Seq of one modern cultivar of pepino and of one accession of the pepino wild relative *S. caripense*. We obtained almost 111 million reads including sequencing of both species. Our transcriptome analysis included *de novo* assembly, structural and functional annotation and comparison with tomato and potato genomes [34, 35], providing us the opportunity to establish a dated phylogeny of the pepino compared with related species. Candidate genes, mainly from tomato agronomic traits of interest have also been found. These genes can provide us an effective comparative approximation to patterns of selection in domestication, and will allow us to select genes useful for the genetic improvement of the pepino. Another important goal is discovery of the high throughput markers (SSR and SNPs). These gene-derived markers are important functionally in that they can provide potential changes in the proteins expressed, as well as an essential tool for the construction of genetic maps and for marker-assisted selection. The rest of the dataset will serve as a public information platform for gene expression and genomics in the pepino and their related species, particularly useful for future studies in pepino, potato, and tomato genomics and breeding.

Results and discussion

Transcriptome sequencing (mRNA-Seq) output and assembly. We performed two Illumina HiSeq-2000 runs, one for the cultivated pepino (*S. muricatum*) cultivar Sweet Long (SL) and one for *S. caripense* (EC-40), one of the key the wild relatives of the pepino [1, 36]. The sequencing of the RNA of three tissues (young leaves, young flowers and mature fruits) from *S. muricatum* generated 58,327,154 raw paired end reads, covering about 11.78 Gb of sequencing raw data (reads with a length of 101 bp). In the case of *S. caripense*, sequencing generated 52,646,045 reads and 10.63 Gb of raw data. Graphical representation of sequence quality is shown in Fig. 1, where the quality scores across all the bases is indicated. All these raw paired-end data have been deposited in

the NCBI Sequence Read Archive. After initial trimming and quality filtering to remove adapters and low-quality data, 33,963,075 clean paired end reads were obtained, encompassing 6.86 Gb of sequencing data in *S. muricatum* and 36,228,181 clean reads and 7.32 Gb of sequencing data in *S. caripense* (Table 1).

The high quality reads of the species *S. muricatum* were used to assemble the transcriptome using the Trinity software. The results of this assembly are shown in Table 2. The length distribution of the transcripts after assembly is shown in Fig. 2. The total of 91,949 (Additional data File S1) contigs were assembled with an average length of 895 bp. It should be noted that more than 63 % (58,465) of the transcripts are between 200 to 766 bp in length and only 1 % of them had a sequence length higher than 3,500 bp. We have selected a subset of the transcripts, the selection based on the most expressed transcript of each Trinity transcript cluster. We obtained 75,832 of the most expressed transcripts (unigenes) (Additional data File S2), with an average length of 704 bp. These sequences were used for all the further analysis. It is worth noting that the number of assembled unigenes obtained is similar or better than that obtained in previous studies using similar technologies – thus demonstrating the quality and potential utility of our work, both in samples preparation and assembling protocol [37, 38].

RNA-Seq offers an opportunity for the analysis of the G/C content (ratio of guanine and cytosine) among unigenes. Owing to the very nature of its bond, the G/C base pair has been considered more stable than the A/T base pair, so during evolution, the variation in G/C content would occur more slowly; however this assertion has been contested [39]. However the G/C content, markedly variable among different organisms, would be an indicator of closeness between species. The percentage G/C of the clean reads was similar between the two species: 41.7 % in *S. muricatum* and 42.5 % in *S. caripense* (Table 1). This G/C content obtained was consistent with values found in other *Solanum* [29]. In particular, in tomato the G/C content found in previous works for cDNA was 40.3% [40] and in potato 43.1% [41]. This demonstrates that our study provides a valid representation of typical Solanaceae transcriptomes, thereby opening up the possibility of using our data for broader comparative studies in this genus.

Functional annotation. To identify *S. muricatum* transcripts potentially encoding proteins with known function, a BLASTX® analysis

was performed sequentially using three proteins databases [42]. The used database order was Swiss-Prot [43], ITAG2.4 from tomato [44] and UniRef90 [45], (e-value cut-off of $1e^{-20}$). Over 65.9 % of the transcripts (49,662) had at least one significant hit. Most of the transcripts with annotation had significant hits in Swiss-Prot (53.7 %), representing 35.2 % of the total of unigenes, an expected result for a manually curated database. The hits obtained in the rest of databases were: ITAG2.4 with a 30.1 % of sequences annotated and UniRef90 with a 4.6 %. These results, represented in Table 3, were similar to those found in others works. Blast results are listed in an additional data File S3.

Using Blast2GO against NR database we recovered gene ontology (GO) terms and enzyme commission numbers (EC) for the most expressed transcripts or unigenes in *S. muricatum*. A total of 197,221 GO terms were assigned to 37,031 transcripts. The unigenes distribution relative to the number of GOs to which they were assigned is shown in additional data File S4. Slightly more than half of the unigenes (50.7 %) have between 1 and 5 GO terms and 12 % have more than 10 GO terms. The maximum number of GO terms annotated in a unigene was 45. Among all the GO terms extracted, 89,060 (45.2 %) belong to the molecular function class (MF), 59,856 (30.3 %) to biological process class (BP) and 48,305 (24.5 %) to cellular components class (CC).

The distribution of annotated transcripts under different GO levels shows a concentration, between levels 4 - 10 in the biological process, between levels 3 - 9 in molecular function and between levels 5 - 8 in cellular component (Fig. 3). The GO levels that ranged between 5 and 15, were 84.1 % for biological processes, 76.0 % for molecular function and 88.1 % of cellular components, indicating good annotation precision (Fig. 3), and that a broad diversity of genes was sampled in our transcriptome. Meanwhile, focusing on GO slim terms for plants, most of the genes were classified into biological process (BP) of cellular processes, metabolic processes, biosynthetic processes and response to stress (Fig. 4). It is worth noting that many sequences were classified in the category of development for different plant structures, like embryo and flower development, pollen-pistil interaction and fruit ripening, all functions related to the three tissues sampled (the full list of these annotation is shown in additional data File S5). The study of these functions can be useful for a precise knowledge of those processes, which have a direct application in breeding. Sequences in the molecular function (MP)

category were assigned to different binding processes, hydrolase activity and catalytic activity, while for cellular component (CC) sequences, we identified the majority of cell and organelle parts represented (Fig. 4 and File S5 in additional data).

The enzyme commission (EC) number is a codification for enzymes, based on the chemical reactions they catalyse [46]. We found a total of 15,337 annotations classified under this scheme involving 12,296 different unigenes (some unigenes have 2 or more EC annotations). Transferases (EC-2) with 6,127 sequences annotated, hydrolases (EC-3) with 4,126 sequences and oxidoreductases (EC-1) with 3,046 sequences were the most important (Fig. 5). Other classes like ligases, lyases and isomerases were represented to a lesser degree. The number of annotated sequences under this scheme was greater than in other studies in the Solanaceae [37, 47]. The complete list of these EC numbers is include in the File S5 in additional data.

The KEGG pathway database is a resource for the systematic analysis of gene functions in terms of networks of genes and molecules in cells and their variants specific to particular organisms [48]. In order to understand the function of the unigenes in pepino, a BLASTX search against the KEGG protein database with a cut-off e value of $1e^{-5}$ was performed. Out of the 75,832 transcripts, 16,027 were annotated in the KEGG pathway database, and assigned to 144 unique pathways. These pathways include amino acid metabolism, sugar metabolism, fatty acid metabolism, as well as biosynthesis of secondary metabolites like flavonoids and terpenoids. Our results show that the largest three pathway groups were purine metabolism, starch and sucrose metabolism, and phenylalanine metabolism (see additional data File S6). Considering that the pepino is largely a dessert fruit in which sugars and bioactive compounds are important for quality [3], we paid attention to the pathways pertaining to starch and sucrose metabolism, and to biosynthesis of carotenoids, anthocyanins, and several vitamins. A considerable number of genes were related to relevant metabolic pathways, including starch and sucrose metabolism (map00500, 727 genes), carotenoid biosynthesis (map00906, 33 genes), anthocyanin biosynthesis (map00942, 31 genes), ascorbate and alderate metabolism (map00053, 123 genes), vitamin B6 metabolism (map00750, 28 genes), retinol metabolism (map00830, 89 genes), thiamine metabolism (map00730, 325 genes), riboflavin metabolism (map00740, 117 genes),

and biotin metabolism (map00780, 98 genes). Finally, we compared the number of genes assigned for every KEGG pathway in our analysis with the analogous genes assigned in tomato and potato genomes. This comparison let us detect significant similarities among the three species (additional data File S6), implying that we have a good representation of the transcriptome. For very few pathways was the number of genes annotated in pepino notably lower than in tomato and potato. This is because some processes may not be properly represented in our samples because they derive from mRNA of three tissues, and we do not have a representation of the whole genome. Other processes instead, are better represented. The table with the results of this comparison is presented in additional data File S6.

Further analysis of these pathways-related genes will improve our understanding of the pepino features, some of them unique and others common with the rest of Solanaceae, and can contribute to enhancing our resources addressed to breeding these species.

Comparison with potato and tomato genomes. All of the most expressed transcripts (75,832) were blasted to the potato genome, resulting in 40,113 (52.9 %) sequences mapped. Furthermore, 37,813 (49.9 %) transcripts of pepino were mapped to the tomato genome. This distribution of the pepino unigenes relative to the potato and tomato genomes was plotted using Circos® software (Fig. 6). This graphical representation provides visual information of the location of the coding regions, but a knowledge of chromosomal realignment and other changes is missing until the development of the sequence of the whole genome of pepino.

We have generated gene model predictions comparing our assembled transcriptome of *S. muricatum* with the tomato genome [49]. This alignment of the unigenes to the tomato genomic DNA were performed using the EST2GENOME® software and the ORFs annotations were carried out using ESTSCAN® software [50] as described in Methods. A large number (48,440) of the most expressed transcripts were predicted to have one ORF (63.9 %). On the other hand we predicted the presence of introns in our unigenes. We found 130,528 in a total of 24,979 unigenes (32.9 %), which means 5.2 introns per unigene, with a maximum number of 19. Knowledge of the positions of these intronic regions is particularly important for SNVs discovery, as it allows us to discard those that are located in the vicinity of an intron,

because that would make it difficult to design the primers to amplify these regions. The annotation results (ORFs, introns, descriptions, GO terms, orthologs, SNVs and SSR) are deposited in additional data File S3 in GFF3 format.

Candidate genes. Several candidate genes related to traits of interest for domestication and breeding were studied. These include genes involved in the inflorescence type, fruit development [51], and synthesis of anthocyanins, chlorogenic acid, saponins, and sucroses, and were evaluated for presence or absence in our assembled transcriptome and compared with the transcripts of *S. caripense* (Table 4). The level of similarity given by score and e-value was also showed. The number of nucleotidic variants between *S. muricatum* and *S. caripense* was also indicated in order to compare differences in both species.

The majority of the genes described in other related species are present in our assembled transcriptome (83.8 %). It should be noted that each of these genes are present too in *S. caripense*. In most cases, there are little differences in these sequences and only 9 are identical among the two species. These results are summarised in File S7 in additional data. It is worth emphasising that the greatest differences between the two species in study were found in genes related to some characters like fruit stripes, anthocyanins and chlorogenic acid synthesis. These differences give a clear idea about the variability and its potential in pepino breeding.

Morphological and fruit composition differences between pepino and wild *S. caripense* are considerable, for example differences related with plant habit, leaf complexity, trailing habit and seediness of the fruits [13, 52]. Here we demonstrate the existence of many differences at genomic level, including genes that are of great interest for breeding. So, we this work will provide the basis for broader studies. Studies where an in-depth and accurate phenotypic characterization can be related to changes at the nucleotide level may be helpful for understanding the genetics of these characters, to find evidence for positive selection in the domestication process, and to how these characters can be used for breeding. For example, in this sense, we have found concentrations of chlorogenic acid (unpublished data), a powerful bioactive molecule with interest in human health as an antioxidant [53, 54], to be much higher in *S. caripense* than in the cultivated *S. muricatum*. Sequence differences found can be used as functional markers for marker assisted breeding in pepino to transfer alleles from the wild *S. caripense*. Furthermore, the

information obtained may be used for tomato or potato improvement in the near future using modern technologies for gene editing like CRISPR/Cas [55], or by transformation using cisgenic approaches [56].

Molecular phylogeny between *Solanaceae* species. We used five genes for a phylogenetic study of pepino and five other Solanaceae crops (potato, tomato, eggplant, capsicum pepper and tobacco). These genes were *waxy* or GBSSI [7, 57], SAMT [58], ADH, β-amylase and CesA [58]. After checking that these genes are represented in our transcriptome, they were concatenated and aligned using ClustalW®. The total length of the sequence analysed was of 9,407 bp including the five genes. Variations were found for a total of 1,809 positions, of which 507 were parsimony-informative, i.e., these sites contain at least two types of nucleotides, and at least two of them occur with a minimum frequency of two. Results of this alignment are presented in additional data File S8.

After alignment, a phylogenetic tree was constructed between the six species using the software MEGA6 [59]. The statistical method used was the maximum likelihood, but results were similar using other methods like, Neighbour Joining, UPGMA and Maximum Parsimony (data not shown). The estimated divergence time of tomato-potato (5.1-7.3 Mya; [60]), was fixed at the intermediate value of 6.2 Mya and was used for time calibration. The constructed tree is represented in Fig. 7 and shows that the ancestors of pepino and of potato and tomato diverged at around 9.26 Mya.

The phylogenetic relationship among the species studied has not always been clear. In this case our results are consistent with previous works such as Spooner *et al.* [6], Wang *et al.* [60] and Garzon-Martinez *et al.* [47] and the divergence times estimated are congruent with data deposited in TimeTree [61], a public knowledge-base of divergence times among organisms, demonstrating the high confidence of this analysis. As shown in Fig. 7, the divergence between all the Solanaceae studied occurred in the last 24 million years during the Miocene epoch. The pepino and the tomato-potato clade shared a common ancestor from which they diverged 9.26 Mya during the Tortonian age in the late Miocene. Other divergence estimates are between eggplant, an African member of the family Solanaceae and the rest of the American *Solanum*, occurred 14 Mya.

SSR and SNV discovery. We performed a general screening on the *S. muricatum* transcriptome for the presence of microsatellites (SSR), analysing its length, type and quality. We focused in the search for di-, tri- and tetra-nucleotide repeats, with a length limited to 20 repetitions. The total of potential SSR yielded was of 1072 in 1049 unigenes; that is, approximately 1.4 % of the transcripts contain SSRs (Table 5). The number of SSRs are slightly lower than expected, or at least lower than obtained in other studies [62, 63]. This may be due to the application of different criteria, but on the other hand, markers obtained should be of better quality. In any case, the number of markers is adequate to develop a high density genetic map, and for genetic diversity and marker assisted breeding studies.

The maximum and minimum lengths of the SSR repeats were 48 and 17 respectively, and the average length was 21 nucleotides. Tri-nucleotide repeats (707) were the most commonly found repetitions in our transcriptome accounting for almost 66% of the SSRs identified. This may occur because tri-nucleotides SSRs do not change the frameshift and mutations had less dramatic effect [64]. The most common motif was AAG (191) representing 17.8% of the tri-nucleotide SSRs; other usual repeats were AG (123) representing 11.5%, and AAC representing the 9.6% of the tri-nucleotide SSRs. Others motifs found in our analysis are summarized in Table 5. All this information with the completed list of SSRs and their characteristics are provided in the additional data File S9.

There is considerable evidence that genic SSRs have important functions [65]. For example, it has been postulated that SSRs may affect the chromatin organization, and also may be related to regulation of gene activity, recombination, and DNA replication, and other functions [66]. Extra-genic SSR markers have several advantages beyond genomic SSRs because they are related to codifying sequences, and thus can be used as candidate genes to study association with phenotypic variation. Also, they can be also useful for genetic diversity studies, as demonstrated for pepino using tomato EST-SSRs [13], for the development of genetic maps and for fingerprinting commercial cultivars, breeding lines or landraces [67].

High throughput sequencing of both transcriptomes has made it possible to obtain a large SNV collection

The variant calling was carried out using the default parameters recommended by the Freebayes software [68], that allows distinguishing

and recognising sequence variations from sequencing errors and mutations introduced during cDNA synthesis. The implementation of several filters described in the Methods has also allowed obtaining markers of potentially high quality, allowing their use in high throughput genotyping platforms [32]. Apart from this, CAPS filter can be especially useful when other methods for SNPs detection are not available.

Applying these criteria, we identified a total of 11,735 SNPs and 766 INDELs in *Solanum muricatum* (SL), and 30,668 SNPs and 1,494 INDELs in *S. caripense* (EC-40), as well as 84,972 SNPs and 4,058 INDELs between both species (interspecific) (Table 6). These values show that both clones present an important degree of heterozygosis, although the highest number of intraclone markers was obtained in *S. caripense*, heterozygosity that makes sense because this wild species is obligated allogamous with a gametophytic self-incompatiblity [18, 69, 70]. Within the detected SNPs, as usual, transitions (62.9 %) were much more abundant than transversions (36.9 %) since transitions are less likely to result in amino acid substitutions, and are therefore more likely to persist as silent substitutions [71]. Within transitions both, A/T and C/T were equally abundant. Such equality remains also for the four transversions types as shown in Table 7. Complete list of these SNPs is provided in additional data File S10.

As a result of this, many SNPs markers were developed that can be readily used in pepino research. These markers exhibit co-dominant inheritance and due to their abundance, they are widely used for different applications, like diversity studies, development of saturated molecular genetic and physical maps, identification of QTL or genes controlling traits of economic importance, marker-assisted selection, or association mapping.

In order to determine their position, heterozygous intra and interspecific SNVs were were located in tomato and potato genome, using the comparison files explained above. This analysis is summed up in Table 8, where we indicate the number of SNVs predicted for every chromosome as well as their hypothetical position and density on chromosomes (using the Circos plot; diagram C in Fig. 6).

Conclusions

This study constitutes the first genomic resource for pepino, a cultigen closely related to tomato and potato [6, 72]. This study is especially important as it may provide a wide array of genomic information that may be useful not only for pepino enhancement but also for tomato and potato breeding, as pepino is part of the tertiary genepool of both crops, and for understanding crop evolution in this group of species. The high quality of the transcriptome presented here, will enhance comparative studies within the genus *Solanum*, and will be useful for future annotations of the *S. muricatum* genome sequence. The detailed annotation provided in this work will facilitate the use of our unigenes for gene discovery, in particular for traits of interest within pepino, (such as soluble solid content, chlorogenic acid content and fruit size). In addition to the pepino, sequencing of the transcriptome of its sister wild relative *S. caripense* has allowed obtaining a large number of molecular markers (SSRs and SNVs), between both species and within them. The filtering process applied in the search of these variants has allowed the selection of the most suitable markers for high throughput genotyping platforms. Our results are another example of how high throughput sequencing technologies can contribute to knowledge on domesticates that have a more limited distribution with closely related species for which the genome sequence is available. Our assembled transcriptome and the large collection of markers found will enhance pepino breeding, facilitate molecular studies in this crop and will be useful to develop the first genetic map of the pepino. Ultimately, the genomic information obtained will be of interest for tomato and potato breeding and for studying genomic changes during evolution and crop domestication in these important crops.

Methods

Plant material. Plant material used consisted of the clonal pepino cultivar Sweet Long (SL) [73] and one clone obtained by vegetative propagation of a single plant of accession EC-40 of *S. caripense*, which was originally collected in Ecuador [52]. Both materials have been maintained in *in vitro* culture at the Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV). SL and EC-40 have contrasting phenotypes for many traits (Fig. 8), some of them interesting for pepino breeding, like fruit size (greater in SL), shape (elongated in SL and round in EC-40) and solid soluble content (SSC) (higher in EC-40)

[13]. Five clonal replicates of each accession were acclimated and grown in a glasshouse in Valencia - Spain (GPS coordinates: lat. 39º29'01" N, long. 0º20'27" W) with quartz sand as substrate and under controlled conditions. From each accession, tissue was sampled from young leaves, flowers in pre-anthesis stage, and mature fruit. All tissues were immediately frozen in liquid nitrogen after being collected and stored at -80ºC until used.

RNA preparation, Illumina paired-end cDNA library construction and sequencing. Total RNA was extracted from each tissue using the TRI reagent (Sigma-Aldrich, St. Louis, USA). RNA integrity was confirmed by agarose electrophoresis and RNA quantification was performed using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Wilmington, USA). We combined equivalent amounts of RNA from each tissue into two pools, one per accession. A total of 10 μg of total RNA for each pool was sent to Macrogen Korea (Seoul, South Korea) for Illumina RNA-seq performed in HiSeq-2000 sequencer (Illumina, San Diego, USA).

The cDNA library was constructed according to the manufacturer's instructions (Illumina/HiSeq-2000 RNA-seq) by Macrogen Korea. Essentially, the mRNA molecules containing poly (A) were purified using Sera-mag Magnetic Oligo (dT) Beads from the RNA samples. A fragmentation buffer was added to break the mRNA into small fragments. Using these fragments as templates, the first strand of cDNA was synthesized. The second strand of cDNA was synthesized using the buffers containing dNTPs, RNase H, and DNA polymerase I. The synthesized cDNA was purified and connected with the sequencing adapters. Finally, a range of cDNA fragments (200±25 bp) were excised from an agarose gel using a gel extraction kit. Then, the library was sequenced using the Illumina/HiSeq-2000 RNA-seq.

These raw sequences are available at the NCBI Sequence Read Archive (SRA) at http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?

DNA sequence processing and *de novo* transcriptome assembly. The pipeline used for the bioinformatics analysis is shown in Fig. 9. After receiving the files with raw data, we used the software FastQC [74] to evaluate the quality of both samples.

In the case of *S. muricatum* Sweet Long we found two sequences overexpressed after initial quality filtering. Blast against databases

(NCBI-GenBank®) showed that these sequences belong to the Pepino mosaic virus (PepMV), although plants were asymptomatic. These reads were eliminated using Bowtie2 [75].

High quality reads are required for better assembling. We performed the following processes: Trimming of adapter contamination, filtering of reads with "N" and trimming of low quality nucleotides Q≥20 using NGS_CRUMS (http://bioinf.comav.upv.es/ngs_crumbs).

We used Trinity software [25] to build the primary assembly. This first assembly was post-processed with the following steps: First, we reduced the redundancy using CAP3 [76]. Then we removed low complex transcripts using DUST score. After that we split some of the subcomponents into new ones making subclusters, using blast and transitivity properties. Finally, we removed low expression transcripts using RSEM (RNA-Seq by Expectation-Maximization) [77]. From the final assembly, we made a subset selecting only the most expressed transcript from each Trinity transcript cluster.

Structural and functional annotation. Annotation of the assembled transcript sequences was performed using the Blastx algorithm [78] against different databases. The order was established prioritizing handmade annotation databases. Accordingly, the databases used were Swiss-Prot [43], ITAG2.4 [44], and UniRef90 [45] in that order. This analysis was released on February 2015. The first analysis compared all transcripts with the first database, the second compared transcripts not paired in the preceding and so on. A typical blast cut-off e-value of $1e^{-20}$ was used.

Additionally, we performed a functional classification of the transcripts following the Gene Ontology (GO) scheme using Blast2GO [79]. This analysis covers three steps as follows, (1) sequence alignment via BLASTX with the NR (Non Redundant) database (cut-off e-value of $1e^{-20}$), (2) gene ontology mapping and (3) functional annotation, including molecular functions, biological processes, and cellular components [79]. In this case, to sum up the functional information of our pepino transcriptome, we performed a plant specific GO slim. Additionally, when possible, Blast2GO gives an Enzyme Commission number (EC number). Meanwhile, KEGG pathways were retrieved from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (version 73.0, January 1, 2015). This KEGG analysis includes a collection of manually

drawn pathway maps representing experimental knowledge on metabolism and various other functions of the cell and the organism.

Comparison with tomato and potato genomes. The whole of the most expressed transcripts were compared to the *S. tuberosum* and *S. lycopersicum* genomes using BLASTN (cut-off value of $1e^{-20}$) in order to obtain the physical position of our assembled sequences. Gene model prediction was performed using the Est2genome software [80] which allows to align EST sequences to genomic DNA sequences with high efficiency. The gene model prediction takes place by sequence homology with the tomato genome. Additionally we used the open reading frame detector ESTScan [50] for annotation of ORFs.

Circos, software that allows visualization of data and information in a circular layout [81], was used to represent our sequences over the tomato and potato reference genomes, which has enabled visual estimation of the distribution of our codifying sequences.

Candidate genes. Taking the pepino transcriptome as a reference database, we evaluated the sequences of several genes associated with breeding characters of interest found in others related species. In total, we selected 12 genes related to fruit shape [51], two related to inflorescence type [82], 11 with the anthocyanins synthesis route [83], 13 related to the synthesis of saponines [84], 4 with the chlorogenic acid synthesis pathway [85], one with sucrose accumulation [86] and one related to fruit stripes [87]. Some of these genes are part of genic families; consequently we evaluated the principal gene and the rest of its family. The total number of sequences evaluated was 115. Description of the genes and their features are shown in Table 4 and in additional data File S6.

Using BLASTN (cut-off of $1e^{-60}$) these genes were compared with the pepino unigenes to determine its presence or absence in our assembled transcriptome. Once defined as part of our transcriptome, they were compared with the transcripts of *S. caripense* in order to recognize nucleotide variants between these two species.

Molecular phylogeny between *Solanaceae* species. Using sequence data available in databases, we chose five nuclear protein-coding genes to investigate phylogenetic relationships within five of the most important Solanaceae crops (potato, tomato, eggplant, pepper and tobacco), in addition to pepino. These genes were, (1) the widely used granule-bound starch synthase gene (*waxy* or GBSSI) [7, 57], (2) the

salicylic acid methyltransferase gene (SAMT) [58], (3) the alcohol dehydrogenase gene (ADH), (4) the β-amylase gene, and (5) the cellulose synthase gene (CesA) [58]. Once isolated, the genes were concatenated one after the other and aligned using ClustalW2, a multiple sequence alignment program [88]. The alignment file generated was used to build a phylogenetic tree using the maximum likelihood distance with 500 bootstrap replications using MEGA6 [59]. Divergence times were estimated with the same program, and the tomato/potato split (5.1–7.3 million years ago) was used for time calibration [60].

SSR and SNV discovery. Mapping reads of *S. caripense* to reference transcriptome of *S. muricatum*. Mining SSRs was carried out using the Sputnik software [89], specially designed for this function. Once the contigs with SSRs were isolated, they were filtered by quality, closeness to introns, number of repetitions and position in the genome of tomato.

SNVs calling (SNPs and INDELS) was performed comparing the assembled transcriptome of *S. muricatum* with the clean reads of both species (*S. muricatum* and its sister wild relative *S. caripense*). We mapped the reads with Bowtie2. For SNV calling we used Freebayes [68]. Several filters, shown in additional data as File S7, were applied in order to maximize the successful validation and its future use in high throughput genotyping platforms. First, filters IV0, IV1 and IV2 were used to select the variants in and between the two species. The filter vks was used to select authentic SNPs on the one hand and INDELS on the other. Other filters were used for optimizing their future use in high throughput genotyping platforms (File S7). Circos [81] was also used for positioning the density (SNV per Mb) and distribution of all these markers over both reference genomes.

Abbreviations

AFLP: Amplified fragment length polymorphism, COS: Conserved ortholog set, CRISPR: Clustered regularly interspaced short palindromic repeats EC: Enzyme commission, EST: Expressed sequence tag, GO: Gene Ontology, KEGG: Kyoto Encyclopedia of Genes and Genomes, Mya: Million years ago, ORF: Open reading frame, QTL: Quantitative trait locus, RAPD: Random amplification of polymorphic DNA, SNP: Single nucleotide polymorphism, SNV: Single nucleotide variant, SSC: Soluble solid content, SSR: Simple sequence repeat.

Authors' contributions

FJH, JB, PZ and SV performed the bioinformatic analyses. JP and SV conceived the study. PG and MP contributed to the data analysis. FJH and MP participated in plant material preparations. PG, GJA and JB contributed to the discussion. FJH, SV and JP drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thankfully acknowledge the computer resources, technical expertise and assistance provided by the PAB (Andalusian Bioinformatics Platform) centre located at the SCBI of the University of Malaga (www.scbi.uma.es). Pietro Gramazio is grateful to the Universitat Politècnica de València for a pre-doctoral (Programa FPI de la UPV-Subprograma 1/2013 call) contract.

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Table 1 Summary of raw and clean reads after processing for *S. muricatum* and *S. caripense*.

	Solanum muricatum	*Solanum caripense*
Total raw reads	58,327,154 x 2	52,646,045 x 2
Total raw reads data size (Gb)	11.78 Gb	10.63 Gb
% G/C	42.0	42.3
Total clean reads	33,963,075 x 2	36,228,181 x 2
Total clean reads data size (Gb)	6.86 Gb	7.32 Gb
% G/C	41.7	42.5

Table 2 Summary of the *Solanum muricatum* transcriptome assembly. After assembly in the first group (Transcripts), and after filtering by level of expression (Most expressed transcripts).

Transcripts	Number	91,949
	Total length	82,353,960
	Average length	895.65
	Maximum length	11,491
Most expressed transcripts	Number	75,832
	Total length	53,411,734
	Average length	704.34
	Maximum length	11,491

Table 3 Functional annotation summary of the pepino sequences over protein databases. First the most expressed transcripts were annotated in Swiss-Prot database. Then, unpaired transcripts in this annotation were evaluated in the next database, ITAG2.4. And finally, the unpaired at this level, were evaluated in the Uniref90 database.

	Number of transcripts	% of total
Annotated in Swiss-Prot	26,688	53.7 %
Annotated in ITAG2.4	14,942	30.1 %
Annotated in UniRef90	2,263	4.6 %
Total annotated in protein databases	43.893	
TOTAL Annotations	49,662	

Table 4 Candidate genes studied affecting traits of importance in different Solanaceae. Traits and genes affecting inflorescence, fruit stripes, fruit shape, anthocyanins route, chlorogenic acid pathway, saponines pathway, and sucrose accumulation are included. More information the Candidate genes section of Material and Methods and in additional data File S7.

Trait	Genes	Features
Inflorescence	Anantha	Gene - F-box protein
	Compound	Transcription factor
Fruit stripes	Fruit Stripes	Transcription factor
Fruit shape	FAS	Intron-regulatory
	Fw2.2	Promoter-regulatory
	Fw3	Promoter-regulatory
	Tonneau	Gene - microtubule
	Wuschel (LC)	SNP in downstream-regulatory
	OVATE	Premature stop
	POS1	Intron-regulatory
	SlCCS52A	Receptor activity
	Sl-IAA17	Gene
	SUN	Transposon insertion-regulatory
	Wee	Gene - Kinase
Anthocyanins pathway	F3'5'H	Gene - Hydroxylase
	Acyltransferase-like	Gene - Acyltransferase
	5GT	Gene - Glucosyltransferase
	ANS	Gene – Anthocyanidin synthase
	DFR	Gene - Dihydroflavonol 4-reductase
	F3H	Gene - Flavanone 3-hydroxylase
	CHI	Gene - Chalcone isomerase
	CHS2	Gene - Chalcone synthase
	CHS3	Gene - Chalcone synthase
	CHS1	Gene - Chalcone synthase
	Acyltransferase-like	Gene - Acyltransferase

Table 4 Cont.

Trait	Genes	Features
Chlorogenic acid pathway	4CL	Gene - 4-Coumarate-CoA ligase
	C3H	Cytochrome P450
	HTC	Gene - Transferase
	HQT	Gene - Transferase
Saponines pathway	Egp#1-1	Gene - Glycosyltransferase
	Egp#1-4	Gene - Glycosyltransferase
	Ptt#1-53	Gene - Glycosyltransferase
	Ptt21	Gene - Glycosyltransferase
	Sgt1-1	Gene - Glycosyltransferase
	Ptt#5-30	Gene - Glycosyltransferase
	Sa#6-15	Gene - Glycosyltransferase
	Sk#7-4	Gene - Glycosyltransferase
	Sk#7-4	Gene - Glycosyltransferase
	Sk#7-5	Gene - Glycosyltransferase
	SaGT4A	Gene - Glycosyltransferase
	SaGT4R	Gene - Glycosyltransferase
	SaGT6	Gene - Glycosyltransferase
Sucrose	TIV1	Gene – Acid invertase

Table 5 Single sequence repeat (SSR) statistics according to the type of motif, the percentage of each motif and the amount of unigenes with SSRs. Complete information about these markers is shown in additional data File S9.

Di-nucleotide motif	Number of Di-SSR	%	Unigenes
AG	123	48.62	
AT	101	39.92	
AC	29	11.46	
Total	253	100	253
Tri-nucleotide motif	Number of Tri-SSR	%	Unigenes
AAG	191	27.02	
AAC	103	14.57	
AAT	101	14.29	
ATC	93	13.15	
ACC	68	9.61	
AGG	56	7.92	
AGC	50	7.07	
ACT, ACG, CCG	45	6.36	
Total	707	100	684
Tetra-nucleotide motif	Number of Tetra-SSR	%	Unigenes
AAAT	28	25.00	
AAAC	17	15.18	
AAAG	16	14.29	
AGAT	10	8.93	
AATC	8	7.14	
Others tetra-nucleotides	33	29.46	
Total	112	100	112

Table 6 Single nucleotide variants statistics for the *S. muricatum* and *S. caripense* transcriptomes.

Species	SNPs	INDELs
Solanum muricatum (SL)	11,735	766
Solanum caripense (EC-40)	30,668	1,494
S. muricatum (SL) vs. *S. caripense* (EC-40)	84,972	4,058

Table 7 Single nucleotide polymorphism (SNPs) statistics. Type and number of transitions and transversions are shown for high quality SNPs identified in each species and between them.

SNPs	Number (%)	SNPs	Number (%)	
Transitions		Transversions		Complex
A<->G	26,684 (31.4)	A<->T	8,514 (10.0)	212
C<->T	26,806 (31.5)	G<->T	8,311 (9.8)	
		C<->G	6,250 (7.3)	
		A<->C	8,288 (9.7)	
Total	53,490 (62.9 %)	Total	31,363 (36.9 %)	212 (0.3 %)

Table 8 Distribution of pepino SNVs on chromosomes of tomato and potato.

	Tomato	Potato
Chromosome 1	2,931	3,031
Chromosome 2	2,529	2,619
Chromosome 3	2,441	2,360
Chromosome 4	1,984	2,151
Chromosome 5	1,548	1,678
Chromosome 6	2,086	2,204
Chromosome 7	1,867	1,845
Chromosome 8	1,628	1,637
Chromosome 9	1,610	1,717
Chromosome 10	1,636	1,656
Chromosome 11	1,530	1,698
Chromosome 12	1,608	1,846
Total	23,501	24,442

Figures

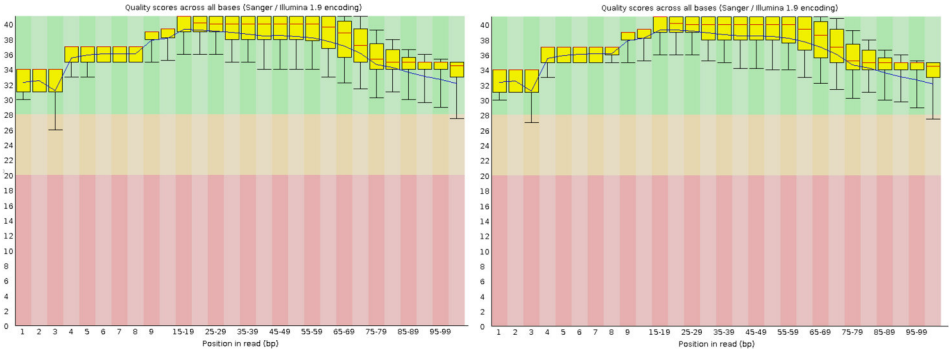

Fig. 1 Boxplots indicating the quality scores across all bases in *S. muricatum* (left) and in *S. caripense* (right). Horizontal axis represents the base position in bp. Vertical axis represents quality score (Q).

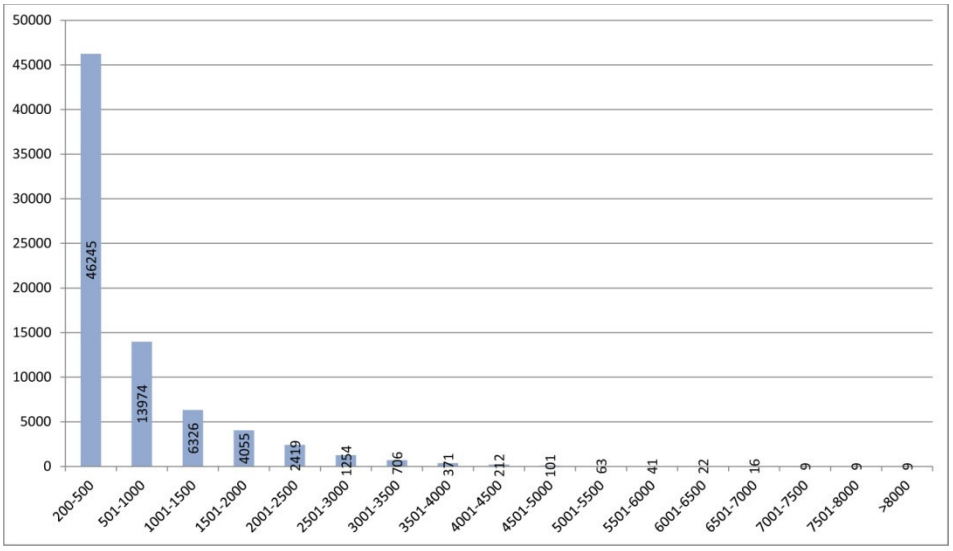

Fig. 2 Length distribution of the pepino (*S. muricatum*) transcriptome most expressed transcripts. Horizontal axis represents the size range of each unigene. Vertical axis represents the number of unigenes for every interval.

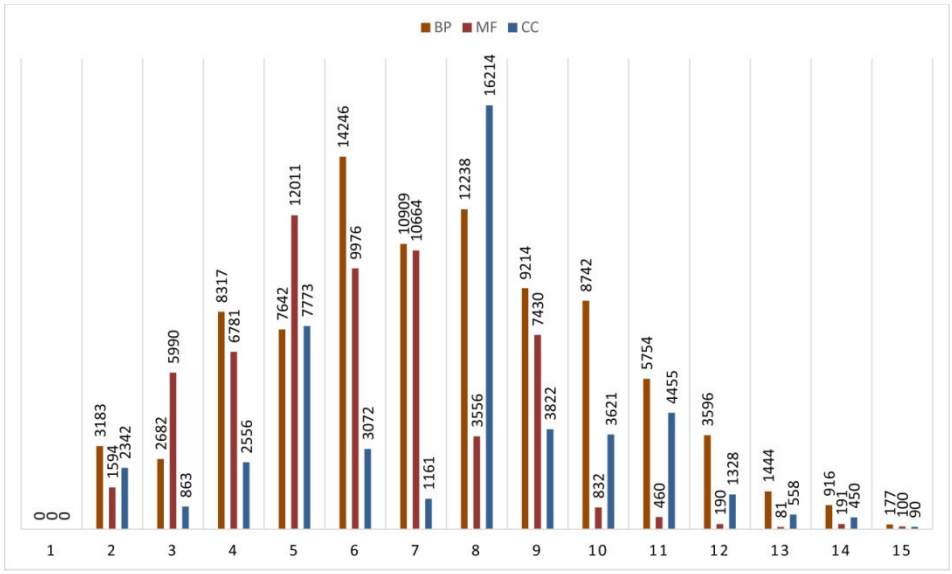

Fig. 3 GO level distribution in each category for the annotated pepino unigenes. X axis represent the GO level and Y axis the number of annotated unigenes. BP = Biological Process, MF = Molecular Function, CC = Cellular Component.

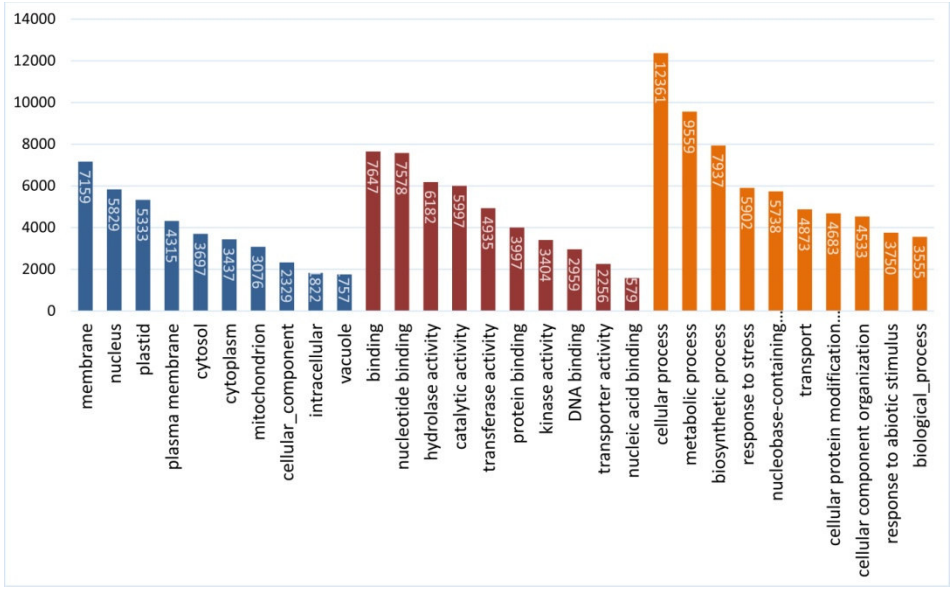

Fig. 4 Gene ontology classification by plant slim term for level 2. The graphic indicates the number of transcripts for every process and functional category, cellular components (left), biological process (middle) and molecular function and (right).

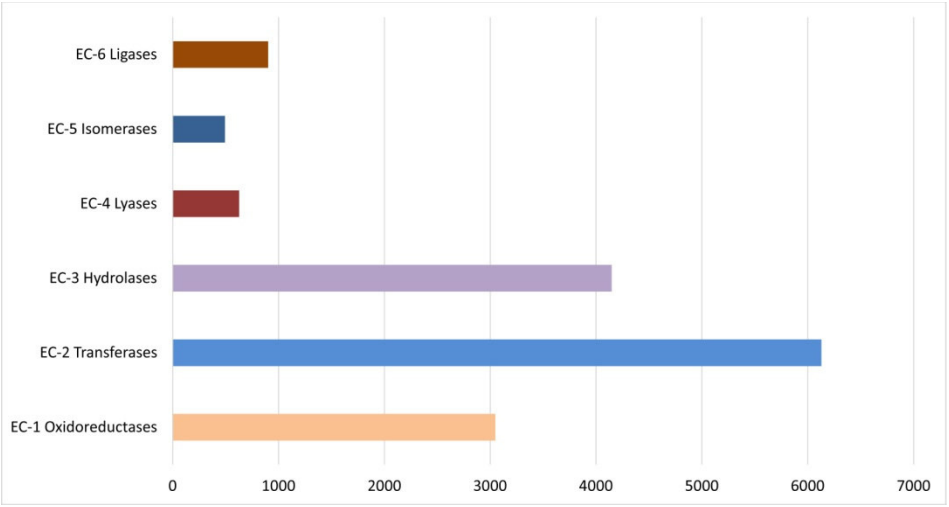

Fig. 5 Number of unigenes for each enzyme commission (EC) category.

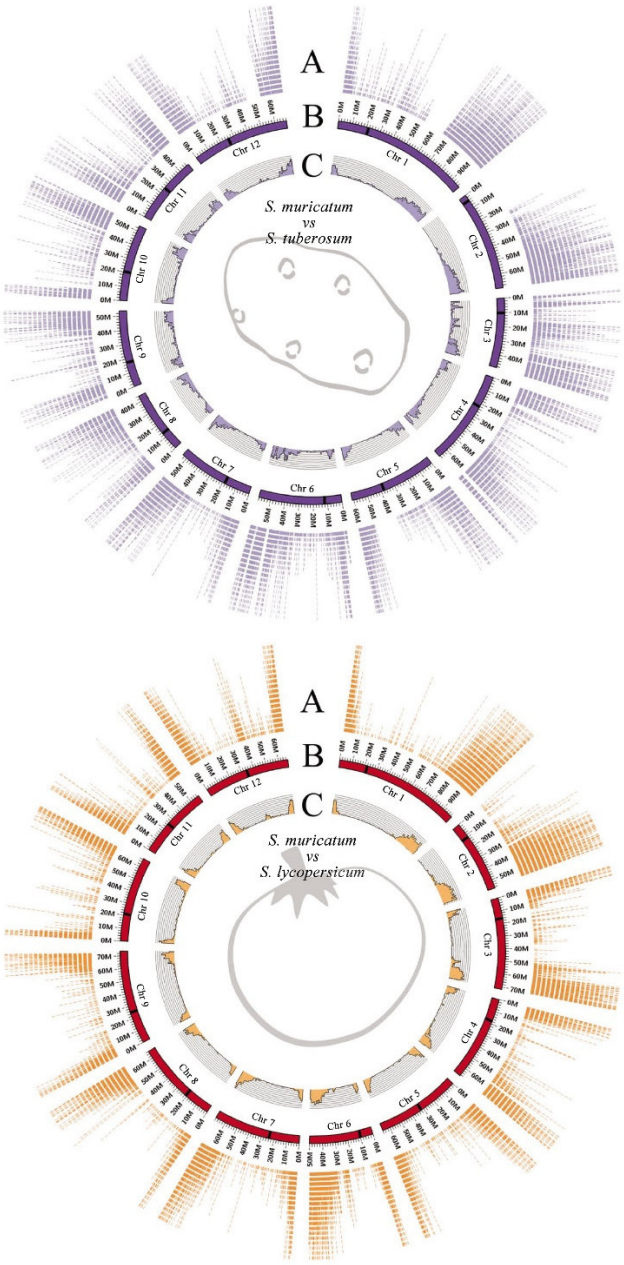

Fig. 6 Graphical representation of the pepino unigenes positions on the chromosomes of potato (top) and tomato (bottom) and SNV distribution/density found on these chromosomes. (A) Distribution of the pepino unigenes on chromosomes, (B) ideograms of the 12 chromosomes, black bar indicates the approximated position of the centromere, (C) SNVs distribution / density, every column indicates the number of SNVs per Mb.

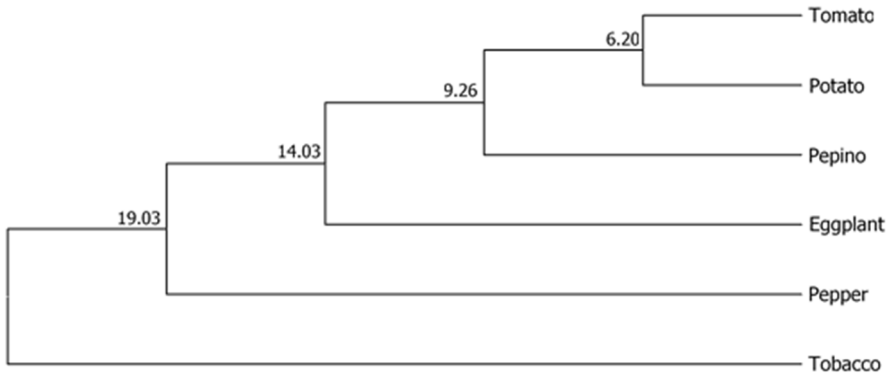

Fig. 7 Phylogenetic relationship among Solanaceae species. The number in the nodes indicates the estimated time of divergence (in millions of years). The length of the branches is proportional to the divergence time. Bootstrap values are not shown as it is 100% in all nodes.

Fig. 8 Fruits of *Solanum muricatum* var. Sweet Long (SL) (left) and of one of the wild relatives, *S. caripense* (EC-40) (right).

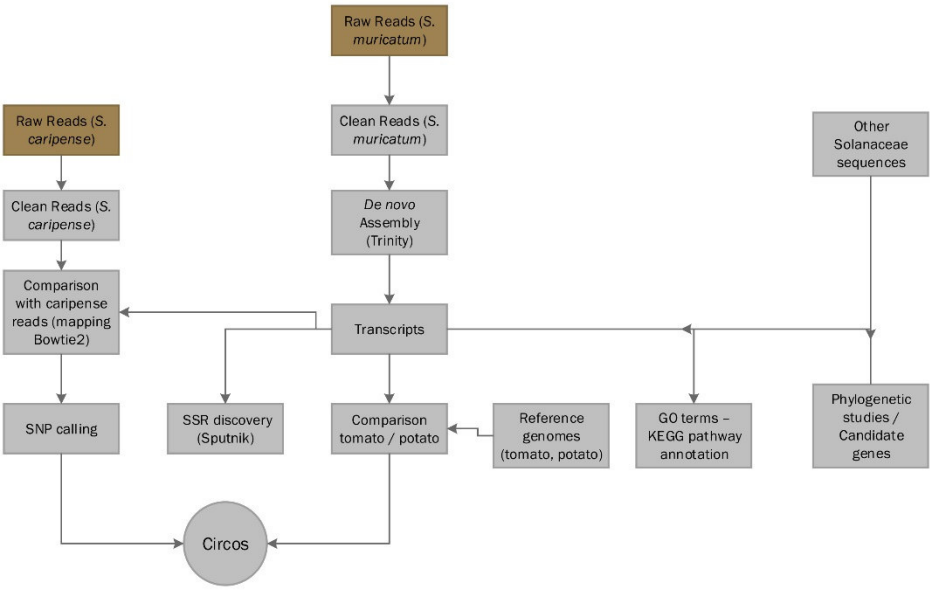

Fig. 9 Schematic representation of the overall sequencing and annotation workflow for the pepino transcriptome.

Supporting information

Additional data - File S1: *Solanum muricatum* assembled transcripts (Fasta format zip comprised). The fasta file provides the sequence of the 91,949 *S. muricatum* transcripts.

Additional data - File S2: *Solanum muricatum* most expressed transcripts or unigenes (Fasta format zip comprised). The fasta file provides the list of the 75,832 most expressed single-copy *S. muricatum* transcripts.

Additional data – File S3: Annotation results in GFF3. All the annotation results (ORFs, introns, descriptions, GO terms, orthologs and markers) provided in the standard format GFF3.

Additional data – File S4: Distribution of GO terms (pdf image). The unigenes distribution regarding the quantity of GO terms to which they are assigned.

Additional data – File S5: GO annotation. GO annotation for the whole *S. muricatum* unigene collection.

Additional data – File S6: KEGG pathway annotation. A zip compressed file with a list of KEGGs pathways, graphics in png format, and a file with a comparison with KEGGs pathways of potato and tomato.

Additional data – File S7: Candidate genes list and features. A Word file with a description of the candidate genes used, the name of the origin sequences, the name of the pepino unigenes and the number of variants in these unigenes between pepino and *S. caripense*.

Additional data – File S8: ClustalW alignment. Text file with multiple alignment from the concatenation of the five genes studied in pepino, tomato, potato, eggplant, pepper and tobacco.

Additional data – File S9: List and features of SSRs. The file provide a list of the SSRs identified in the pepino transcripts, including information about the type and numbers of repetitions, and in which transcript they are present.

Additional data – File S10_1: SNV calling filters, list and features – Part 1. A zip compressed file with the first part of a list of all the SNVs identified in the pepino transcripts, including their positions and the nucleotide changes.

Additional data – File S10_2: SNV calling filters, list and features – Part 2. A zip compressed file with the second part of a list of all the SNVs identified in the pepino transcripts, including their positions and the nucleotide changes.

3.4.- Fruit composition diversity in local and modern pepino (*Solanum muricatum*) varieties and wild related species

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Submitted to Food Chemistry.

ABSTRACT

Pepino (*Solanum muricatum*) is an Andean crop cultivated for its juicy fruits. Despite its potential for diversification of horticultural production, little is known about the chemical composition of pepino and its diversity. We have evaluated the contents in dry matter, protein, β-carotene, chlorophylls and seven minerals in the fruits of 15 pepino varieties and in six accessions of wild relatives. For all traits we found highly significant differences, of several fold in most cases, among accessions in the collection. Average values for the composition traits evaluated were similar to other vegetables having the same uses, like melon and cucumber, but the contents in phenolics were much higher, with values ranging between 50.9 and 123.6 mg/100 g. Wild species had significantly higher average contents for all traits than the cultivated pepino, revealing that they are sources of variation of great interest for pepino quality breeding. Modern varieties of pepino had significantly lower concentrations of protein, P, K, and Zn than local varieties, indicating that a likely nutrient dilution effect during the breeding process, and were less diverse in a multivariate principal components analysis. Most of the significant intra-group correlations among traits were positive, which is of interest for the development of varieties with higher contents in several nutrients and bioactive compounds. The results reveal that one serving (200 g) of selected pepino varieties may make a significant contribution of P, K, Fe and Cu (>6% of the recommended dietary allowances or adequate intake) and also can also represent almost 30% of the average daily intake of phenolics. The information of chemical composition of pepino, including the remarkable high content in phenolics, will be of interest for the enhancement of this neglected crop. Also, the high diversity for composition indicates that there are ample prospects for the development of new pepino varieties with improved fruit contents in nutrient and bioactive compounds.

Keywords: antioxidants, breeding, diversity, minerals, pigments, *Solanum muricatum*, wild species

1. Introduction

Despite the potential of underutilized crops for human nutrition and for the diversification of agriculture (Mayes et al., 2012), very frequently little information is available on the diversity for chemical composition available in neglected crops and in their wild relatives (Toledo & Burlingame, 2006). The evaluation of the content of nutrients and bioactive compounds may contribute to the enhancement of neglected crops, as this may result in the discovery of significant or high levels for certain nutrients or bioactive compounds that can stimulate their demand. Also, knowledge of the diversity in the crop is of interest for selection and for breeding (Wricke & Weber, 1986). In addition, as has occurred in many major crops (Fernie, Tadmor, & Zamir, 2006), the evaluation of closely related species can also allow the identification of sources of variation for its utilization in breeding programmes aimed at improving the nutritional and functional quality of neglected crops.

The pepino (*Solanum muricatum* Aiton) is a little known crop from the Andean region cultivated for its fruits and is phylogenetically closely related to tomato and potato (Spooner, Anderson, & Jansen, 1993). Despite being a major crop during pre-Columbian times, as revealed by the Spanish chroniclers and the multiple ancient pottery representations discovered, it was largely substituted by other Old World crops and became a neglected crop (Prohens, Ruiz, & Nuez, 1996). However, during the recent decades there has been a renewed interest for pepino cultivation, in particular for diversification of horticultural production, both in its region of origin and in other countries from tropical, subtropical and temperate regions (Levy, Kedar, & Levy, 2006; Kola, 2010; Rodríguez-Burruezo, Prohens, & Fita, 2011; Ge, He, Zhang, Wang, & Li, 2014; Muñoz, Peruzé, Balzarini, Bruno, & Salvatierra, 2015). The pepino fruit, which normally weighs between 150 and 300 g and is typically round, ellipsoid or elongated (Herraiz et al., 2015a), has some attractive characteristics for consumers, like yellow skin covered by purple stripes, intense aroma and yellow juicy flesh with a mild sweet taste (Rodríguez-Burruezo, Prohens, & Fita, 2011). The pepino fruit generally is consumed when fully ripe in the same way than melon, i.e., as a refreshing dessert fruit, although less sweet (Prohens et al., 2005). A less common use is when unripe in the same way than cucumber; in fact its name in Spanish is “pepino dulce”, which means “sweet cucumber”, while

the English name "pepino" was directly taken from the Spanish word for cucumber (Prohens, Ruiz, & Nuez, 1996). Regarding its properties, pepino has been attributed some beneficial properties for human-health, like anti-inflammatory, anticarcinogenic, and antidiabetic (Hsu, Guo, Wang, & Yin, 2011; Shathish & Guruvaypoorappan, 2014), which can contribute to increasing its demand (Lähteenmäki, 2008).

The composition of pepino has been barely studied and most studies involve only one or very few varieties (Redgwell & Turner, 1986; Fresquet et al., 2001; Prono-Widayat, Schreiner, Huyskens-Keil, & Lüdders, 2003; Prohens et al., 2005; Huyskens-Keil, Prono-Widayat, Lüdders, & Schreiner, 2006; Kola, 2010; Özcan & Arslan, 2011). These works showed that pepino has a high moisture content (normally above 90%), a soluble solids content usually between 5% and 8%, a low content in sugars and organic acids (commonly below 4% and 0.5%, respectively), and a significant content of vitamin C (generally between 30 and 80 mg/100 g). Studies involving a larger number of varieties mostly focused on proximate composition traits, like soluble solids content or acidity, or vitamin C content (Rodríguez-Burruezo, Prohens, & Nuez, 2002), revealing a large variation within the cultivated species that could be exploited for selection and breeding (Rodríguez-Burruezo, Prohens, & Fita, 2011).

Wild species have been extensively used for introgression breeding in many vegetable crops, including composition traits (Kole, 2011). The pepino and its closest wild relatives form part of *Solanum* section *Basarthrum*, which includes 15 species (Anderson & Jansen, 1998; Anderson, Martine, Prohens, & Nuez, 2006). Within this section, pepino is the only member of series *Muricata* and hybridizes easily with several species of series *Caripensia* (Anderson, 1979; Prohens, Anderson, Rodríguez-Burruezo, & Nuez, 2003; Rodríguez-Burruezo, Prohens, & Fita, 2011). In particular, hybrids with *S. caripense* Humb. & Bonpl. ex Dun. and *S. tabanoense* Correll are highly fertile and it is possible to obtain backcrosses to pepino (Prohens, Anderson, Rodríguez-Burruezo, & Nuez, 2003; Rodríguez-Burruezo, Prohens, & Fita, 2011). Few works have been done on the composition, other than proximate composition traits, of these wild genetic resources for pepino breeding. Both species present a higher content in soluble solids, acidity and vitamin C than cultivated pepino (Prohens, Anderson, Rodríguez-Burruezo, & Nuez, 2003; Prohens et al., 2005; Rodríguez-Burruezo, Prohens, & Fita, 2011) and have been

used in backcross breeding programmes of pepino aimed at improving the fruit quality (Rodríguez-Burruezo, Prohens, & Fita, 2011). Also, another wild species (*S. trachycarpum* Bitter & Sodiro), may be of special interest for pepino breeding as it grows in dry areas (Anderson, Martine, Prohens, & Nuez, 2006), which may be associated to higher dry matter and concentration of nutrients in the fruit. However, the potential of wild species for pepino breeding for increased content in most nutrients and bioactive compounds is largely unknown and no studies exist on their content in protein, phenolics, pigments, and minerals.

Selection and breeding programmes in pepino have mostly been performed in countries outside of the Andean region, where most of the diversity exists (Blanca et al., 2007; Herraiz et al., 2015a), and have mostly concentrated on yield, taste and adaptation to intensive production systems (Prohens & Nuez, 1999; Prohens, Anderson, Rodríguez-Burruezo, & Nuez, 2003; Levy, Kedar, & Levy, 2006; Rodríguez-Burruezo, Prohens, & Fita, 2011). As a result of these breeding programmes several modern varieties have been obtained that are mostly adapted to the new cultivation conditions and environments in which pepino has been introduced (Rodríguez-Burruezo, Prohens, & Fita, 2011; Herraiz et al., 2015a). In a previous study we demonstrated that modern breeding resulted in a reduction in the genetic diversity in the modern varieties and also in significant morphological changes compared to local varieties from the Andean region (Herraiz et al., 2015a). In other fruits and vegetables, modern breeding has been linked to a decrease in the concentration of nutrients (Davis, 2009). In the case of pepino, no information exists on the effect of breeding and selection on chemical composition traits that were not the target of the selection programmes. We consider that it is important to evaluate the impact of modern breeding on nutrients and bioactive compounds of pepino, as this may have important implications for the consumers as well as for establishing new breeding objectives (Rodríguez-Burruezo, Prohens, & Fita, 2011).

In this work, we study the content in dry matter, protein, total phenolics, β-carotene, chlorophylls and minerals in a collection of pepino, including local and modern varieties, as well as in related species. We evaluate the diversity, differences among groups, relationships among accessions and traits, and the contribution to the diet resulting from pepino consumption. The information obtained will be of interest for the

enhancement of pepino and also for the selection and development of new pepino varieties with improved composition.

2. Material and Methods

2.1. Plant material and cultivation conditions

Twenty-one accessions, corresponding to 15 varieties of pepino and six accessions of wild relatives were used for the present study (Table 1). Pepino materials included seven local varieties from the Andean region and eight modern varieties obtained through selection and breeding programmes in different countries. Wild relatives consisted in four accessions of the widespread *S. caripense* and one accession of *S. tabanoense* and *S. trachycarpum* (Table 1). Previous morphological and molecular characterization of most of these materials revealed that they encompass a wide genetic diversity (Blanca et al., 2007; Herraiz et al., 2015a).

For each accession, five plants were clonally micropropagated (Cavusoglu & Sululoglu, 2013) and cultivated in order to obtain fruits. Plants were cultivated in a greenhouse with hydroponic facilities (quartz sand benches) in Valencia (Spain) in order to avoid experimental error arising from variation in plant nutrition and differences in the amount of water available to individual plants. Plants were distributed according to a completely randomized design and spaced 1.7 m between rows and 0.4 m apart in the row. A drip irrigation system using pressure compensating emitters were used for providing the nutrient solution, which had the following final concentration of the main anions and cations (resulting from the ions present in the irrigation water plus those added with the soluble fertilizers): 11.47 mM NO_3^-, 1.00 mM NH_4^+, 1.50 mM $H_2PO_4^-$, 6.75 mM K^+, 3.25 mM Ca^{2+}, 2.50 mM Mg^{2+} and 2.82 mM SO_4^{2-}. Microminerals were supplied by adding the following salts to the irrigation water: 50 μM H_3BO_3, 10 μM FeEDTA, 4.5 μM $MnCl_2$, 3.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$ and 0.1 μM $(NH_4)_6Mo_7O_{24}$. In order to stimulate fruit set, flowers were mechanically vibrated, and for self-incompatible wild species *S. caripense* and *S. tabanoense* manual pollinations were performed using pollen from other genotypes in order to obtain fruits.

2.2. Preparation of samples

Five samples (replications) per accession were taken, with each replication corresponding to fruits of one of the five plants included in the experiment. Fruits were collected when fully ripe. This stage is determined by the fruit having reached the final size and displaying the typical pepino yellow background colour covered by purple/brownish stripes and releasing an intense aroma (Herraiz et al., 2015b). After harvesting, fruits were brought to the laboratory, where they were washed, peeled and cut into longitudinal slices. The fruit slices were weighted and frozen in N_2 and stored at -80ºC until lyophilised. Freeze-dried tissue corresponding to the fruits of each individual plant was bulked and powdered to form each of the samples.

2.3. Analytical methods

Dry matter was determined using the fruit samples weight before and after lyophilisation using the formula 100 x (dry weight/fresh weight). Protein content was calculated as N x 6.25 from the N content values determined with the Kjeldahl method. Total phenolics (g/100 g) were determined according to the Folin-Ciocalteu procedure (Singleton & Rossi, 1965) after extraction with acetone (70% v/v) and acetic acid (0.5% v/v). Absorbance was measured after at 750 nm and caffeic acid (Sigma-Aldrich Chemie) was used as a standard. For β-carotene determination, samples were extracted with ethanol:hexane (4:3 v/v) in darkness. After separation of the hexane phase, β-carotene contents were determined by measuring absorbance at 450 nm. Chlorophylls *a* and *b* and total chlorophyll were measured spectrometrically after extraction with acetone (80% v/v) according to Wellburn (1994).

For the analysis of minerals, 2 g of the liophylised samples were calcined in a furnace at 450ºC for 2 h. Subsequently they were weighted and dissolved in 2 mL of HCl. The mixture was heated until vapors appeared, after which immediately several mL of distilled water were added. After filtration, the extract volume was brought to 100 mL with distilled water. The following methodologies were used for the different minerals: P was determined by spectrometry using the molibdovanadate method, K by flame photometry, and Ca, Mg, Fe, Cu and Zn by atomic absorption spectrophotometry. All results of composition determinations are reported on a 100 g fresh weight basis.

2.4. Data analysis

Data for each composition trait were analyzed using a one-way factorial analysis of variance (ANOVA) and least significant difference (LSD) values were calculated. The average and standard error (SE) were calculated from accession means for pepino local varieties, pepino modern varieties and wild relatives accessions as well as for the whole collection. Significance of pairwise differences among averages for pepino local varieties, pepino modern varieties and wild relatives were calculated with *t*-tests. Given that differences among averages of the three groups of accessions for the traits measured could result in overestimated results for the correlations between traits, pairwise correlations were calculated based on within-group residuals of accession means (i.e., intra-group correlation). Principal components analysis (PCA) for all accessions and for pepino accessions only were performed for standardized composition trait using Euclidean distances among accessions. The contribution (in percentage) of one serving (200 g) of pepino to the daily Recommended Dietary Allowances (RDA) for protein, vitamin A, and all minerals except K and the Adequate Intake (AI) for K were calculated according to the values for adult males and females of RDA and AI provided by Food and Nutrition Board (2011).

3. Results

3.1. Composition

A great diversity, with highly significant differences ($P<0.0001$) among the set of accessions studied, was observed for all composition traits studied (Tables 2 and 3). Differences of several-fold, with a minimum of 3.3-fold for dry matter content and a maximum of 111.3-fold for chlorophyll *a* were observed in the collection. When the comparison is restricted to cultivated pepino, these differences are of a minimum of 1.5-fold for dry matter content and a maximum of 15.5-fold for chlorophyll *a*. For all traits, the wild species presented higher average values than the cultivated species with average values significantly higher than those of pepino local or modern varieties (Tables 2 and 3). In fact, except for β-carotene, chlorophyll *b* and Fe contents, there is no overlap between values observed in cultivated and wild species (Tables 2 and 3).

Dry matter content ranged between 5.95 and 8.08 g/100 g in pepino and between 10.50 and 17.28 g/100 g in the wild species, with the

highest value corresponding to the single *S. trachycarpum* accession (E-34) (Table 2). No significant differences were observed for average values between local and modern varieties of pepino. For protein content the values for the cultivated pepino varied between 0.365 and 0.652 g/100 g in pepino and 1.247 and 2.027 g/100 g in the wild species (Table 2). Local pepino varieties presented significantly higher contents than modern varieties, with the former having an average content 6.3% higher than the latter. In fact, all modern varieties had protein contents below 0.5 g/100 g, while five out of the seven local varieties presented protein contents above this value (Table 2).

Total phenolics ranged between 50.9 and 123.6 mg/100g in pepino and between 175.4 and 287.6 mg/100 g in the wild species. β-carotene values were much lower with values between 48.8 and 166.1 μg/100 g in pepino and 159.2 and 641.8 μg/100 in the wild species. Chlorophyll *a* content was generally higher than that of chlorophyll *b* in all accessions, with an average ratio of 1.72. The ranges of variation were large in pepino, with total chlorophyll content between 0.112 and 1.234 mg/100 g. This wide range was due to an odd accession (RP-1) with very low contents in chloropylls. In this respect, this pepino accession had total chlorophyll content 2.5-fold lower than that of the pepino accession ranking second for lowest chlorophyll values. For wild species, the range of total chlorophyll content ranged from 1.374 to 6.888 mg/100 g. No significant differences were observed between local and modern varieties for any of the antioxidants and pigments evaluated (Table 2).

Among the macrominerals, K presented the highest concentration, with an average value of 180.6 mg/100 g in the set of accessions (Table 3). P was the second mineral with highest content values, with an average content of 22.01 mg/100 g, followed by Ca and Mg, with average values of 7.01 and 4.98 mg/100 g, respectively. For microminerals, the highest average concentration was for Fe (0.262 mg/100 g), followed by Cu (0.262 mg/100 g) and Zn (0.172 mg/100 g). As occurred for the rest of traits average values of wild species for all minerals were much higher than those of the cultivated species, with differences ranging from 2.15-fold for Mg to 4.50 for Zn. For all minerals, important differences were observed in the set of accessions and also among pepino or wild accessions (Table 3). For example, for K the range in cultivated pepino was between 49.9 and 176.9 mg/100 g, while in the wild species, the range varied between 212.2 and 432.1 mg/100 g. For Cu, the relative variation was very large in

the cultivated pepino with contents varying from 0.004 to 0.047 mg/100 g, although in absolute values it was larger in the wild species, in which it ranged between 0.053 and 0.131 mg/100 g. Although the ranges of variation between local and modern pepino varieties overlapped for all minerals, on average the local varieties presented significantly higher contents in P (33.9%), K (52.3%) and Zn (61.2%) than modern varieties (Table 3).

3.2. Correlations among traits

A total of 41 pairwise correlations, calculated from the within-group residuals of accessions means, were significant (Table 4). Dry matter and protein were positively correlated and both of them presented positive correlations with β-carotene, as well as with minerals P, Mg and Zn. Dry matter was also positively correlated with total phenolics, while protein content with K. Total phenolics presented positive correlations with minerals Ca, Fe and Zn (Table 4). The pigments β-carotene, chlorophylls *a* and *b*, and total chlorophylls were positively intercorrelated, and all of them were also correlated with K and Mg contents. β-carotene was positively correlated with P, while the chlorophylls were negatively correlated with Cu. Regarding correlations among minerals, P was positively correlated with Mg and Zn, K was positively correlated with Mg and negatively with Fe and Cu, and Mg presented a positive correlation with Zn. Finally, the three microminerals (Fe, Cu and Zn) were positively intercorrelated.

3.3. Principal components analysis

The first component of the PCA with all accessions accounted for 84.0% of the variation and had an eigenvalue of 11.76, while the second component barely accounted for 9.2% of the variation, with an eigenvalue of 1.28 (Table 5). All the composition traits were positively correlated with the first component, with values between 0.230 (for Cu) and 0.286 (for Protein). The second PCA component presented high positive (>0.2) correlations with β-carotene, the three chlorophyll measures, and K, and high negative (<-0.2) correlations with Ca, and especially Fe and Cu (Table 5). The PCA plot with all accessions shows that the first component clearly separates the wild species, with highly positive values for the first component from the pepino accessions, which had negative values for this first component. The second component does not separate the different groups, although most pepino and wild accessions present positive values.

The PCA plot with all accessions also reveals that wild species have a greater dispersion than pepino in both the first and second components.

The first and second components of an additional PCA which included only pepino varieties accounted for 37.4% and 24.6% of the total variation, respectively. Eigenvalues for the first and second components were 5.23 and 3.44 respectively. The first component was positively correlated with all traits, except with Ca, Fe and Cu, which presented small absolute values (<0.08 in all cases). The second component presented very high positive correlations with Ca (0.508), Fe (0.514) and Cu (0.498), while it presented a highly negative correlation with K (-0.302). The PCA graph revealed that local varieties of pepino presented a greater dispersion than modern varieties in both the first and second components. All modern varieties presented negative values for the first component, while local varieties presented a wide range of values, with one variety presenting highly positive values (OV-8), three varieties intermediate positive values (CH2-22, Col-1 and OT-1), two with values close to 0 (37-A and PT-154) and one with highly negative values (RP-1). When considering the second component, the local varieties were separated in three groups, with 37-A having highly positive values, PT-154 and RP-1 moderate negative values and the rest of accessions presenting values close to 0. The modern varieties were separated in two clusters, one with positive values for the second component (El Camino, Puzol and Valencia) and another with negative values, which included the rest of varieties.

3.4. Contribution to RDA/IA

The comparison of the nutrient values contained in one serving (200 g) of pepino and the nutrients included in the daily Recommended Dietary Allowances (RDAs) and Adequate Intake (AI) by the Food and Nutrition Board (2011) is presented in Table 6. The results reveal that on average one serving of pepino makes a low contribution (<3%) to the protein, vitamin A, Ca, Mg, and Zn RDAs for both male and female adults and to the Fe RDA for females (Table 6). However, for P, K and Cu for adults of both sexes and Fe for adult males, one serving of pepino provides a moderate contribution (3-6%) to the RDA (and AI for K). When considering the best pepino accession for each of the nutrients, the contribution is low (<3%) for protein, Ca and Mg for both sexes and Zn for males, moderate (3-6%) for vitamin A, Fe and Zn for adult females,

and considerable (>6%) for P, K and Cu for both sexes and for Fe for adult males (Table 6).

4. Discussion

Selection and breeding for pepino varieties with improved content in nutrients and bioactive compounds is an important objective for the enhancement of this neglected crop (Rodríguez-Burruezo, Prohens, & Fita, 2011). This is the first work in which a large diversity of cultivated pepino and some of its closest wild relatives has been examined for traits such as protein content, β-carotene, total phenolics, and content in minerals. Therefore, it represents an important contribution to the identification of sources of variation for selection and breeding programmes.

The ranges of values obtained by us are in agreement with previous studies in which the composition of one or a few varieties of pepino has been studied (Redgwell & Turner, 1986; Fresquet et al., 2001; Prono-Widayat, Schreiner, Huyskens-Keil, & Lüdders, 2003; Prohens et al., 2005; Huyskens-Keil, Prono-Widayat, Lüdders, & Schreiner, 2006; Kola, 2010; Özcan & Arslan, 2011), confirming that pepino has a high content of water and a low protein content. Compared to other fleshy fruits with uses similar to those of pepino, like melon (as a fresh fruit) or cucumber (for using salads) (Prohens, Ruiz, & Nuez, 1996), it has similar contents of dry matter, protein, and minerals (Ekholm et al., 2007; Maynard & Hochmuth, 2007; Maietti et al., 2012). However, the content of total phenolics is much higher than that of both melon and cucumber (Fu et al., 2011; Ji et al., 2011; Maietti et al., 2012). Compared to other solanaceous berries, it presents a somewhat higher phenolics content than tomato (Luthria, Mukhopadhyay, & Krizek, 2006) or eggplant (Prohens, Rodríguez-Burruezo, Raigón, & Nuez, 2007), suggesting that it has a high antioxidant capacity (Chun et al., 2005). The content of β-carotene it is also similar to that of honeydew melons, although much lower than that of the cantaloupe type (Laur & Tian, 2011), and higher than that of standard commercial types of cucumber (Cuevas, Song, Staub, & Simon, 2010). Regarding the content in chlorophylls, it is similar to that of non-green fleshed melons (Reid, Lee, Pratt, & Chichester, 1970) and lower than that of cucumber (Chen & Yang, 2012). These comparisons suggest that for the traits we have evaluated, the pepino presents an overall

composition similar to that of melon and cucumber, although with a much higher content in phenolics. This specific difference in phenolics concentration may be of relevance for the promotion and enhancement of pepino, as there is an increasing demand for vegetables with higher contents in bioactive compounds (Lähteenmäki, 2008; Diamanti, Battino, & Mezzetti, 2011).

The results reveal that there is a great diversity for pepino composition, matching the results obtained for morphological traits and molecular markers (Blanca et al., 2007; Herraiz et al., 2015a; Muñoz, Pertuzé, Balzarini, Bruno, & Salvatierra, 2015), indicating that there are ample opportunities for selection and breeding. Considerable differences were found between the composition of cultivated pepino and its wild relatives. Compared to the cultivated pepino, wild species presented a higher dry matter content, as well as higher concentrations for the rest of traits studied, than the cultivated pepino. Other studies have found that wild relatives *S. caripense* and *S. tabanoense* present higher concentrations in dry matter than the cultivated species (Prohens et al., 2005). Amazingly, although the dry matter content of wild species was higher than that of the cultivated pepino, it only accounted partially for the larger values observed in the rest of nutrients for the wild species. In this respect, the ratio between the average content in dry matter between wild relatives and cultivated pepino was lower (in some cases much lower) than that for the rest of traits. For example, while on average the dry matter content was less than two-fold higher in the wild species compared to the cultivated pepino, for β-carotene, chlorophylls, and Zn was greater than four-fold higher. This suggests that the pepino wild relatives that we have evaluated, which are cross-compatible with pepino (Anderson, 1979; Prohens, Anderson, Rodríguez-Burruezo, & Nuez, 2003; Rodríguez-Burruezo, Prohens, & Fita, 2011), may represent a very useful source of variation for pepino breeding. In particular, *S. trachycarpum* presented a dry matter content almost three-fold higher than that of pepino. This species is from dry areas (Anderson, Martine, Prohens, & Nuez, 2006) and probably has acquired the capacity to accumulate higher contents of nutrients in the fruit than other species, even when grown under the same conditions in which the supply of water is not a limiting factor. This makes *S. trachycarpum* an interesting genetic resource for pepino breeding, not only because it can help increasing the pepino fruit quality, but also for adaptation to drought.

Within the cultivated pepino there have been also many differences among accessions for the composition traits. Large variations for composition traits within the cultivated species have also been observed for other crops of the same family, like tomato, eggplant, or tree tomato (Raigón, Prohens, Muñoz-Falcón, & Nuez, 2008; Acosta-Quezada et al., 2015; Figàs et al., 2015). In pepino, these differences have always been greater for composition traits than for dry matter content, indicating that considerable genetic differences exist in the capacity to accumulate certain nutrients or bioactive compounds and therefore clonal selection can be successfully applied (Rodríguez-Burruezo, Prohens, & Fita, 2011). This suggests that some pepino clones, like OV-8, which ranks first for total phenolics, β-carotene, chlorophylls, P and Zn and presents high or intermediate values for the rest of traits, would be a good candidate for selection of a clone with high content in nutrients and bioactive compounds. In some cases, the differences have been of more than 10-fold within the cultivated species, like for chlorophyll *a* and total chlorophylls and Cu. For all traits a continuous range of variation has been found, but in the case of chlorophyll content one accession (RP-1) presented very low contents of both chlorophyll *a* and *b*, suggesting that it may be a mutant for deficit of chlorophyll content in the fruit flesh. In other crops, like cucumber or melon, mutants for low chlorophyll content have been described (Cuevas, Song, Staub, & Simon, 2010; Dogimont, 2011), and in the case of pepino this could be of interest for selecting varieties with flesh with higher yellow chroma and luminosity.

There is evidence that modern breeding has resulted in a reduced concentration of nutrients in modern varieties of fruits and vegetables as a result of the so-called "dilution effect" attributable to the higher yields of modern varieties (Davis, 2009). In our case we have found a significantly lower content in protein, P, K and Zn in the modern pepino varieties compared to the local varieties, which may be a consequence of the selection for high yield of the modern pepino varieties (Rodríguez-Burruezo, Prohens, & Fita, 2011). In a previous study, we also found that modern breeding resulted in the selection of clones presenting considerable morphological differences with local varieties (Herraiz et al., 2015a), probably resulting from selection for adaptation to new environments (Rodríguez-Burruezo, Prohens, & Fita, 2011). The multivariate analyses we have performed also confirm that, as occurred for morphological traits and molecular data (Herraiz et al., 2015a), modern pepino varieties have a lower diversity for composition profile

than local varieties. In consequence, we suggest that present and future breeding programmes should also take into consideration the nutrient composition in addition to yield and organoleptic quality, as has been done in other crops (Diamanti, Battino, & Mezzetti, 2011)

Results obtained for within-group correlations are of interest, as they may result from a pleiotropic effect and in consequence may result in indirect selection of one trait when a correlated trait is selected for (Wricke & Weber, 1986). In our case, most of the significant correlations observed have been positive, which may is advantageous for selecting for increased concentration of nutrients and bioactive compounds in pepino. For example, selection for high content of phenolics, which is increasingly an important breeding objective in fruits and vegetables (Kaushik et al., 2015), may result in indirect selection for increased β-carotene, P, Mg and Zn contents, which is desirable in order to improve the nutrient composition of pepino fruits. Also, an expected positive correlation was between chlorophylls and Mg, as chlorophyll molecules contain a Mg ion. Regarding the most important negative correlations observed, Cu is known to induce chlorophyll loss (Ouzounidou, 1996), which may account for the negative correlation of Cu with chlorophyll content may result from the know effect of Cu on chlorophyll loss.

Discovering or highlighting outstanding composition properties is of great relevance for the enhancement of new crops, as consumers increasingly value this information (Botonaki, Polymeros, Tsakiridou, & Mattas, 2006). The comparison of the composition values obtained with the RDA/AI (Food and Nutrient Board, 2011) for the different nutrients studied shows that pepino is a good source of P, K, Fe and Cu, which could be exploited for promoting this crop. In particular, one serving of some selected accessions could provide more than 10% of the RDA of Cu for both sexes or Fe for males. For total phenolics, there is no RDA/AI; however, the consumption in different European populations has been estimated at around 800-900 mg/day on average (Ovaskainen et al., 2008; Tresserra-Rimbau et al., 2013). On average, one serving of 200 g of pepino could represent around 20% of the daily consumption of total phenolics, which is a considerable contribution. Furthermore, if the varieties with higher content are used, this percentage could increase to almost 30%. Given the proven benefits on human health of dietary phenolics (Del Rio et al., 2013), this clearly indicates that the high content in phenolics of pepino may make it an attractive fruit for health-concerned

consumers (Lähteenmäki, 2008). The high content in phenolics may also be related to the healthy properties attributed to pepino (Hsu, Guo, Wang, & Yin, 2011; Shathish & Guruvaypoorappan, 2014).

5. Conclusions

Our results provide information on the composition and diversity of pepino fruit for dry matter, total phenolics, β-carotene, chlorophylls and minerals. Overall the results showed that pepino is a highly diverse crop for fruit composition, indicating that there is a high potential for selection and breeding. Also, wild related species represent interesting sources of variation for pepino breeding, as they presented much higher values than those present in the cultivated species. The fact that modern varieties of pepino presented less diversity for fruit composition and lower contents in protein, P, K, and Zn than local varieties suggests that modern breeding programmes should take into account the content in nutrients in order to develop new varieties better adapted to the demand for vegetables with increased contents in nutrients and bioactive compounds. Finally, the high content in phenolics of the pepino may be exploited for its promotion as a healthy fruit. All this information may help in the enhancement of the pepino crop.

Acknowledgements

Pietro Gramazio is grateful to Universitat Politècnica de Valencia for a pre-doctoral (Programa FPI de la UPV-Subprograma 1/2013 call) contract.

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Table 1
Plant materials, origin and fruit characteristics of the pepino varieties used.

Accession	Code	Species	Country of origin / breeding	Fruit length (cm)	Fruit width (cm)	Fruit length/width ratio
Local pepino varieties						
37-A	37	*S. muricatum*	Ecuador	7.3±0.2	4.1±0.1	1.79±0.05
Col-1	Co	*S. muricatum*	Colombia	7.3±0.2	7.9±0.2	0.93±0.03
CH2-22	CH	*S. muricatum*	Chile	7.8±0.9	7.7±1.0	1.03±0.04
OT-1	OT	*S. muricatum*	Ecuador	6.0±0.3	5.5±0.3	1.09±0.02
OV-8	OV	*S. muricatum*	Chile	5.7±0.5	5.6±0.1	1.03±0.09
PT-154	PT	*S. muricatum*	Peru	7.9±0.6	11.1±1.2	0.72±0.03
RP-1	RP	*S. muricatum*	Ecuador	5.6±0.4	8.1±0.4	0.71±0.07
Modern pepino varieties						
El Camino	EC	*S. muricatum*	New Zealand	7.6±0.7	6.0±0.7	1.27±0.04
Kawi	Ka	*S. muricatum*	New Zealand	14.5±0.9	9.4±0.6	1.55±0.03
Puzol	Pu	*S. muricatum*	Spain	10.9±0.4	6.8±0.3	1.60±0.08
Quito	Qu	*S. muricatum*	UK	6.6±0.4	6.1±0.4	1.08±0.01
Sweet Long	SL	*S. muricatum*	Spain	10.8±0.5	5.7±0.3	1.89±0.08
Sweet Round	SR	*S. muricatum*	Spain	7.2±0.4	8.3±0.3	0.87±0.05
Turia	Tu	*S. muricatum*	Spain	15.5±0.7	7.4±0.1	2.08±0.08
Valencia	Va	*S. muricatum*	Spain	11.6±1.2	5.3±0.6	2.21±0.20
Wild relatives						
BIRM/S 1034	c1	*S. caripense*	Ecuador	2.8±0.1	2.7±0.1	1.01±0.01
E-7	c2	*S. caripense*	Ecuador	3.5±0.1	3.6±0.1	0.97±0.02
EC-40	c3	*S. caripense*	Ecuador	2.8±0.2	2.8±0.1	1.01±0.03
QL-013	c4	*S. caripense*	Ecuador	3.2±0.1	2.9±0.1	1.10±0.02
E-257	ta	*S. tabanoense*	Ecuador	4.6±0.1	3.7±0.1	1.26±0.03
E-34	tr	*S. trachycarpum*	Ecuador	2.5±0.1	2.1±0.1	1.18±0.03

Values are expressed as mean ± S.E. of 5 independent samples for each variety.

Table 2

Mean values for dry matter, protein content, total phenolics, β-carotene, and chlorophylls on a fresh weight basis of 15 accessions of local and modern pepino varieties and six accessions of wild relatives. Average±SE for the three groups of accessions (local, modern, wild) and for the global mean, as well as values of the *F*-test for differences among accessions and least significant differences (LSD, P=0.05) are also provided.

Accession	Dry matter (g/100 g)	Protein (g/100 g)	Total phenolics (mg/100 g)	β-carotene (µg/100 g)	Chlorophyll *a* (mg/100 g)	Chlorophyll *b* (mg/100 g)	Total chlorophyll (mg/100 g)
Local pepino varieties							
37-A	5.95	0.565	107.2	82.6	0.448	0.218	0.665
Col-1	7.61	0.589	78.6	93.3	0.469	0.297	0.765
CH2-22	8.08	0.565	73.1	133.0	0.332	0.143	0.474
OT-1	7.00	0.652	103.3	107.3	0.385	0.256	0.641
OV-8	7.17	0.649	123.6	166.1	0.619	0.616	1.234
PT-154	6.22	0.448	87.5	113.3	0.226	0.221	0.447
RP-1	6.03	0.399	70.3	55.9	0.040	0.072	0.112
Average	6.87±0.31	0.552±0.036	91.9±7.5	107.4±13.5	0.360±0.070	0.2603±0.066	0.620±0.130
Modern pepino varieties							
El Camino	7.30	0.497	96.7	91.2	0.338	0.283	0.620
Kawi	5.26	0.401	49.4	40.0	0.429	0.385	0.813
Puzol	6.91	0.386	82.1	109.7	0.402	0.269	0.670
Quito	6.14	0.411	72.4	75.3	0.394	0.515	0.909
Sweet Long	6.35	0.365	114.2	90.6	0.318	0.224	0.542
Sweet Round	7.30	0.489	84.9	118.9	0.470	0.393	0.863
Turia	5.93	0.423	50.9	103.2	0.481	0.359	0.840
Valencia	6.51	0.399	89.9	48.8	0.180	0.105	0.285
Average	6.46±0.25	0.421±0.017	80.1±7.8	84.7±9.99	0.377±0.035	0.317±0.044	0.693±0.074
Wild relatives							
BIRM/S 1034	12.09	1.511	199.3	399.0	3.087	1.588	4.674
E-7	12.45	1.379	287.6	159.2	0.865	0.510	1.374
EC-40	10.50	1.247	215.9	483.6	4.193	2.696	6.888
QL-013	12.63	1.786	205.3	554.7	2.852	0.913	3.765
E-257	11.12	1.607	175.4	479.0	3.422	1.087	4.508
E-34	17.28	2.027	284.5	641.8	4.447	3.070	7.515
Average	12.68±0.98	1.593±0.115	228.0±19.2	452.9±67.6	3.144±0.522	1.644±0.419	4.787±0.906

Table 2 cont.

Accession	Dry matter (g/100 g)	Protein (g/100 g)	Total phenolics (mg/100 g)	β-carotene (µg/100 g)	Chlorophyll *a* (mg/100 g)	Chlorophyll *b* (mg/100 g)	Total chlorophyll (mg/100 g)
Global mean	8.37	0.800	126.3	197.4	1.162	0.677	1.838
Prob. *F*-test	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
LSD (P=0.05)	1.13	0.190	29.6	92.5	0.408	0.118	0.690
Prob. values for *t*-test for averages comparison							
Local vs. modern	0.3239	0.0049	0.2975	0.1926	0.8270	0.4775	0.6216
Local vs. wild	<0.0001	<0.0001	<0.0001	0.0002	0.0001	0.0047	0.0004
Modern vs. wild	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0032	0.0002

Table 3

Mean values for minerals content on a fresh weight basis of 15 accessions of local and modern pepino varieties and six accessions of wild relatives. Average±SE for the three groups of accessions (local, modern, wild) and for the global mean, as well as values of the *F*-test for differences among accessions and least significant differences (LSD, P=0.05) are also provided.

Accession	P (mg/100 g)	K (mg/100 g)	Ca (mg/100 g)	Mg (mg/100 g)	Fe (mg/100 g)	Cu (mg/100 g)	Zn (mg/100 g)
Local pepino varieties							
37-A	13.84	96.1	8.94	3.26	0.431	0.047	0.117
Col-1	14.06	167.2	4.26	4.65	0.173	0.023	0.118
CH2-22	15.52	176.9	4.27	4.40	0.182	0.027	0.123
OT-1	20.25	166.7	4.85	4.71	0.133	0.021	0.114
OV-8	25.54	164.6	3.75	3.79	0.188	0.016	0.142
PT-154	14.93	138.1	3.40	3.71	0.157	0.007	0.083
RP-1	10.66	127.7	2.74	3.60	0.124	0.004	0.057
Average	16.40±1.87	148.2±10.9	4.60±0.77	4.02±0.21	0.198±0.04	0.021±0.005	0.108±0.011
Modern pepino varieties							
El Camino	15.38	67.6	8.62	3.78	0.301	0.036	0.078
Kawi	13.04	131.4	5.04	3.65	0.143	0.022	0.081
Puzol	12.34	49.9	8.12	3.49	0.292	0.033	0.075
Quito	12.19	119.1	3.20	3.46	0.119	0.008	0.072
Sweet Long	9.79	115.1	5.04	3.19	0.123	0.007	0.059
Sweet Round	9.00	115.1	3.05	2.83	0.101	0.011	0.050
Turia	12.34	119.3	4.46	4.11	0.112	0.018	0.061
Valencia	13.88	60.9	7.37	3.52	0.249	0.043	0.063
Average	12.25±0.73	97.3±11.4	5.61±0.77	3.50±0.14	0.180±0.03	0.022±0.005	0.067±0.004
Wild relatives							
BIRM/S 1034	41.20	320.8	9.94	6.90	0.365	0.084	0.387
E-7	36.58	212.2	13.53	6.76	0.587	0.123	0.411
EC-40	36.01	354.7	12.91	7.86	0.303	0.059	0.229
QL-013	46.55	336.2	9.24	7.41	0.531	0.131	0.475
E-257	40.84	320.9	9.39	7.76	0.371	0.054	0.296
E-34	48.17	432.1	15.13	11.70	0.528	0.065	0.532
Average	41.56±2.04	329.5±28.9	11.69±1.02	8.07±0.75	0.447±0.047	0.086±0.014	0.388±0.046

Table 3 cont.

Accession	P (mg/100 g)	K (mg/100 g)	Ca (mg/100 g)	Mg (mg/100 g)	Fe (mg/100 g)	Cu (mg/100 g)	Zn (mg/100 g)
Global mean	22.01	180.6	7.01	4.98	0.262	0.040	0.172
Prob. *F*-test	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
LSD (P=0.05)	4.58	34.8	1.85	0.91	0.095	0.027	0.041
Prob. values for *t*-test for averages comparison							
Local vs. modern	0.0480	0.0069	0.3701	0.0567	0.7155	0.8240	0.0025
Local vs. wild	<0.0001	<0.0001	0.0001	0.0002	0.0019	0.0006	<0.0001
Modern vs. wild	<0.0001	<0.0001	0.0004	<0.0001	0.0003	0.0004	<0.0001

Table 4

Pairwise Pearson linear correlations based on within-group residuals of accession means (n=21) for the composition traits studied.

	Protein	Total phenolics	β-carotene	Chlorophyll a	Chloropyll b	Total chlorophyll	P	K	Ca	Mg	Fe	Cu	Zn
Dry matter	0.781***	0.549***	0.448*	0.239ns	0.389ns	0.318ns	0.503*	0.425ns	0.330ns	0.749***	0.323ns	0.051ns	0.772***
Protein		0.233ns	0.658**	0.376ns	0.267ns	0.340 ns	0.767***	0.532*	0.108ns	0.672***	0.305ns	0.086ns	0.797***
Total phenolics			-0.120ns	-0.196ns	0.176ns	-0.030ns	0.219ns	-0.137ns	0.594**	0.253ns	0.506*	0.238ns	0.471*
β-carotene				0.858***	0.636***	0.788***	0.558**	0.782***	-0.052ns	0.614**	-0.139ns	-0.362ns	0.252ns
Chlorophyll a					0.852***	0.970***	0.318ns	0.793***	0.067ns	0.605***	-0.315ns	-0.606**	-0.070ns
Chlorophyll b						0.954***	0.246ns	0.733***	0.266ns	0.684***	-0.246ns	-0.574**	0.035ns
Total chlorophyll							0.297ns	0.796***	0.162ns	0.665***	-0.295ns	-0.615***	-0.024ns
P								0.403ns	0.087ns	0.474*	0.219ns	0.146ns	0.647**
K									-0.242ns	0.704***	-0.486*	-0.567**	0.214ns
Ca										0.336ns	0.723***	0.330ns	0.226ns
Mg											0.034ns	-0.311ns	0.468*
Fe												0.749***	0.547*
Cu													0.463*

ns, *, **, *** indicate non-significant, or significant at P=0.05, 0.01 and 0.001, respectively.

Table 5

Correlation coefficients between fruit composition traits and the two first principal components of a PCA analysis for all the accessions (pepino and wild relatives) and for pepino accessions only.

Composition trait	All accessions (pepino and wild)		Cultivated pepino only	
	First component	Second component	First component	Second component
Dry matter	0.281	-0.042	0.240	0.086
Protein	0.286	-0.033	0.387	0.104
Total phenolics	0.271	-0.178	0.182	0.227
β-carotene	0.274	0.205	0.341	-0.031
Chlorophyll a	0.272	0.275	0.316	0.019
Chlorophyll b	0.250	0.373	0.249	-0.161
Total chlorophyll	0.268	0.316	0.297	-0.077
P	0.282	-0.038	0.356	0.104
K	0.272	0.238	0.276	-0.302
Ca	0.250	-0.230	-0.077	0.508
Mg	0.281	0.117	0.220	-0.034
Fe	0.237	-0.471	-0.003	0.514
Cu	0.230	-0.485	0.005	0.498
Zn	0.278	-0.163	0.369	0.155
Eigenvalue	11.76	1.28	5.23	3.44
Variance explained (%)	84.0	9.2	37.4	24.6

Table 6

Contribution to the daily Recommended dietary allowances (RDAs) for protein, vitamin A and all minerals studied except K, and Adequate intake (AI) for K (Food and Nutrition Board, 2011) from a serving size (200 g) of pepino considering the average for all accessions as well as the pepino accession with highest value for each nutrient. For vitamin A we have considered that it is synthesized exclusively from β-carotene. Data are based only on cultivated pepino.

	Daily RDA/AI		Contribution of one serving (200 g) to daily RDA/AI (%)			
			Pepino average		Pepino accession with highest value	
Nutrient	Males[a]	Females[a]	Males	Females	Males	Females
Protein (g)	56	46	1.7	2.1	2.3	2.8
Vitamin A[b] (μg)	10800	8400	1.8	2.3	3.1	4.0
P (mg)	700	700	4.1	4.1	7.3	7.3
K (mg)	4700	4700	5.2	5.2	7.5	7.5
Ca (mg)	1000	1000	1.0	1.0	1.8	1.8
Mg (mg)	420	320	1.8	2.3	2.2	2.9
Fe (mg)	8	18	4.7	2.1	10.1	4.8
Cu (mg)	0.9	0.9	4.8	4.8	10.5	10.5
Zn (mg)	11	8	1.6	2.2	2.6	3.5

[a]Values corresponding to adult males and females in the range of 19-50 y. For Mg the value indicated corresponds to the range 31-50 y, which is slightly higher than that for adults of 19-50 y.

[b]Expressed as β-carotene equivalents.

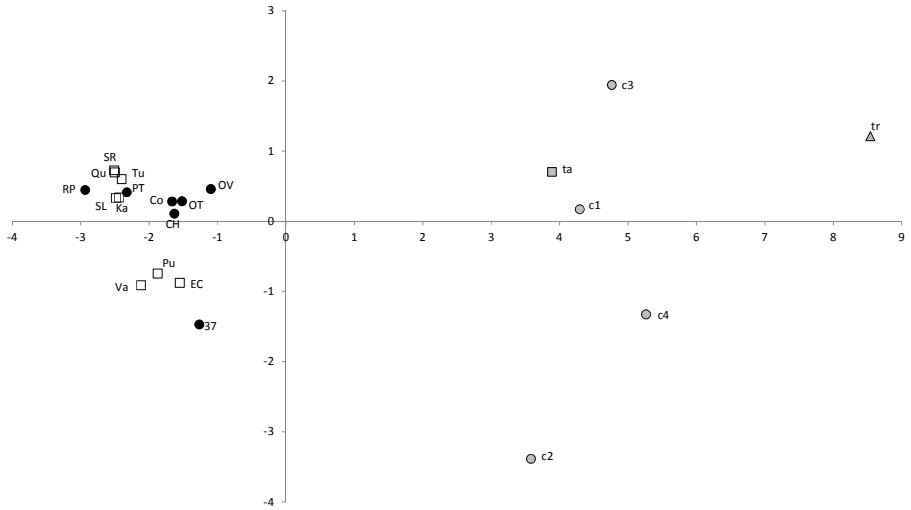

Fig. 1

Principal components analysis scatterplot against the first (X-axis) and second (Y-axis) principal components of 21 pepino and wild relatives accessions based on 14 fruit composition traits. First and second components account, respectively, for 84.0% and 9.2% of the total variation. The different groups of accessions are represented by different symbols: pepino local accessions (solid circle), pepino modern accessions (open square), and wild *S. caripense* (grey circle), *S. tabanoense* (grey square) and *S. trachycarpum* (grey triangle). See Table 1 for the codes of individual accessions.

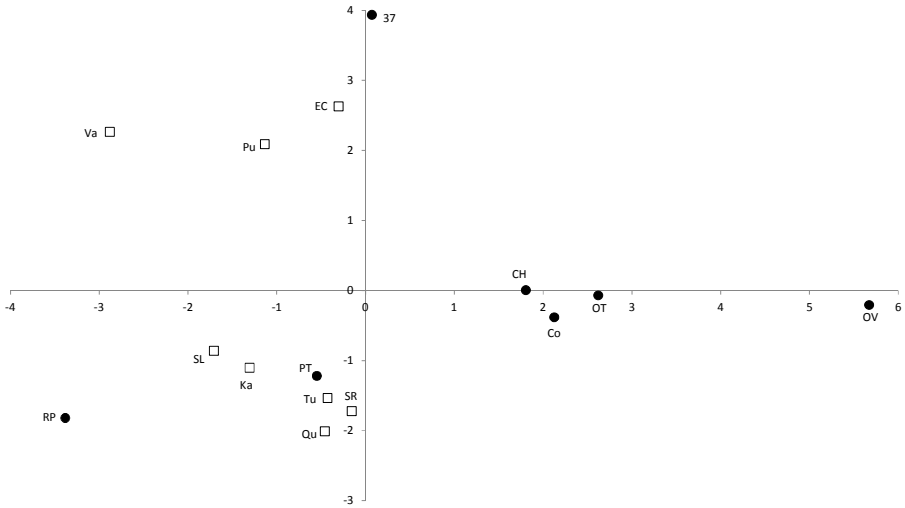

Fig 2

Principal components analysis scatterplot against the first (X-axis) and second (Y-axis) principal components of 15 pepino accessions based on 14 fruit composition traits. First and second components account, respectively, for 37.4% and 24.6% of the total variation. The different groups of accessions are represented by different symbols: local accessions (solid circle), modern accessions (open square). See Table 1 for the codes of individual accessions.

3.5.- Phenolic profile and biological activities of the fruit of pepino (*Solanum muricatum*) and its wild relative *S. caripense*

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Abbreviations

ORAC: Oxygen Radical Absorbance Capacity; TRC: Total Reducing Capacity; Amu: Atomic Mass Unit.

Submitted to Journal of Agricultural and Food Chemistry.

Abstract

Pepino (*Solanum muricatum*) is an edible and juicy fruit native to the Andean region for which little information is available on its phenolic composition and bioactive properties. Four pepino varieties (37-A, El Camino, Puzol and Valencia) and one accession (E-7) of its close wild relative *S. caripense* were characterized by HPLC-DAD-MSn/ESI, and 24 hydroxycinnamic acid derivatives were detected (5 to 16 compounds per variety and accession). The major phenolics in the varieties were chlorogenic acids and derivatives, while in *S. caripense* a caffeoyl-synapoyl-quinic acid was the major compound. The in vitro antioxidant capacity (DPPH and ORAC tests) was higher in *S. caripense*. Pepino and *S. caripense* extracts did not show any toxicity on macrophage cells and the raw extracts inhibited NO production of LPS-stimulated macrophages by 36% (El Camino) to 67% (37-A). No single variety ranked high simultaneously for hydroxycinnamic acids content, and biological activity. We suggest the screening of large collections of germplasm or the use of complementary crosses between Puzol (high for hydroxycinnamic acids and biological activity) and *S. caripense* E-7 to select and breed pepino varieties with enhanced properties.

Keywords: antioxidants, biological activity, HPLC-DAD-MSn/ESI, macrophages,

1. Introduction

Pepino (*Solanum muricatum* Aiton), also known as pepino dulce, is an herbaceous crop native to the Andean region cultivated for its edible, mild-sweet and juicy fruits, which may be very variable in fruit size, shape and colour (Herraiz et al. 2015). Fruits of most commercially important varieties generally weigh between 80 and 250 g, and are round to elongate in shape, with a yellow skin with purple longitudinal stripes. During the last decades there has been a growing interest for pepino cultivation both in the Andean region as well as in several other countries, as pepino is considered as a crop with potential for diversification of horticultural production (Rodríguez-Burruezo, Prohens, & Fita, 2011).

Apart from its attractive morphology, aroma and flavour, the fruit of pepino presents antioxidant, antidiabetic, anti-inflammatory and antitumoral activities (Hsu, Guo, Wang, & Yin, 2011; Shathish & Guruvayoorappan, 2014; Sudha, Sangeetha Priya, Shree, Babu, & Vadivukkarasi, 2012). In this respect, an important feature for the

enhancement and increase of the demand of exotic fruit crops like pepino is having knowledge on composition in biologically active constituents and the discovery of properties that may be of interest for human health. Although it is known that pepino contains significant amounts of vitamin C (Rodríguez-Burruezo et al., 2011), for phenolic compounds, which have a main role in the bioactive properties of other *Solanum* fruits (Kaushik et al., 2015), there are very few studies in pepino (Di Scala et al., 2011; Hsu et al., 2011; Wu, Meyer, Whitaker, Litt, & Kennelly, 2013). In this respect, it has been found that the content in phenolics in pepino fruit is much higher than that of vitamin C (Di Scala et al., 2011; Hsu et al., 2011), indicating that they may have an important role in its bioactive properties. Regarding the phenolics profile, Hsu et al. (2011) using HPLC separation detected five phenolic acids and four flavonoids, while Wu et al. (2013) used LC-TOF-MS methods to study the phenolic profiles of several *Solanum* species, including pepino, and were able to detect eight hydroxycinnamic acid derivatives and one flavonoid in the pepino fruit. All these studies used only one variety and therefore little information exists on the diversity of pepino phenolics.

Up to now, most of the breeding efforts in pepino have been devoted to improving yield, resistance to diseases, and fruit flavor and aroma (Rodríguez-Burruezo et al., 2011). However, up to now no comprehensive studies exist on the diversity for phenolics compounds and their concentration in the pepino fruit, which is of major relevance for breeding for increased content in phenolics. Also, breeding for other associated fruit quality properties, like antioxidant activity and biological activity, as well as studying their relationship with the content in phenolics would be of great relevance for the enhancement of this crop. However, again, no information is available on the diversity for these traits, as all studies are based on a single variety (Hsu et al., 2011; Shathish & Guruvayoorappan, 2014; Sudha et al., 2012).

In this work, we determine the phenolic profile and content of pepino fruits using HPLC-DAD-MSn/ESI, and study the antioxidant and biological (anti-inflammatory) activities of a set of pepino varieties representative of the diversity of this crop. We also include one *S. caripense* accession, which is a close wild relative of pepino (Blanca et al., 2007) that has been used for pepino breeding (Rodríguez-Burruezo et al., 2011). The information obtained will provide relevant information on the phenolic profile and composition of pepino fruits and will be of great interest for the selection, breeding and enhancement of this crop.

2. Material and Methods

2.1. Plant Material

Four accessions of pepino and one of *S. caripense* previously characterized at the morphological and molecular levels (Herraiz et al. 2015) were used for the present study. Pepino accessions were selected as representative of the diversity of pepino, while the *S. caripense* accession was included as representative of a wild relative of interest for pepino breeding (Rodríguez-Burruezo et al., 2011). Main characteristics of these accessions can be consulted in Table 1 and a picture of them is shown in Figure 1. Five clonal replicates of each accession were transplanted to a glasshouse at Valencia-Spain in January 2014 and were cultivated using the standard techniques for pepino cultivation in Mediterranean climates (Nuez & Ruiz, 1996). Manual pollinations were performed on self-incompatible *S. caripense* plants (Rodríguez-Burruezo et al., 2011) using pollen from another *S. caripense* accession in order to obtain fruit set. Further details on growing conditions can be consulted elsewhere (Herraiz et al. 2015).

2.2. Sample preparation

Five fruit samples, each of which consisted of at least three fruits from one of the five clonal plants of each accession, were used for the analyses. Fruits were harvested when ripe and cut in slices, frozen in liquid N2, and stored at -80ºC until lyophilized. Powdered tissue of the different fruits harvested of each plant was bulked and thoroughly mixed to form a sample.

2.3. Phenolic composition

Subsamples of the lyophilized fruit (100 mg) were extracted with 1.5 mL of methanol:water:formic acid (70:29:1, v:v:v), vortexed and sonicated in an ultrasonic bath for 60 min. The samples were kept at 4 °C overnight and sonicated again for 60 min. A centrifugation was performed for 10 min at 10000 rpm and the supernatant was filtered through a 22 µm PVDF filter before HPLC-DAD-MSn/ESI analysis.

Chromatographic analyses were carried out on a Kinetex column (5 µm, C18, 100 A, 150 x 4.6 mm; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents: 1 % acetic acid in water (A) and acetonitrile (B), starting with 1% B followed by 15 % B in 15 min, 30 % B at 30 min, maintained in 30 % B at 40 min, changing to 95 % B at 45 min, maintained in 95 % B at 50 min, decreasing to the initial conditions of 1 % B at 55 min and 1 % B at 60 min. The flow rate was 800 µL min-1, and the

injection volume 5 μL. The HPLC-DAD-MSn/ESI analyses were carried out as in Sánchez-Rodríguez et al (Sánchez-Rodríguez, Ruiz, Ferreres, & Moreno, 2012).

The identification of the peaks was obtained analyzing both the UV-vis spectra, as well as the extracted-ion chromatograms of the ion current at m/z values corresponding to the [M-H]- ions of the compounds and their fragmentation. Quantification of the identified analytes was performed using the external standard method with calibration graphs at the wavelength corresponding to their maximum absorbance (320 nm for hydroxycinnamic acids and 360 nm for flavonoids).

2.4. Antioxidant activity

Antioxidant activity was measured using three different methods: 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging capacity, oxygen radical absorbance capacity (ORAC), adapted to microscale according to Mena et al (2011), and total reducing capacity (TRC) using the Folin-Ciocalteu reagent.

The DPPH assay was performed with 96-well microplates (Nunc) in an Infinite M200 Tecan microplate reader. The reaction starts by adding 2 μL of the diluted sample to the well containing the stock solution (250 μL). ORAC assay was performed according to Ou, Hampsch-Woodill, & Prior (2001). Standard curves of the antioxidant Trolox were used to express both ORAC and DPPH results, as mM Trolox / 100 g dry weight.

Total reducing capacity (TRC) was determined according to the Folin-Ciocalteu procedure as indicated in Plazas et al. (2014). Caffeic acid (Sigma-Aldrich Chemie) was used as a standard and total reducing capacity was expressed as caffeic acid equivalents in g/kg of dry weight.

2.5. Anti-inflammatory activity

Subsamples of 500 mg of lyophilized fruit were homogenized in 4 mL of methanol and extracted in an ultrasonic bath during 30 minutes. Extracted samples were centrifuged at 2000 rpm for 5 minutes, and the supernatant was collected and filtered through 0.2-μm sterile PTFE filters. Extract dilutions of 1:10 in sterile phosphate buffered saline (PBS) were prepared for each of the samples.

The murine macrophage cell line RAW 264.7 (ECACC, Salisbury, UK) was used for the in vitro biological activity experiments using the methodology indicated in Plazas et al. (2014). Basically, the effect of each extract (raw extract or 1:1 and 1:10) on cell viability was evaluated with the

3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl-tetrazolium bromide (MTT) assay and the anti-inflammatory activity of the extracts was evaluated through the inhibition of the production of the free radical NO in stimulated murine macrophages.

2.6. Data analysis

A Euclidean distance matrix based on absence (0)/presence (1) of the phenolic compounds detected by HPLC-DAD-MSn/ESI was computed for clustering analysis by using the UPGMA (unweighted pair group method with aritmethic mean) method. Average values and standard errors were calculated for each accession for the quantitative data obtained. For viability and NO inhibition tests, significance of the differences compared to the control were evaluated with a Dunnett's t-test. Varieties were ranked for their total contents in phenolics, antioxidant and biological activity traits.

3. Results

3.1. Phenolic composition

Based on retention times, UV spectra, [M-H]- and mass fragmentation and comparison with available data in the literature (Clifford, Johnston, Knight, & Kuhnert, 2003; Clifford, Knight, & Kuhnert, 2005; Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010; Sánchez-Rodríguez et al., 2012), a total of 24 phenolic compounds were identified in the five accessions of *S. muricatum* and *S. caripense* (Table 2). All the compounds detected corresponded to hydroxycinnamic acid derivatives (Table 2). The chromatogram at 320 nm shows a high abundance in most varieties of peak 10, corresponding to 5-caffeoyl-quinic acid (Figure 2). Peak 11 shows, like peak 10, a deprotonated molecular ion m/z 353, but the MS fragmentation pattern reveals that it corresponds to 4-caffeoyl-quinic acid30. Peaks 2 and 14 were identified as other caffeoyl-quinic acids, by means of MS2 of their deprotonated molecular ion (m/z 353), giving a base peak at m/z 191. The compound 2 also gave a relative intense ion at m/z 179, however in the compound 14 this ion is undetectable. According to Clifford et al. (2003) they can be labeled as the 3-caffeoyl-quinic acid and 5-caffeoylquinic acid isomer, respectively. Di-caffeoyl-quinic acids were detected at retention times of 15 min (peak 1), 18 min (peak 5), 20 min (peak 19) and 33 min (peak 22), with deprotonated molecular ion m/z 515 (Sánchez-Rodríguez et al., 2012). Peaks 3, 4, 6 and 9 were identified as caffeoyl-hexosides (m/z

341), with similar fragmentation patterns. Peak 8 corresponded to caffeoyl-di-hexoside (m/z 503), and peak 18 (m/z 441) caffeoyl-hexoside derivatives because their MS fragmentation gave a m/z 341 ion (deprotonated caffeoyl-hexosides). The compound 23 (m/z 433) should be a caffeoyl derivative because of its MS fragmentation at m/z 179 (deprotonated caffeic acid) and 135 (179-44). Feruloyl-hexosides (compounds 7, 13 and 17, m/z 355), feruloyl-di-hexosides (15 and 21, m/z 517) and p-coumaroyl-di-hexoside (peak 12, m/z 487) were also identified. We also detected sinapoyl derivatives, with m/z 547 (peak 16, sinapoyl-di-hexoside), and peak 20 (m/z 577), its MS fragmentation gave the m/z 415 ([(M-H)-162]-, loss of the caffeoyl-radical), 353 (deprotonated caffeoyl quinic acid, [(M-H)-224]-) as well as the 224 (neutral sinapic acid) and 191 (deprotonated quinic acid). The m/z 559 (peak 24) is a caffeoyl-sinapoyl-quinic acid with a MS fragmentation including a loss of 162 amu (caffeoyl-radical) to give m/z 397, the deprotonated sinapic acid (m/z 223) and the ion at m/z 173 ([quinic acid-H-18]-). Out of the 24 identified compounds, 3-caffeoyl-quinic acid, caffeoyl-hexose IV, 5-caffeoylquinic acid and 4-caffeoyl-quinic acid were present in all the varieties. Some compounds were specific of variety: compounds 14, 16 and 20 were specific to El Camino, compounds 17 and 18 to Valencia, compounds 1 and 4 to *S. caripense* E-7, and compound 19 to Puzol, while no compounds were specific to 37-A (Table 2). The dendrogam obtained based on presence/absence of the 24 phenolic compounds reveals two major groups: one constituted by the wild *S. caripense* E-7 and the primitive pepino accession 37-A, while the other includes the three modern pepino cultivars (El Camino, Puzol and Valencia) (Figure 3).

Thirteen out of the 24 compounds were present in sufficient quantities to be quantified. (Table 3) whilst in some cases, the concentrations were lower than the limit of quantification. Total content of hydroxycinnamic acids ranged between 1.11 mg/g (37-A) to 2.35 mg/g (Valencia). In pepino accessions two dicaffeoylquinic acids, namely the isomers 3-caffeoyl-quinic acid (accession 37-A) and 5-caffeoyl-quinic acid (accessions El Camino, Puzol and Valencia) were the major compounds. For *S. caripense* E-7 the major hydroxycinnamic acid derivative was a caffeoyl-synapoyl-quinic acid (Table 3), which was also the second most abundant compound in 37-A. For the rest of pepino varieties, the second major compound was feruloyl-dihexose (El Camino), feruloyl-hexose (Puzol) and p-coumaroyl-di-hexose (Valencia).

3.2. Antioxidant activity

Significant differences have been found among the five accessions studied, with a range of variation of 3.3, 1.6 and 1.9-fold for the ORAC, DPPH and TRC assays, respectively (Table 4). ORAC values have been always higher (on average 4.7-fold) than those of DPPH, despite being measured in the same units (µmol Trolox /g). The highest values for the three methods have been obtained for *S. caripense* accession E-7. ORAC values of E-7 have been more than two-fold greater than the pepino accession 37-A, which presented values 1.6-fold higher than Valencia (Table 4). For DPPH free radical scavenging capacity, the differences between *S. caripense* E-7 and the pepino accessions has been much lower, and in fact pepino variety Puzol presented values similar to those of E-7 (Table 4). Puzol variety had DPPH values 1.6 fold higher than those of El Camino, which was the variety with lowest values for this antioxidant parameter. Finally, for TRC all the pepino varieties presented significantly lower values than those of *S. caripense* E-7. In this case, the pepino variety with highest values was 37-A, with values 1.5 fold higher than Puzol, which was the variety with the lowest value for this parameter (Table 4).

3.3. Biological activity

No significant differences were observed for raw (1:1) and diluted (1:10) extracts of pepino and *S. caripense* on cell viability of macrophage cells, revealing a lack of toxicity on these cells of any of the extracts. All raw extracts demonstrated a significant inhibition of the NO production of the macrophage cells (Figure 4). The highest NO production inhibition was caused by pepino accession 37-A, with a 67% of inhibition with respect to the control, while the rest of accessions had a similar performance, with inhibition values ranging from 36% (El Camino) to 41% (Puzol). The 1:10 dilutions of pepino accessions 37-A and Valencia also presented significant inhibition of the NO production, but the values were much lower (always below 10%) than those of raw extracts (Figure 4). The 1:10 dilutions for the rest of accessions (El Camino, Puzol, and *S. caripense* E-7) did not present significant inhibition of NO production.

3.4. Selection of varieties for phenolic content and biological activities

When varieties are ranked for their total content in total hydroxycinnamic acids, ORAC, DPPH and TRC antioxidant activities and inhibition of NO production in stimulated macrophage cells we did not find a single variety ranking high for all traits considered (Table 5). On the other hand, one variety (El Camino) generally presented low ranks, with an intermediate rank (3) for hydroxycinnamic acids content and a low

rank (4 or 5) for the antioxidant traits and NO production inhibition. Pepino variety Valencia, which ranked first for hydroxycinnamic acids content, also presented intermediate or low ranks for the rest of traits (Table 5). *Solanum caripense* E-7 had the highest ranks for the three antioxidant measures, but presented a low rank for hydroxycinnamic acids content and an intermediate rank for NO production inhibition. Pepino accession 37-A ranked first for NO production inhibition and second for ORAC and TRC antioxidant measures, but presented the lowest rank for hydroxycinnamic acids content and a low rank for DPPH antioxidant activity. Finally, pepino accession Puzol ranked second for hydroxycinnamic acids content, DPPH antioxidant activity and NO production inhibition, with an intermediate rank for ORAC and the lowest rank for TRC (Table 5).

4. Discussion

The HPLC-DAD-MSn/ESI technique, which is very efficient for detecting and identifying phenolic compounds of plant extracts, has allowed detecting 24 hydroxycinnamic acid derivatives in the pepino flesh. The election of the method is based on previous experiences (Ferreres et al., 2011; Sánchez-Rodríguez et al., 2012), and we have observed that methanol improves the phenolic acid ionization in the LC-MS compared to ethanol, which extracts more sugars from the plant matrix and therefore is less indicated for these type of studies. This increases substantially the number of phenolic metabolites detected up to now in pepino (Hsu et al., 2011; Wu et al., 2013), with a number of phenolic metabolites similar to those detected in tomato using the same technique (Sánchez-Rodríguez et al., 2012), significantly improving the phytochemical characterization of pepino varieties.

All the phenolic compounds detected corresponded to hydroxycinnamic acid derivatives and no flavonoids were identified. This indicates that the pepino flesh is more similar in phenolic composition to eggplant, whose phenolic fraction is mostly constituted by hydroxycinnamic acid derivatives (Alarcón-Flores, Romero-González, Vidal, & Frenich, 2013; Prohens et al., 2013), than tomato, which also presents relevant quantities of flavonoids (Alarcón-Flores et al., 2013; Sánchez-Rodríguez et al., 2012). Our results are in agreement with the results of Wu et al. (2013), who found that hydroxycinnamic acid derivatives were the major phenolic compounds of pepino flesh. However, Hsu et al. (2011) reported significant levels of flavonoids such as

myricetin, naringenin, quercetin and rutin in aqueous and ethanolic extracts of pepino. These discrepancies may be caused by differences in the plant material used and/or the extraction and detection methodology (Häkkinen & Törrönen, 2000; Stalikas, 2007).

The high diversity found among the five accessions used for the profile of phenolic acids is coincident with the high genetic diversity of the pepino and its wild relatives (Blanca et al., 2007; Herraiz et al. 2015). Only five compounds are universal to all the accessions and eight compounds are specific of accession, which indicates that as in other *Solanum* fruit species, like eggplant (Wu et al., 2013), fruit phenolic acids profile may be useful for chemotaxonomy in the pepino group. Amazingly, the wild *S. caripense* E-7 and the primitive pepino cultivar 37-A have less phenolic compounds and lower concentration than the modern varieties, which is in contrast to what has been found in eggplant and tomato, in which the domestication and breeding processes have reduced the content in phenolics (Meyer et al., 2015; Prohens, Rodríguez-Burruezo, Raigón, & Nuez, 2007; Willits et al., 2005).

The predominant phenolic compounds of pepino, as it occurs in many vegetables (Kaushik et al., 2015), have been the chlorogenic, acid isomers caffeoylquinic acid and 3-caffeoyl-quinic acid. However, for *S. caripense* E-7 the major compound has been caffeoyl-sinapoyl-quinic acid, which is characteristic of Robusta coffee (Jaiswal, Patras, Eravuchira, & Kuhnert, 2010). This suggests that important biochemical differences must exist in the pathway of synthesis of phenolic acids between pepino and *S. caripense*.

The three antioxidant measures taken involve hydrogen atom transfer (ORAC) or electron-transfer (DPPH and TRC) reactions (Hwang, Kim, Park, Lee, & Kim, 2014). ORAC values of pepino samples have been much higher than those of DPPH, an observation also found in other fruits like in Citrus (Gironés-Vilaplana, Moreno, & García-Viguera, 2014). The antioxidant capacity of pepino varieties depend both on the antioxidant activity of each phenolic compound, as well as the concentration present, the possible synergisms and the method employed. ORAC method employs a more hydrosoluble environment than DPPH, suitable for compounds as the hydroxycinnamic acids of pepino samples. By comparison with other fruits and vegetables, ORAC values are intermediate-high (Speisky, López-Alarcón, Gómez, Fuentes, & Sandoval-Acuña, 2012; Wu et al., 2004). The Folin-Ciocalteu method measures the total reducing capacity (TRC) (Huang, Ou, & Prior, 2005). In our case the

antioxidant values measured by the TRC method using the Folin-Ciocalteu reagent revealed that the antioxidant activity of pepino is comparable to that of eggplant (Stommel & Whitaker, 2003) which has a high antioxidant capacity (Morales-Soto et al., 2014). These data indicate pepino presents high values for antioxidant capacity and may make a significant contribution of antioxidants intake in the diet.

Pepino is a cultivated edible species, while *S. caripense* is occasionally harvested from the wild for its sweet fruits (Rodríguez-Burruezo et al., 2011). The extracts, even when not diluted, of both species did not affect viability of macrophage cells, which is an indication of a lack of citotoxicity (Ferrari, Fornasiero, & Isetta, 1990). The lack of cytotoxicity of *S. caripense* is in contrast wild relatives of the genus *Solanum*, which are cytotoxic due to their high contents of glycoalkaloids and other antinutritional compounds (Cárdenas et al., 2015; Plazas et al., 2014), and therefore facilitates its use in breeding of the cultivated pepino (Rodríguez-Burruezo et al., 2011). Pepino and *S. caripense* raw extracts inhibited significantly the production of NO in LPS-stimulated macrophages, suggesting that they have an in vivo anti-inflammatory effect (Wang & Mazza, 2002).

The results obtained have failed to identify a single accession with high values for the traits studied. This is probably a consequence that the different traits studied measure different aspects of the fruit quality of the samples. In this respect, the hydroxycinnamic acids contribute to antioxidant activity (Razzaghi-Asl, Garrido, Khazraei, Borges, & Firuzi, 2013), but other antioxidant compounds present in the pepino flesh, like vitamin C or carotenoids (Di Scala et al., 2011; Sudha et al., 2012) may also play a role in antioxidant activity. At the same time, the different antioxidant measures, due to the different nature of the chemical reactions involved, may give considerable differences in the results (Huang et al., 2005). Also, inhibition of NO production in LPS-stimulated macrophages does not exclusively depend on phenolics or antioxidant activity, as other bioactive compounds may be involved (Thilakarathna & Rupasinghe, 2012). Therefore, if a pepino variety with high values for the different types of traits observed (content in phenolics) is desired, we suggest either the screening of large collections of materials, or the intercrossing of materials with complementary desirable characteristics.

5. Conclusions

The fruit of pepino and its wild relative *S. caripense* present significant quantities of phenolic acid derivatives, as well a remarkable antioxidant and biological activity, which may be related to its properties beneficial for health. The phenolic fraction of the fruit flesh of pepino and its wild relative *S. caripense* is mostly constituted by hydroxycinnamic acid derivatives; although modern pepino varieties have a different and richer profile of phenolic compounds than the wild *S. caripense* and the primitive pepino materials. Different accessions have ranked first for hydroxycinnamic acid content (modern pepino variety Valencia), antioxidant activity measures (*S. caripense* E-7) and biological activity (primitive pepino variety 37-A). This suggests that selection of larger collections or the development of breeding programmes will have to be undertaken if varieties with high values are desired for the three types of traits measured here. Our results provide relevant information of the phenolics composition, antioxidant and biological activities of a representation of the diversity of pepino and of its wild relative *S. caripense*. This information will contribute to the enhancement of this neglected crop.

6. Acknowledgements

The authors express their gratitude to the Spanish Ministry of Economy and Competitiveness (MINECO) and the Iberian-American Programme for Cooperation and Development (CYTED) (Ref. 112RT0460) - CORNUCOPIA Thematic Network (URL: redcornucopia.org). We are also grateful to Mariola Plazas and Pietro Gramazio for their technical assistance.

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TABLES

Table 1. Pepino (*S. muricatum*) and its wild relative *S. caripense* accessions used in the present study and main fruit characteristics

Accession	Origin	Main use	Fruit shape	Fruit weight (g)	Soluble solids content (%)
S. muricatum					
37-A	Ecuador	Fresh fruit	Conical	72±9	5.4±0.5
El Camino	New Zealand	Fresh fruit	Heart-shaped	127±12	6.7±0.5
Puzol	Spain	Salads	Ellipsoid	213±24	7.2±0.4
Valencia	Spain	Fresh fruit	Elongated	192±22	7.6±0.6
S. caripense					
E-7	Ecuador	Occasionally picked for its sweet fruits	Round	19±2	10.1±0.9

Values are expressed as mean ± S.E. of 5 independent samples for each variety.

Table 2. Rt, MS: $[M-H]^-$, $MS^2[M-H]^-$ and relative abundance (%) of phenolic compounds identified (marked by an X) in fruit samples of pepino (*S. muricatum*) and its wild relative *S. caripense* samples

Peak	Compound	Rt	$[M-H]^-$	$MS2[M-H]^-$, *m/z* (%)	37-A	El Camino	Puzol	Valencia	E-7
1	di-caffeoyl-quinic acid I	15.2	515	353 (54), 191(100)					X
2	3-Caffeoyl-quinic acid	16.4	353	191 (100), 179 (42)	X	X	X	X	X
3	Caffeoyl-hexoside I	17.4	341	179 (100), 135 (21)		X	X	X	X
4	Caffeoyl-hexoside II	17.6	341	179 (64), 135 (9)		X	X	X	
5	di-caffeoyl-quinic acid II	18.4	515	353 (78), 191 (88)					X
6	Caffeoyl-hexoside III	18.4	341	179 (100), 135 (19)		X	X	X	
7	Feruloyl-hexoside	19.2	355	193 (100), 175 (53)			X		
8	Caffeoyl-di-hexoside	19.3	503	341 (36), 179 (100)		X	X	X	
9	Caffeoyl-hexose IV	19.8	341	179 (100), 135 (12)	X	X	X	X	X
10	5-Caffeoyl-quinic acid	20.6	353	191 (100)	X	X	X	X	X
11	4-Caffeoyl-quinic acid	20.7	353	173 (100)	X	X	X	X	X
12	*p*-Coumaroyl-di-hexoside	22.0	487	325 (14), 163 (100)		X	X	X	X
13	Feruloyl-hexoside	22.3	355	193 (100), 175 (56)		X	X	X	
14	Caffeoyl-quinic acid isomer	23.0	353	191 (100)		X			
15	Feruloyl-dihexoside	23.2	517	235 (50), 193 (100), 175 (76)		X	X	X	
16	Sinapoyl-di-hexoside	23.4	547	265 (82), 324 (43), 223 (28)		X			
17	Feruloyl-hexoside	24.4	355	193 (100), 175 (19)				X	
18	Caffeoyl-hexoside derivative	27.8	441	341 (100)				X	
19	di-caffeoyl-quinic acid	30.6	515	353 (100), 191 (12)			X		
20	Sinapoyl-quinic acid derivative	31.1	577	415 (100), 353 (13), 191 (9)		X			
21	Feruloyl-di-hexoside derivative	31.7	517	323 (100), 193 (41), 179 (19)			X		
22	di-caffeoyl-quinic acid	32.9	515	353 (100), 191 (2)		X	X	X	
23	Caffeoyl-hexoside derivative	34.6	423	179 (100), 135 (28)				X	
24	Caffeoyl-sinapoyl-quinic acid	35.7	559	397 (100), 223 (18), 173 (3)	X	X	X	X	X

Table 3. Compounds quantified (mg/g d.w.) in fruit samples of pepino (*S. muricatum*) and its wild relative *S. caripense* samples by HPLC-DAD

		S. muricatum				***S. caripense***
Peak	Compound	37-A	El Camino	Puzol	Valencia	E-7
2	3-Caffeoyl-quinic acid	0.90 ± 0.31	< LOQ	< LOQ	< LOQ	< LOQ
9	Caffeoyl-hexose IV	0.07 ± 0.01	< LOQ	< LOQ	< LOQ	< LOQ
10	5-Caffeoyl-quinic acid	< LOQ	0.89 ± 0.46	1.44 ± 0.24	1.38 ± 0.35	0.19 ± 0.03
11	4-Caffeoyl-quinic acid	0.03 ± 0.01	< LOQ	< LOQ	< LOQ	< LOQ
12	*p*-Coumaroyl-di-hexose	n.d.	0.06 ± 0.01	0.14 ± 0.04	0.41 ± 0.05	0.06 ± 0.01
13	Feruloyl-hexose	n.d.	0.14 ± 0.04	0.28 ± 0.08	< LOQ	n.d.
14	5-Caffeoyl-quinic acid isomer	n.d.	0.03 ± 0.01	n.d.	n.d.	n.d.
15	Feruloyl-dihexose	n.d.	0.26 ± 0.04	0.16 ± 0.02	0.37 ± 0.07	n.d.
16	Sinapoyl-di-hexose	n.d.	0.05 ± 0.01	n.d.	n.d.	n.d.
18	Caffeoyl-hexose derivative	n.d.	n.d.	n.d.	0.06 ± 0.01	n.d.
19	Di-caffeoyl-quinic acid	n.d.	< LOQ	0.06 ± 0.01	< LOQ	n.d.
20	Sinapoyl-quinic acid derivative	n.d.	0.05 ± 0.02	n.d.	n.d.	n.d.
24	Caffeoyl-sinapoyl-quinic acid	0.10 ± 0.02	0.04 ± 0.01	0.09 ± 0.01	0.13 ± 0.02	1.13 ± 0.15
	Total hydroxycinnamic acids	1.11 ± 0.08	1.51 ± 0.07	2.17 ± 0.07	2.35 ± 0.10	1.37 ± 0.06

n.d.: not detected, < LOQ: detected but present at concentrations lower than the limit of quantification (LOQ). Values are expressed as mean±SE of five independent samples for each variety.

Table 4. Antioxidant activity of fruit samples of pepino (*S. muricatum*) and its wild relative *S. caripense* samples using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging capacity, oxygen radical absorbance capacity (ORAC), and total reducing capacity (TRC) based on the Folin-Ciocalteu reagent method

Accession	ORAC (μmol Trolox/g d.w.)	DPPH (μmol Trolox/g d.w.)	FCR (μmol caffeic acid/g d.w.)
S. muricatum			
37-A	83.5 ± 7.0	26.1 ± 3.4	99.2 ± 11.7
El Camino	76.0 ± 2.6	22.2 ± 1.5	73.6 ± 1.7
Puzol	80.9 ± 8.2	34.5 ± 2.1	66.2 ± 4.8
Valencia	51.9 ± 11.9	29.2 ± 2.1	76.8 ± 6.7
S. caripense			
E-7	170.7 ± 22.9	36.3 ± 2.9	127.9 ± 4.8

Values are expressed as mean±SE of five independent samples for each variety.

Table 5. Ranking (ordered from highest to lowest) for total content of hydroxycinnamic acids, antioxidant activity measures (ORAC, DPPH, and TRC), and inhibition of NO production in the raw extracts (1:1) of lyophilized samples of the different accessions studied of pepino (*S. muricatum*) and its wild relative *S. caripense*, and sum of ranks for each accession

Accession	Hydroxycynnamic acids	ORAC	DPPH	TRC	NO inhibition
S. muricatum					
37-A	5	2	4	2	1
El Camino	3	4	5	4	5
Puzol	2	3	2	5	2
Valencia	1	5	3	3	4
S. caripense					
E-7	4	1	1	1	3

FIGURES

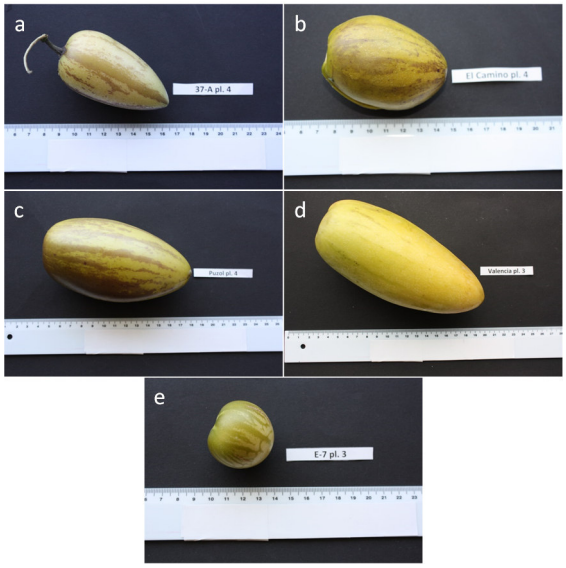
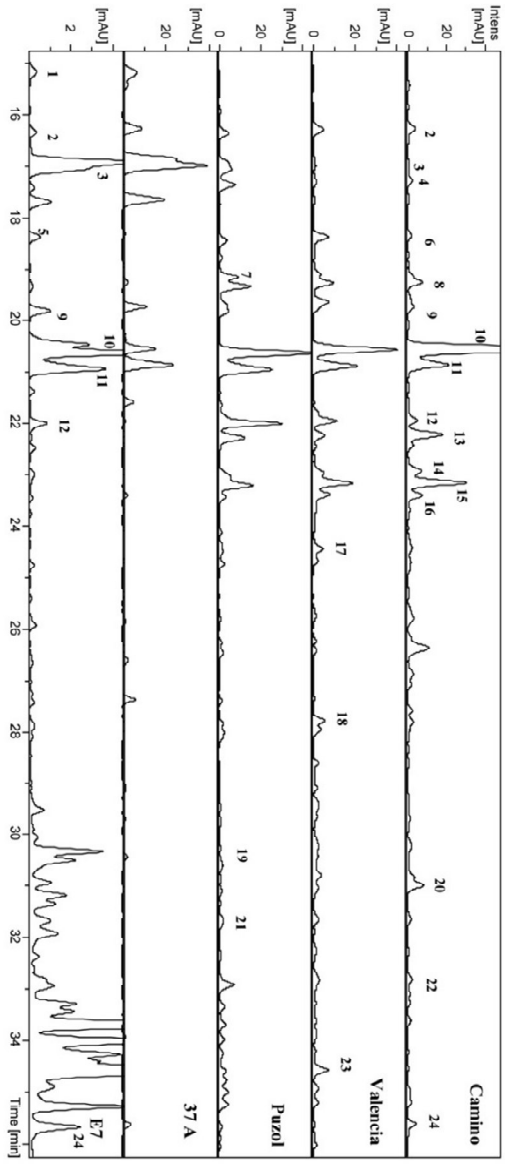


Figure 1. Fruit samples of the pepino (*S. muricatum*) and *S. caripense* accessions used. Pepino accessions correspond to 37-A (a), El Camino (b), Puzol (c), and Valencia (d), while *S. caripense* accession is E-7 (e). Scale is in cm.

Figure 2. Chromatogram obtained from reversed-phase LC-MS/MS analysis of pepino varieties. Numbers in bold correspond to the peaks identified and described in Tables 2 and 3.



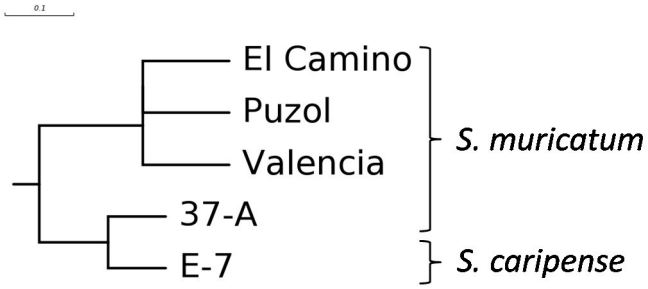


Figure 3. Euclidean distance-based UPGMA phenogram of four pepino accessions (37-A, El Camino, Puzol and Valencia) and one *S. caripense* accession (E-7) according to the absence/presence of 24 phenolic compounds detected by HPLC-DAD-MS^n/ESI**.**

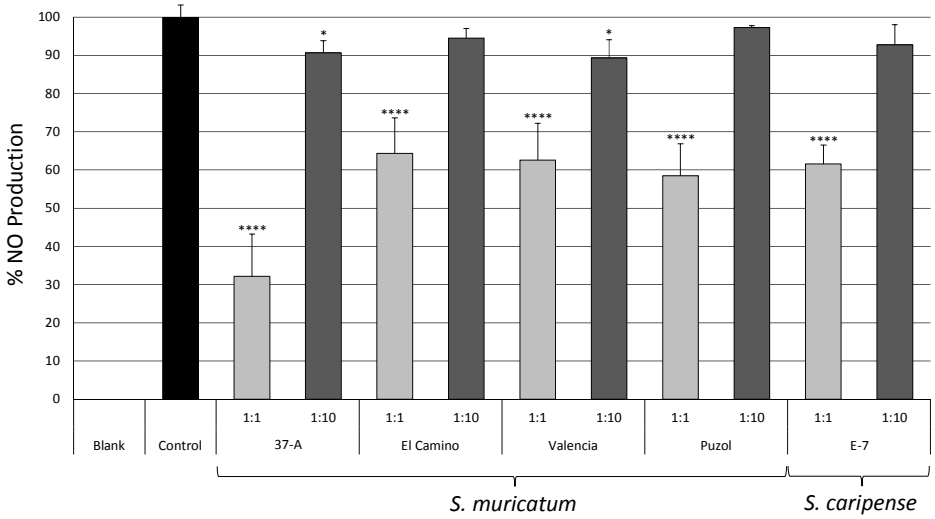


Figure 4. Percentage of NO production of RAW 264.7 macrophages incubated in raw (1:1; light grey columns) and diluted (1:10; dark grey columns) methanolic extracts of pepino and *S. caripense* accessions. Bars represent ± SE of the mean. Columns tagged with asterisks indicate that the mean values are significantly different from the control (**** and * indicate significance at P values of 0.0001 and 0.05, respectively) according to the Dunnett's multiple comparison test.

DISCUSIÓN GENERAL

4.- DISCUSIÓN GENERAL:

Desde el inicio de la agricultura los seres humanos favorecieron la dispersión de cultivos entre distintas zonas, lo que provocó que existiese una gran variabilidad de especies cultivadas en cada territorio. Esta tendencia se invirtió en las últimas décadas por diversos motivos, principalmente corporativistas, produciéndose paulatinamente una reducción de esta variabilidad. En la actualidad la responsabilidad de nutrir a un alto porcentaje de la población mundial recae en unas pocas especies, siendo un grupo de cereales como trigo, maíz y arroz, los responsables de la mayor cantidad de aporte calórico, mientras que un grupo más amplio de hortalizas complementan la dieta aportando otros nutrientes como vitaminas, minerales y fibra.

En este contexto, la ampliación del espectro de cultivos, ya sea recuperando los abandonados o introduciendo cultivos de otras zonas resulta beneficioso en numerosos aspectos, favoreciendo a consumidores, agricultores y mercados.

Son los bancos de germoplasma los centros encargados de conservar tanto la variabilidad perdida de algunos cultivos, como la variabilidad de especies potencialmente interesantes para ser introducidas en otras áreas. A parte de la conservación, son objetivos prioritarios de los bancos de germoplasma, la evaluación y caracterización de estos materiales, así como el desarrollo de claves y descriptores para llevar a cabo esta tarea. En el caso del pepino dulce, el COMAV en colaboración con la FAO desarrolló una lista de descriptores para la caracterización morfológica del pepino dulce (Prohens et al., 2004), pero se hace necesario disponer de descriptores fenológicos adaptados a esta especie como el que se ha desarrollado en esta tesis (Herraiz et al., 2015a).

Por otro lado los marcadores moleculares son una herramienta de alto valor para el estudio de esta variabilidad conservada en los bancos de germoplasma. Existen varios criterios para decidir qué marcador molecular es el más apropiado para estudiar esta variabilidad, pero casi siempre la elección viene determinada por restricciones ya sean económicas o técnicas. Estas restricciones se hacen todavía más patentes cuando la especie a estudiar no es muy importante a nivel económico y/o ha sido poco estudiada a nivel genómico. Este es el caso del pepino dulce, donde hasta la actualidad, su variabilidad había sido estudiada empleando marcadores que no requerían un conocimiento previo del genoma, como

RFLPs (Spooner et al., 1993), AFLPs o analizando las secuencias de algún gen en concreto, como la 3-metilcrotonil-CoA carboxilasa (Blanca et al., 2007).

En la presente tesis se incluye el primer trabajo que emplea marcadores SSR en pepino dulce, que junto con una caracterización morfológica, pretende estudiar la variabilidad en una colección de entradas de la especie cultivada y especies relacionadas (Herraiz et al., 2015b). Estos marcadores moleculares han sido desarrollados inicialmente en tomate, especie para la cual hay una enorme cantidad de información a nivel genómico y que se han conseguido transferir al pepino dulce.

El desarrollo en los últimos años de las denominadas tecnologías de secuenciación masiva, que permiten la obtención de millones de secuencias de ADN de manera rápida y cada vez más económica, ha permitido la consecución de logros científicos de gran importancia. Uno de los trabajos incluidos en esta tesis ha sido la secuenciación del primer transcriptoma de pepino dulce empleado la tecnología Illumina HiSeq-2000. La secuenciación de la especie silvestre *S. caripense*, muy cercana filogeneticamente, ha servido para obtener miles de marcadores SNVs (SNPs e INDELs) de alta calidad que presentan múltiples aplicaciones de interés para la mejora del pepino dulce.

En la actualidad, los consumidores demandan alimentos más saludables, y sabrosos. Es por ello que uno de los objetivos de mejora más importantes a día de hoy en numerosas especies es el contenido en compuestos bioactivos, es la denominada nutracéutica. En la presente tesis, se presenta un trabajo que evalúa la composición de nutricional de la colección de entradas de pepino dulce y especies silvestres relacionadas (Herraiz et al., 2015d), así como otro trabajo donde se estudia el perfil de compuestos fenólicos en 5 entradas, su capacidad antioxidante y la respuesta a la adicción de extractos de frutos de esas entradas en cultivos celulares de macrófagos murinos sometidos a estrés oxidativo (Herraiz et al., 2015e).

4.1.- Desarrollo de un descriptor fenológico basado en la escala BBCH

La mayoría de las Solanáceas cultivadas se reproducen mediante semillas, las excepciones a esta norma son la patata y el pepino dulce que habitualmente se reproducen vegetativamente (Prohens et al., 2005; Cavusoglu y Sulusoglu, 2013). A pesar de esta similitud entre patata y pepino dulce, existen numerosas diferencias fenológicas, siendo la principal que la patata se cultiva por sus tubérculos y no por sus frutos. Para las Solanáceas cultivadas por sus frutos ya existe desde hace años una clave BBCH de caracterización fenológica (Feller et al., 1995), así como una clave específica de patata (Hack et al., 1993), es por este motivo por el que se hace necesario el desarrollo de una clave de caracterización fenológica adaptada al pepino dulce como la aquí presentada.

La escala de caracterización BBCH consiste en una descripción de los estados fenológicos de un cultivo bajo unas condiciones estándar. Se basa en un código decimal, siendo 10 los estadios principales. En el caso del pepino dulce se inicia con la germinación y/o el inicio del enraizamiento de los esquejes (estadio 0) y finaliza con la senescencia de la planta (estadio 9). Entre estos dos estadios se desarrolla todo el periodo de cultivo que incluye el desarrollo vegetativo, floración y fructificación.

En el trabajo presentado se realiza una descripción y explicación de cada uno de esos estadios, así como un ejemplo donde la aplicación de esta escala fenológica permitió caracterizar fenológicamente una colección de entradas de pepino dulce y especies silvestres relacionadas, pudiendo determinar entre otros aspectos cuál es el momento óptimo de recolección de cada entrada.

El conocer al detalle los distintos estados fenológicos por los que pasa un cultivo es de gran importancia tanto para agricultores, técnicos y mejoradores, para por ejemplo determinar cuándo se aconseja aportar una dosis de abonado, o cuándo deja de ser eficaz un tratamiento fitosanitario, para la tasación de un cultivo por aseguradoras, etc.

4.2.- Caracterización morfológica y molecular de una colección de entradas de pepino dulce y especies relacionadas

En el artículo presentado se demuestra cómo los marcadores moleculares pueden contribuir de manera complementaria y sinérgica con los descriptores morfológicos a estudiar la variabilidad de una colección de entradas de pepino dulce y especies silvestres relacionadas (Herraiz et al., 2015b). Disponer de esta información es de gran importancia para los mejoradores sobre todo en especies tan poco estudiadas como el pepino dulce.

En el artículo se describen cuáles son los caracteres morfológicos más discriminantes entre las entradas, y cuáles los más invariables. Las mayores diferencias se establecen entre las formas cultivadas y las silvestres. Dentro de las entradas de la especie cultivada, la mayor variabilidad se encuentra en las variedades locales, frente a las variedades modernas, mucho más homogéneas.

Los marcadores moleculares SSR no hacen más que afianzar los resultados obtenidos con la caracterización morfológica, confirmándose como unos de los mejores marcadores para una correcta caracterización de colecciones de germoplasma. Se obtuvo una correlación elevada entre las matrices de distancias obtenidas por las dos estrategias. De los 20 marcadores SSR de tomate evaluados, 14 pudieron transferirse a pepino dulce, obteniendo a parte de la amplificación del fragmento esperado, un polimorfismo, en ocasiones elevado. Esta elevada transferibilidad podría permitir emplear la ingente cantidad de marcadores desarrollados en tomate en pepino dulce y otras especies de la sección *Basarthrum*, con numerosas aplicaciones.

Con los datos obtenidos se observa una elevada heterocigosidad en toda la colección, algo esperado teniendo en cuenta trabajos previos y conociendo de qué manera se obtuvieron las variedades modernas (Rodríguez-Burruezo et al., 2011). También es destacable que debido a su propagación vegetativa, no se produce la fijación de alelos a la que conllevaría una autofecundación continuada. Nuevamente las variedades locales son más heterogéneas demostrándose la existencia de un cuello de botella genético en la mejora moderna de este cultivo. En el caso de las especies silvestres esta heterocigosidad no ha sido tan grande como la esperada. Estas especies presentan en ocasiones una alogamia estricta determinada genéticamente, pero quizá los pequeños tamaños poblacionales y/o la propagación de estas entradas en los bancos de

germoplasma ha ocasionado una reducción de la variabilidad al partir de pocos individuos. Aún así estas especies presentan una elevada diversidad y representan una importante fuente de variación para la mejora de la especie cultivada.

4.3.- Ensamblaje *de novo* y análisis del transcriptoma de pepino dulce

En la publicación presentada se hace patente cómo el empleo de las técnicas de secuenciación masiva de nueva generación, puede contribuir a estudios genómicos en especies poco cultivadas o de poca importancia económica, permitiendo la detección de un gran número de transcritos, su anotación, así como la identificación de un gran número de marcadores moleculares de alta calidad.

La secuenciación se llevó a cabo empleando la tecnología Illumina HiSeq-2000, en una mezcla de ARN de tres tejidos de la especie cultivada *S. muricatum* (Sweet Long) y de la especie silvestre *S. caripense* (EC-40). Inicialmente se obtuvieron 58,327,154 de lecturas paired-end para *S. muricatum* y 52,646,045 para *S. caripense*.

Las lecturas de *S. muricatum* tras su limpieza, filtrado y posterior ensamblaje, resultaron en un total de 75,832 unigenes con una longitud media de 704 pb. Es importante mencionar que hasta la publicación de este trabajo solamente había 126 secuencias depositadas en la base de datos del NCBI, todas procedentes de un mismo trabajo (Blanca et al., 2007) donde se estudió las implicaciones en la evolución y domesticación estudiando variaciones en un gen en varias entradas de pepino dulce.

Estos unigenes obtenidos se anotaron mediante comparación en bases de datos de proteínas, obteniendo que en más de un 65 % de las mismas se podía determinar su función. Además se empleó el programa Blast2GO (Conesa et al., 2005) para asignar, siempre que fue posible, a cada unigen un término GO, que determina su ontología según tres categorías: (1) si tiene una función molecular, (2) si forma parte de un proceso biológico o (3) si está localizado en un componente celular. Así mismo, la anotación del programa Blast2GO asigna, siempre que sea posible, un número EC (Enzyme Commission Number), que viene a ser una clasificación numérica de los enzimas basada en las reacciones químicas que catalizan. De igual manera se obtuvo la asignación de los enzimas a las distintas rutas metabólicas empleando la base de datos KEGG.

La comparación de las secuencias de varios genes candidatos para caracteres de interés descritos en otras especies, entre la especie cultivada (*S. muricatum*) y la silvestre (*S. caripense*) ha permitido observar diferencias a nivel nucleotídico en esos genes. Estas diferencias genéticas pueden emplearse como marcadores moleculares para la introgresión de genes de la especie silvestre a la cultivada, o asumiendo que *S. caripense* es el ancestro silvestre del pepino dulce, estudiar los procesos de selección que desencadenaron en la domesticación del cultivo.

Para realizar un análisis filogenético se emplearon cinco genes, presentes en los unigenes del transcriptoma de pepino dulce y en otras cinco especies de Solanáceas. Se analizaron las variaciones en estos cinco genes, resultando un árbol filogénetico que demostró que el pepino dulce, divergió hace aproximadamente 9 millones de años de un ancestro común con la patata y el tomate, y se confirma que son estás las dos Solanáceas más próximas al pepino dulce, algo que ya afirmaron otros autores basándose en otras metodologías (Spooner et al., 1993; Sarkinen et al., 2013).

La obtención de marcadores moleculares de manera masiva es uno de los grandes avances logrados con las técnicas de secuenciación masiva. En el caso del pepino dulce, debido a su escasa información de secuencia previa, el desarrollo masivo de marcadores estaba muy limitado. En este transcriptoma publicado se identificaron *in silico* 1,072 marcadores SSR en 1,049 unigenes, y su comparación con las lecturas de *S. caripense* permitió identificar 89,030 marcadores SNV mayormente SNPs variables entre ambas especies, así como otros miles intraespecíficos en ambas especies.

Estos marcadores tanto SSRs como SNVs, presenta numerosas ventajas, principalmente su codominancia y su abundancia. Sus principales aplicaciones serán, ampliar los estudios de diversidad realizados hasta la fecha, desarrollar mapas genéticos y físicos, identificación de QTLs y mapeo por asociación, así como en selección asistida por marcadores.

4.4.- Caracterización de compuestos de valor nutricional en la colección de entradas de pepino dulce y especies relacionadas

La demanda de hortalizas con un mayor contenido en compuestos bioactivos está propiciando el desarrollo de programas de mejora dirigidos a aumentar el contenido en estas sustancias. Estudios previos en

pepino dulce confirmaban las bondades de esta fruta en varios aspectos, como su efecto hipotensivo (Redgwell y Turner, 1986), diurético (Sánchez-Vega, 1992), antitumoral (Ren y Tang, 1999), antidiabético (Hsu et al., 2011) y antiinflamatorio (Shathish y Guruvayoorappan, 2014), así como un alto contenido en vitaminas, minerales y compuestos antioxidantes (Redgwell y Turner, 1986; Pluda et al., 1993a; Sanchez et al., 2000). Son varios los estudios que evalúan el contenido en estas sustancias, pero casi todos incluían un número reducido de variedades.

En uno de los trabajos presentados en esta tesis (Herraiz et al., 2015d), se evalúa el contenido en materia seca, proteínas, antioxidantes, pigmentos (β-caroteno) y minerales en la colección de entradas de pepino dulce y especies silvestres relacionadas incluida en los otros trabajos anteriores.

En líneas generales el pepino dulce presenta contenidos similares de materia seca, proteínas y β-caroteno que otras especies como el melón o el pepino. En cambio, el contenido de fenoles totales es mucho mayor que en otras especies como tomate o berenjena, sugiriendo una mayor capacidad antioxidante que puede tener interés a la hora de promocionar el cultivo en vista a su introducción en nuevos mercados.

También se han encontrado diferencias significativas entre la especie cultivada *S. muricatum* y el resto de especies silvestres evaluadas. Generalmente, las especies silvestres presentan un mayor contenido en todos los caracteres estudiados y se confirman como materiales interesantes para la mejora de la especie cultivada. Destaca por ejemplo la entrada de la especie *S. trachycarpum* que acumula más de 3 veces más de materia seca que las entradas de pepino dulce, haciendo esta entrada interesante no solamente para mejorar la calidad organoléptica del fruto del pepino dulce, sino también para su resistencia a la sequía.

Dentro de la especie cultivada también hay diferencias importantes en todos los caracteres evaluados, con la excepción del contenido en materia seca que es bastante homogéneo. Se observan evidencias de que la mejora moderna ha producido una reducción en la cantidad de nutrientes con respecto a las variedades locales, es lo que se denomina "efecto dilución" (Davis, 2009) que se ha demostrado en numerosas especies. En el caso del pepino dulce este "efecto dilución" de nutrientes es más evidente en proteínas, y algunos minerales como fósforo, potasio y cinc.

En este trabajo también se ha visto que existen unas correlaciones positivas entre caracteres estudiados que pueden resultar en un efecto pleiotrópico y como consecuencia la selección para un carácter puede acarrear una selección indirecta para otro. La mayoría de los caracteres están correlacionados entre sí, siendo esto una enorme ventaja para el mejorador. Así por ejemplo, una selección para un contenido elevado de compuestos fenólicos en pepino dulce, nos está seleccionando indirectamente para un aumento del contenido de β-caroteno, de fósforo, de magnesio y de cinc. Correlaciones de este tipo también existen entre contenido en clorofila y magnesio, y de manera inversa entre el contenido en clorofila y el cobre.

En definitiva este trabajo ha servido para confirmar y determinar el elevado contenido en determinados nutrientes beneficiosos en el pepino dulce, confirmando que es un alimento muy saludable. Destaca principalmente por su contenido en fósforo, potasio, hierro y cobre, ayudando su consumo a alcanzar la dosis diaria recomendada de estos minerales. Y sobre todo destaca por su contenido en polifenoles, que pueden suponer hasta un 20% de la dosis diaria recomendada.

A raíz de estos resultados se planteó la posibilidad de estudiar el perfil de polifenoles en 4 entradas de pepino dulce y una entrada de *S. caripense*, se estudió también la capacidad antioxidante de las mismas y finalmente, la respuesta que provoca la adicción de un extracto de zumo de pepino dulce a células de macrófagos de ratón sometidas a estrés oxidativo. Todo este trabajo se presenta en el último trabajo de esta tesis (Herraiz et al, 2015e).

Se detectaron 24 metabolitos fenólicos derivados del ácido hidroxicinámico usando HPLC-DAD-ESI-MS^n, esto es un número significativamente más alto de los detectados en trabajos previos usando técnicas similares (Hsu et al., 2011; Wu et al., 2013). Cabe indicar que es un número similar a los encontrados en tomate usando también HPLC-DAD-ESI-MS^n (Sánchez-Rodríguez et al., 2012). Sorprendentemente no se han encontrado flavonoides, cosa que sí ocurre en tomate, lo que demuestra que el contenido en pulpa es mucho más parecido en compuestos fenólicos a la berenjena (Prohens et al., 2013) que al tomate (Sánchez-Rodríguez et al., 2012; Slimestad y Verheul, 2009; Alarcón-Flores et al., 2013). Algunos autores sí han encontrado flavonoides en la pulpa de pepino dulce (Hsu et al., 2011; Wu et al., 2013), pero estas

diferencias podrían deberse a utilizar otras variedades y otros procedimientos de extracción.

El perfil de polifenoles de las 5 accesiones fue muy diferente. De los 24 compuestos detectados, sólo 5 son comunes a las 5 accesiones, y 8 de ellos son específicos de accesión. Este grado de divergencia entre entradas en su perfil de polifenoles permitió realizar un estudio de quimiotaxonomía o quimiosistemática. Mediante un dendrograma basado en el método UPGMA se permitió agrupar a las variedades modernas (Valencia, El Camino y Puzol) según su perfil bioquímico de polifenoles. Por otro lado, la variedad local 37-A y la entrada de la especie silvestre *S. caripense* (E-7) se agrupan en otro conglomerado. Esto es coincidente con lo obtenido en el trabajo de caracterización morfológica y molecular (Herraiz et al., 2015b), donde en el PCA y PCoA la entrada 37-A se localizaba entre la especie cultivada y las especies silvestres, considerándose una variedad primitiva, donde el proceso de domesticación no está muy avanzado, o bien ha sufrido introgresiones recientes de alguna especie silvestre. Estas últimas entradas (37-A y E-7) presentan un menor número de compuestos fenólicos y a menor concentración que las variedades modernas, al contrario de lo ocurrido a raíz de la domesticación del tomate y la berenjena donde se produjo una dilución de nutrientes (Willits et al., 2005; Prohens et al., 2007; Meyer et al., 2015).

Los compuestos fenólicos predominantes en pepino dulce, tal y como ocurre en mucho vegetales (Kaushik et al., 2015) han sido los isómeros del ácido clorogénico, el ácido 5-cafeoilquínico y el ácido 3-cafeoilquínico. En cambio en la especie *S. caripense* el más abundante fue el ácido cafeoil-sinapoilquínino, que es característico del tipo de café robusta (*Coffea robusta*) (Jaiswal et al., 2010). Este hecho sugiere que existen diferencias en la ruta de síntesis de los ácidos fenólicos entre *S. muricatum* y *S. caripense*.

El resumen de los tres métodos empleados para determinar la capacidad antioxidante de las entradas estudiadas, indica que todas ellas presentan una capacidad antioxidante similar a la de la berenjena (Stommel y Whitaker, 2003), la cual se considera que tiene una elevada capacidad (Morales-Soto et al., 2014). Por lo que el consumo del pepino dulce contribuiría considerablemente a alcanzar la dosis diaria recomendable de estos compuestos.

Los extractos de zumo de estas entradas sobre los cultivos de macrófagos no mostraron citotoxicidad, ni siquiera sin diluir. Además estos extractos reducen significativamente la producción de óxido nítrico (NO) en células sometidas a un estrés oxidativo mediante lipopolisacaridos (LPS). Se ha sugerido que las sustancias que reducen la producción de NO en células estresadas tienen un efecto antiinflamatorio in vivo (Wang y Mazza, 2002).

4.5.- Contribución de esta tesis a la mejora y valorización del pepino dulce

Los mercados demandan una mayor variedad de frutas y hortalizas por lo que la introducción de nuevos cultivos es de interés. El estudio de nuevas especies, sus diferencias con el resto y las propiedades que puedan aportar, son esenciales para el éxito de estos nuevos cultivos.

El pepino dulce es una fruta que ha conseguido introducirse con éxito en varios países, donde es un producto muy valorado. Junto con esto, el desarrollo en los últimos años de variedades adaptadas al área de cultivo mediterránea, con una gran facilidad de manejo y una elevada calidad organoléptica, es lo que lo convierte en un cultivo prometedor para nuestra agricultura.

En este sentido se hacía necesario el desarrollo de herramientas de descripción fenológica y una caracterización detallada de los recursos genéticos disponibles, como la que se ha llevado a cabo en esta tesis. La secuenciación del transcriptoma proporciona herramientas que ayudarán en el conocimiento y facilitará el manejo de esta especie, y por último, se ha hecho especial énfasis en la correcta caracterización de su composición nutracéutica, ya que es el alto contenido en estos compuestos bioactivos su mayor virtud y lo que la diferencia del resto.

La concatenación de estos trabajos aquí presentados, reuniendo estudios en distintos aspectos, como el fenológico, morfológico, molecular, genómico, nutricional y nutracéutico, proporciona una base que permitirá el desarrollo de nuevas variedades adaptadas a nuestra área de cultivo, con una elevada calidad organoléptica y nutracéutica. Esta información, junto con una adecuada promoción de sus cualidades, permitirá el avance en la mejora genética de este cultivo y lo podrá situar en una posición de ventaja para competir por estar en las mesas de los consumidores europeos.

CONCLUSIONES GENERALES

5.- CONCLUSIONES GENERALES:

1.- La escala de caracterización fenológica BBCH, ha permitido identificar y clasificar los diferentes estados de desarrollo del pepino dulce; además se ha mostrado adecuada para detectar diferencias en caracteres de interés entre variedades evaluadas en un mismo estado fenológico.

2.- La caracterización usando descriptores morfológicos y marcadores microsatélites transferidos de tomate, ha revelado una enorme variabilidad en las entradas estudiadas, tanto en las de pepino dulce como en la de especies silvestres relacionadas.

3.- Empleando estás herramientas morfológicas y moleculares se ha podido diferenciar las especies silvestres de las entradas de la especie cultivada, y dentro de esta, se puede diferenciar las variedades modernas de las tradicionales. A este respecto las variedades modernas presentan menor diversidad que las tradicionales.

4.- La secuenciación del primer transcriptoma de pepino dulce ha permitido enriquecer enormemente la información genómica disponible en esta especie, siendo también de utilidad para sus especies cercanas, patata y tomate.

5.- La elevada calidad del transcriptoma presentado en esta tesis ha permitido llevar a cabo estudios comparativos en el género *Solanum*. La detallada anotación permitirá identificar genes que controlen caracteres de interés agronómico, y la gran cantidad de marcadores moleculares identificados serán de gran utilidad para la mejora del pepino dulce y especies cercanas.

6.- Se ha encontrado una elevada variabilidad en la composición nutricional en la colección de entradas estudiada, sugiriendo que existe un alto potencial de mejora en las mismas para estos caracteres, principalmente en las especies silvestres analizadas.

7.- Los elevados contenidos en polifenoles encontrados, así como de otros compuestos bioactivos, pueden ayudar a la introducción del pepino dulce en nuevos mercados preocupados por una alimentación más saludable.

8.- Tanto el pepino dulce, como la especie silvestre *S. caripense*, presentan una elevada actividad antioxidante y biológica. El perfil de

compuestos fenólicos difiere notablemente entre una especie y otra, así como entre variedades de la especie cultivada.

9.- Se han encontrado fuentes de variación para los parámetros de interés nutracéutico estudiados (contenido en polifenoles, actividad antioxidante y actividad biológica), habiéndose determinado que para una mejora combinada de estos tres parámetros sería necesario evaluar nuevas entradas de pepino dulce, o realizar cruzamientos complementarios entre las ya analizadas.

10.- El pepino dulce es una especie muy variable, al igual que sus especies silvestres relacionadas, a nivel morfológico, molecular y nutracético. Las herramientas desarrolladas y la información obtenida serán de utilidad en la mejora genética de esta especie y en el desarrollo de nuevos cultivares mejorados.

BIBLIOGRAFÍA GENERAL

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