Escuela Técnica Superior de Ingeniería Agronómica y de Montes

Departamento de Genética

Variabilidad genética en *Agropyron cristatum* y su uso en la mejora del trigo

Genetic variability in *Agropyron cristatum* and its use in wheat breeding

Tesis presentada por D. Alejandro Copete Parada para optar al título de

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Fdo. **Alejandro Copete Parada**

VºBº de la Directora
Fdo. Prof. Dra. Dña. Adoración Cabrera Caballero
Catedrática de la Universidad
E.T.S.I.A.M.

VºBº del Codirector
Fdo. Dr. D. Roberto Moreno Pinel
Investigador
E.T.S.I.A.M.

Universidad de Córdoba

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AUTOR: *Alejandro Copete Parada*

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Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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TÍTULO DE LA TESIS:

Variabilidad genética en *Agropyron cristatum* y su uso en la mejora del trigo

DOCTORANDO: D. Alejandro Copete Parada

INFORME RAZONADO DEL LOS DIRECTORES DE LA TESIS
(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La presente Tesis doctoral se ha realizado dentro del Programa de Doctorado "Biociencias y Ciencias Agroalimentarias" de la Universidad de Córdoba y se ha desarrollado en el marco del proyecto AGL2014-52445-R. La Tesis plantea como objetivo general estudiar la variabilidad genética en *Agropyron cristatum* y utilizar esta especie como recurso genético del trigo harinero con el fin último de ampliar la base genética de este cultivo. Durante esta Tesis se ha realizado una caracterización citológica, molecular y agro-morfológica de una colección mundial de accesiones de *A. cristatum*; se ha determinado la localización cromosómica de genes que confieren resistencia a oídio en *A. cristatum*; se ha identificado y localizado un gen de respuesta a la vernalización; se ha establecido un mapa comparativo entre trigo y *A. cristatum* y se han desarrollado introgresiones de *A. cristatum* en trigo harinero.

La Tesis se ha desarrollado de acuerdo con los plazos marcados, lo que ha permitido que hasta el momento ya se hayan publicado dos trabajos en revistas SCI y que haya otros dos manuscritos en preparación. Por todo ello, la tesis cumple los requisitos de calidad necesarios para su exposición y defensa pública.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de octubre de 2018

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Culmino una etapa más, pero comienzo otra nueva, la cual vendrá llena de desafíos, retos, metas y alegrías.

Muchas gracias a todos/as por todo!!

Alejandro Copete Parada

Abstract

Wheat is one of the most important crops of the world as it constitutes one of the main sources of food. The evolution of agriculture during the last century and the search for high-yield varieties have narrowed the genetic bases of crop, including wheat. This process originated an important genetic drift producing gradual disappearance of genes from crop fields. The Triticeae tribe species, which wheat (*Triticum* spp.) belongs, is an excellent genetic-diversity source to obtain new allelic variations required for breeding programs.

Wheat is one of the crops in which closely species and wild species have contributed with beneficial traits in the development of new varieties, mainly by providing genes for resistance to diseases and plagues. Among the species of the tribe Triticeae, *Agropyron cristatum* (L.) Gaertner (genome P) presents many interesting agronomic traits that can be transferred to wheat by crossing. The main objective of this PhD thesis is to evaluate the genetic variability in *A. cristatum* and to use this species as a genetic source in common wheat to increase the genetic basis of wheat. The general objective is approached from different specific objectives.

In chapter I, an *A. cristatum* world's collection of 115 accessions was characterized, the cytological, molecular and agronomic results obtained showed that there is high variability in this species. Diploid, tetraploid and hexaploid accessions were found, being the tetraploids the most frequent. A correlation between the geographic distribution and the ploidy level was obtained. The capillary electrophoresis evaluation using 6 short-sequence DNA repeats (SSR) showed high levels of polymorphism and 166 different alleles varying between 84 and 256 bp were obtained. The polymorphic information content (PIC) of these markers presents a range between 0.579 to 0.968. The high genetic variability detected is reflected in the high phenotypic variability of the collection, with differences between accessions for length and width of the spike and number of spikelets per spike.

In chapter II, the addition lines of *A. cristatum* in common wheat (*T. aestivum* L.) were evaluated for resistance to fungal diseases showing that the resistance to powdery mildew (*Blumeria graminis* F. tritici) in seedlings and adult plants stages is determined by genes located in both the chromosome arms 2PL and 6PL. A set of Conserved Orthologous Set (COS) molecular markers specific for both chromosome arms was developed. They were

used for the detection of introgressions of both chromosome arms in wheat genetic background.

In Chapter III the gametocide action of the gene/s from *Aegilops cylindrica* Host was used to induce genetic exchanges between the wheat genome and the genome of *A. cristatum.* Introgression lines were obtained in wheat background for chromosomes 4P, 5P and 6P. These lines were characterized cytologically by Fluorescence In Situ Hybridization (FISH) and by molecular markers. In addition, a set of COS markers specific for chromosomes 4P and 5P were developed, which, together with the specific COS markers of 6P chromosome mentioned in chapter II, were used to detect the chromosome introgressions obtained.

In Chapter IV, a candidate gene for the response to vernalization was identified and characterized (*VRN-P1*) in the “Ruff” variety of *A. cristatum.* The coding region alignment of the *VRN-P1* sequence of *A. cristatum* showed a similarity of 95% and 96% with the *VRN-A1* gene of wheat and *VRN-H1* gene of barley, respectively. The location of the *VRN-P1* gene in the 5PL chromosome arm showed that this gene is orthologous to wheat and barley *VRN1* gene. Additionally, the phylogenetic analysis of the deduced protein in species belonging to the Poaceae family revealed a closely genetic relationship between *VRN-P1* and the *VRN1* gene.

The localized COS markers, together with the *VRN-P1* gene, have allowed to establish a comparative mapping between *T. aestivum* and *A. cristatum*. These results indicate that the macrosynteny is well conserved between the chromosomes of both species, which suggests that the introgression of *A. cristatum* genes in the wheat genome is possible.

Keywords: *Agropyron cristatum*, COS, Genetic diversity, FISH, Introgression, Molecular markers, *Triticum aestivum*.

Resumen

El trigo es uno de los cultivos más importantes del mundo ya que constituye una de las principales fuentes de alimento. La evolución de la agricultura durante el siglo pasado y la búsqueda de variedades de alto rendimiento ha llevado al estrechamiento de la base genética de los cultivos, incluido el trigo. Este proceso produjo una deriva muy importante de genes causando su desaparición gradual de los campos de cultivo. Las especies de la tribu Triticeae, a la que pertenece el trigo (*Triticum* spp.), representan una excelente fuente de diversidad genética de la que extraer nuevas variaciones alélicas requeridas en los programas de mejora. El trigo es uno de los cultivos en los que las especies silvestres y relacionadas han contribuido con caracteres beneficiosos en el desarrollo de nuevas variedades, principalmente proporcionando genes de resistencia a enfermedades y plagas. Entre las especies de la tribu Triticeae, *Agropyron cristatum* (L) Gaertner (genoma P) presenta numerosos caracteres de interés agronómico que pueden transferirse al trigo mediante cruzamiento. La presente Tesis doctoral plantea como objetivo general evaluar la variabilidad genética en *A. cristatum* y utilizar esta especie como recurso genético del trigo harinero con el fin último de ampliar la base genética de este cultivo. Este objetivo se aborda desde diferentes objetivos específicos.

En el capítulo I se ha caracterizado una colección mundial de 115 accesiones de *A. cristatum* y los resultados citológicos, moleculares y agronómicos revelan que existe una gran variabilidad en esta especie. Se han encontrado accesiones diploides, tetraploides y hexaploides, siendo las tetraploides las más frecuentes, observándose una correlación entre la distribución geográfica y el nivel de ploidía. La evaluación por electroforesis capilar empleando 6 marcadores microsatélites (SSR) muestran altos niveles de polimorfismo, generándose 166 alelos diferentes con un rango entre 84 y 256 pb. El contenido de información polimórfica (PIC) de dichos marcadores presenta un rango entre 0.579 a 0.968. La alta variabilidad genética detectada se refleja en la gran variabilidad fenotípica de la colección encontrándose diferencias significativas entre accesiones para los caracteres longitud y ancho de la espiga y número de espiguillas por espiga.

En el capítulo II se evalúan las líneas de adición de *A. cristatum* en trigo común (*T. aestivum* L.) para resistencia a enfermedades fúngicas demostrándose que la resistencia a oídio (*Blumeria graminis* f. sp. *tritici*) en plántula y planta adulta está determinada por genes localizados en los brazos cromosómicos 2PL y 6PL. Se han desarrollado un conjunto de marcadores COS (Conserved Ortologous Set) específicos para ambos brazos cromosómicos,

útiles en la detección de introgresiones de ambos brazos cromosómicos en el fondo genético de trigo.

En el capítulo III se ha utilizado la acción gametocida del gen/es de *Aegilops cylindrica* Host para inducir intercambio entre el genoma de trigo y el genoma de *A. cristatum* obteniéndose líneas de introgresión en trigo para los cromosomas 4P, 5P y 6P. Estas líneas se han caracterizado citológicamente mediante hibridación *in situ* fluorescente (FISH) y mediante marcadores moleculares. De igual forma, se han desarrollado un conjunto de marcadores COS específicos para los cromosomas 4P y 5P, que junto con los marcadores COS específicos del cromosoma 6P mencionados en el capítulo II fueron útiles para detectar las introgresiones cromosómicas obtenidas.

En el capítulo IV se ha identificado y caracterizado un gen candidato de respuesta a la vernalización (*VRN-P1*) en la variedad "Ruff" de *A. cristatum.* El alineamiento de la región codificante de la secuencia *VRN-P1* de *A. cristatum* presenta una similitud del 95% y 96% con el gen *VRN-A1* de trigo y *VRN-H1* de cebada, respectivamente. La localización del gen *VRN-P1* en el brazo cromosómico 5PL indica que este gen es ortólogo al *VRN1* de trigo y cebada. Del mismo modo, el análisis filogenético de la proteína deducida en especies pertenecientes a la familia Poaceae, reveló una relación genética muy cercana entre *VRN-P1* y el gen *VRN1*.

Los marcadores COS localizados, junto con el gen *VRN-P1,* han permitido establecer un mapeo comparativo entre *T. aestivum* y *A. cristatum.* Los resultados obtenidos indican que se conserva la macrosintenia entre los cromosomas de ambas especies lo que sugiere que es posible la introgression de genes de *A. cristatum* en el genoma de trigo.

Palabras clave: *Agropyron cristatum*, COS, Diversidad genética, FISH, Introgresión, Marcadores moleculares, *Triticum aestivum*.

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Introducción general

El trigo

El trigo es uno de los cultivos de cereales, rico en carbohidratos, proteínas y minerales, que junto con el arroz y el maíz, constituyen la base alimenticia de la mayor parte de la población mundial. En 2016 se cultivaron en el mundo más de 220 millones de hectáreas de trigo con una producción de 759 millones de toneladas, siendo Asia y Europa los continentes con mayor superficie cultivada. España ocupó el vigésimo tercer lugar, con una siembra de 2.18 millones de hectáreas, alcanzando una producción de 6.43 millones de toneladas entre trigo duro y harinero (FAO 2017). Andalucía obtuvo la segunda mayor producción de trigo en España y ocupa el primer lugar en producción de trigo duro (58.2%) y sexto en trigo blando (8%) (FAO 2017).

El éxito del trigo como cultivo de importancia mundial en la alimentación se debe en gran medida a su adaptabilidad a un amplio rango de condiciones climáticas. Este hecho se atribuye en parte a su naturaleza alohexaploide, originada como resultado de dos eventos de hibridación. El primero de ellos se estima que ocurrió hace varios cientos de miles de años a partir del cruzamiento entre dos especies diploides silvestres: *Triticum urartu* Thum. ex. Gandil (2n=2x=14, AA) y una especie de la sección Sitopsis de *Triticum* que se considera relacionada con *Aegilops speltoides* Tausch (2n=2x=14=, BB). Como resultado de esta hibridación se originó el alotetraploide *Triticum turgidum* Linneo (2n=4x=28, AABB),un ancestro del trigo duro (*T. turgidum* sp. *durum*) cultivado para producción de pasta hoy en día. El segundo evento de hibridación ocurrió entre *T. turgidum* y la especie diploide *Aegilops tauschii* Cosson (2n=2x=14, DD) que dio lugar al trigo alohexaploide (2n=6x=42, AABBDD) cultivado actualmente como trigo harinero (*Triticum aestivum* Linneo). El trigo harinero o trigo común, con 21 pares de cromosomas es estructuralmente un alohexaploide, con tres juegos homeólogos de cromosomas cada uno con 7 pares cromosómicos pertenecientes a los genomios o subgenomios A, B y D. Sin embargo, el trigo se comporta meióticamente como un diploide debido a la acción de los genes *Ph* que impiden el apareamiento de cromosomas homeólogos (Martínez-Pérez et al. 1999).

Los recursos genéticos del trigo

El aumento de la población actual y la reducción de la tierra cultivable constituyen factores determinantes para formular propuestas de solución que garanticen la seguridad alimentaria mundial. La revolución verde iniciada a mediados del siglo XX se caracterizó por el aumento de los rendimientos en los cultivos que incrementaron la producción por unidad de área a partir del ejercicio de una agricultura de alta intensidad basada en el uso elevado de insumos (Pingali 2012). Se impuso la utilización de variedades homogéneas genéticamente, por lo que buena parte del material genético presente en las variedades locales se perdió o quedó confinado a espacios reducidos. La pérdida o reducción de la variabilidad genética en las especies cultivadas puede limitar y comprometer la obtención de variedades adaptadas a nuevas condiciones ambientales futuras. Los avances de un programa de mejora sólo pueden conseguirse cuando se cuenta con variabilidad genética para los caracteres deseables. Actualmente se están desarrollando diversas iniciativas europeas y nacionales que tratan de incrementar la variabilidad genética de las especies cultivadas para adaptar la agricultura al cambio climático haciendo uso de las especies emparentadas con las cultivadas. Una de estas iniciativas pretende garantizar la seguridad alimentaria mediante la recolección, conservación, evaluación y uso de especies cercanas a las cultivadas para incrementar la base genética de 29 cultivos (entre ellos el trigo) incluidas en el Anexo 1 del Tratado Internacional sobre los Recursos Fitogenéticos para la Alimentación y la Agricultura (Dempewolf et al. 2014). Otras iniciativas pretenden desbloquear el potencial de la diversidad de los cultivos y especies relacionadas presentes en bancos de germoplasma existentes hoy en día, como por ejemplo “Diversity Seek” (DivSeek) (Meyer 2015).

Las especies emparentadas a las cultivadas se agrupan mediante el sistema de clasificación formulado por Harlan y De Wet en 1971, que permite establecer diferencias entre las especies silvestres de las cultivadas, en consideración con la facilidad del intercambio de genes. Las especies que pertenecen al acervo genético primario son aquellas que presentan genomios homólogos, por lo tanto pueden cruzarse directamente con el cultivo. El acervo primario en el trigo incluye variedades hexaploides cultivadas y las especies silvestres diploides donadores de los subgenomas A, B y D del trigo hexaploide, como *T. urartu, Ae. speltoides* y *Ae. tauschii.* La transferencia de genes del acervo genético primario al cultivo suele ser

sencilla y puede realizarse mediante cruzamiento directo, recombinación homóloga, retrocruzamiento y selección.

El acervo genético secundario del trigo lo constituyen los parientes poliploides de las especies *Triticum* y *Aegilops* que comparten al menos un genomio con el trigo. La transferencia de genes por recombinación homóloga es posible si el gen está localizado también en el mismo grupo homeólogo. Este grupo también incluye la especie tetraploide *T. timopheevii* Zhuk. y las especies diploides de *Aegilops* de la sección *Sitopsis* que contiene el genoma S y que están relacionadas con el genomio B de trigo.

El acervo genético terciario incluye especies cuyo parentesco es aún más lejano y los genomios de estas especies tienen un menor grado de homología con el trigo. Entre ellas se encuentran, por ejemplo, especies pertenecientes a los géneros *Hordeum, Secale* y *Agropyron*. En este caso deben emplearse otras estrategias en un programa de introgresión puesto que la transferencia de genes no se puede lograr a través de la recombinación homóloga (Kumar-Chaudhary et al. 2014).

Las especies incluidas en los acervos genéticos secundario y terciario suelen presentar diversas barreras precigóticas y postcigóticas que dificultan la transferencia de genes de dichas especies al trigo. Las barreras precigóticas previenen la unión de los gametos para formar un cigoto y pueden deberse a causas como la germinación inadecuada del polen o la inhibición del crecimiento del tubo polínico en el estilo (Khush y Brar 1992). Las barreras reproductivas postcigóticas conducen a anormalidades después de la fertilización causando inviabilidad del híbrido o híbridos débiles, aborto de embriones en etapas jóvenes o esterilidad causada por falta de apareamiento cromosómico debido a la falta de sintenia (Khush y Brar 1992; Prohens et al. 2017). Para superar estas barreras y evitar el aborto embrionario y obtener híbridos sexualmente viables, los mejoradores han desarrollado diversas técnicas como el rescate de embriones inmaduros, la fertilización *in vitro* o la fusión de protoplastos.

La introgresión de genes a través de la hibridación interespecífica, particularmente en especies más lejanas evolutivamente, a menudo se dificulta por una limitada recombinación entre los cromosomas de la especie donadora y la cultivada. Para superar esta barrera se han

desarrollado varios métodos que promueven la rotura de cromosomas y el intercambio de genes. Los tratamientos con irradiación han sido utilizados para generar introgresiones, la primera obtenida usando este método fue llevada a cabo por Sears (1956) para transferir un gen de resistencia a roya de la hoja de *Ae. umbellulata* Zhuk al trigo.

El apareamiento de cromosomas homólogos y la recombinación en el trigo están regulados por el gen *Ph1,* el principal regulador del apareamiento de cromosomas durante la meiosis. El locus está localizado en el brazo largo del cromosoma 5B (Feldman y Sears 1981; Qu et al. 1998; Roberts et al. 1999). Este gen mantiene la estabilidad del genoma y evita el apareamiento entre cromosomas homeólogos. Sin embargo, el uso de trigos mutantes para el gen *Ph1* facilita la introgresión de genes provenientes de parientes lejanos mediante recombinación entre cromosomas homeólogos. Sears (1977) fue el primero en utilizar los mutantes *Ph1* para introgresar material genético de especies más lejanas evolutivamente en el genoma del trigo (Sears 1977).

La introgresión de genes de una especie donadora al trigo también se puede llevar a cabo mediante cromosomas gametocidas. En 1988, Endo publica un sistema genético único en el trigo denominado sistema gametocida (*Gc*) en el que ciertos cromosomas de especies del género *Aegilops* que contienen genes *Gc* inducen roturas cromosómicas en los cromosomas de trigo. Cuando el cromosoma gametocida se encuentra en el fondo genético del trigo en monosomía, la planta produce dos tipos de gametos, uno con cromosomas Gc y el otro sin ellos. Las roturas cromosómicas ocurren solamente en este último caso. Tales reestructuraciones cromosómicas pueden ser semiletales y dar lugar a cigotos viables que contienen cambios estructurales cromosómicos. Este método se ha utilizado para producir translocaciones entre distintas especies y el trigo como por ejemplo, cebada y centeno (Masoudi-Nejad et al. 2002; Endo 2009).

Uno de los retos a superar en los programas de introgresión es la "carga de ligamiento". A menudo los fragmentos introgresados pueden contener genes que confieren un fenotipo no deseado y que están ligados al gen/es que controlan los caracteres deseados. El uso de mutantes del gen *Ph1* permite reducir el tamaño de los fragmentos introgresados reduciendo de este modo la carga de ligamiento y la eliminación de genes no deseados. No obstante, el

uso de especies silvestres ha permitido significantes avances en la mejora del trigo para numerosos caracteres, particularmente para aquellos que tienen un control genético sencillo tales como la resistencia o tolerancia a enfermedades y plagas (Hajjar y Hodgkin 2007; Maxted y Kell 2009).

Algunos ejemplos que demuestran el éxito de la hibridación interespecífica en la obtención de nuevas variedades de trigo son la introgresión del gen *Lr19* de resistencia a la roya de la hoja de *Agropyron elongatum* (Host) Beauvois en trigo (Sharman y Knott 1966) que además también incrementa el rendimiento (Bedő y Láng, 2015) o la transferencia de resistencia a oídio (gen *Pm21*) mediante la translocación 6VS-6AL procedente de *Haynaldia villosa* (L.) Schur. (Chen et al. 1995). Un ejemplo bien conocido es la introgresión en trigo es la translocación 1RS/1BL. El brazo corto del cromosoma 1R de centeno contiene genes que aumentan el rendimiento de grano y proporcionan resistencia a las principales enfermedades causadas por roya, incluyendo los genes *Lr29*/*Yr26* de resistencia a roya de la hoja y roya amarilla, una mejor adaptación y tolerancia al estrés, mayor biomasa y un incremento en el peso del grano (Zarco-Hernández et al. 2005). Se ha estimado que entre 1991 y 1995, el 45% de las 505 variedades de trigo harinero cultivadas en 17 países lleva esta translocación trigo-centeno (Rabinovich 1998).

Método de detección de introgresiones

Las técnicas citogenéticas constituyen una herramienta útil que facilita no sólo la detección de introgresiones sino también la selección de plantas con números cromosómicos equilibrados. La Hibridación In situ Fluorescente (FISH: Fluorescence *in situ* Hybridization) es una técnica basada en la complementariedad de pares de bases del ADN y emplea moléculas fluorescentes para localizar fragmentos de ADN sobre una preparación de cromosomas. Una variante de esta técnica es la hibridación *in situ* genómica (GISH: Genomic *in situ* Hybridization) en la que se usa el ADN genómico total de una especie como sonda.

Los marcadores moleculares constituyen una herramienta valiosa en los programas de mejora ya que permiten la selección de los genotipos deseados. Presentan una serie de ventajas que no poseen otro tipo de marcadores (como los morfológicos), entre ellas, ser prácticamente ilimitados en número y que no se afectan por el medio ambiente ni por el estado de desarrollo

de la planta (Collard et al. 2005). Además, se pueden trabajar muestras biológicas pequeñas y facilitan la evaluación en estados de desarrollo temprano, reduciendo tiempo, dinero y por tanto aumentando la eficiencia de los programas de mejora (Agarwal et al. 2008).

Actualmente los marcadores moleculares más utilizados se basan en la reacción en cadena de la polimerasa (PCR: Polimerase Chain Reaction). Entre ellos, los marcadores microsatélites (SSR: Simple Sequence Repeat) son secuencias cortas repetidas en tándem, muy abundantes en el genoma, poseen una gran diversidad alélica y tienen una buena reproducibilidad (Saghai-Maroof et al. 1994). Gracias a la secuenciación del genoma se ha podido hacer uso de marcadores SNPs (Single Nucleotide Polymorphism) que son variaciones nucleotídicas únicas en una secuencia genómica, son los marcadores moleculares más abundantes en el genoma y son muy adecuados para la construcción de mapas genéticos de alta densidad y estudios de diversidad (Agarwal et al. 2008).

Las tecnologías de secuenciación han evolucionado notablemente de modo que se ha logrado secuenciar el genoma completo de especies cultivadas como el maíz, arroz y más recientemente el trigo (Zimin et al. 2017; International Wheat Genome Sequencing Consortium, 2018). La sintenia que existe entre las especies de la tribu Triticeae y en general en los cereales (Moore et al. 1995; Gale y Devos 1998) posibilita la transferencia de marcadores moleculares entre especies. Entre ellos, los marcadores COS (Conserved Orthologous Set) desarrollados en arroz y transferidos al trigo son marcadores altamente conservados y apropiados para estudios de mapeo comparativo (Quraishi et al. 2009). Además, constituyen una herramienta importante para el análisis de la variabilidad genética y para la selección asistida por marcadores en los programas de mejora.

Agropyron cristatum

El género *Agropyron* pertenece, como el trigo, a la tribu Triticeae *y* agrupa un complejo de especies que se caracterizan por presentar polinización cruzada, aspecto cespitoso y condición perenne, cuyo genoma básico se denomina P (Asay y Jensen 1996). *A. cristatum* (L.) Gaertner es la especie más representativa de este género y fue descrita por Linneo por primera vez con el nombre *Triticum cristatum*. La denominación latina *cristatum* significa "crestado" y

alude a su inflorescencia en forma de cresta (Johnson y Smith 1972). Dentro de la especie existen biotipos diploides (2n=2x=14), tetraploides (2n=4x=28) y hexaploides (2n=6x=42), cuyo número básico de cromosomas es x=7.

A. cristatum se originó en el interior de Europa y Asia (Dewey 1984) y actualmente su distribución es muy amplia. En América del Norte ha sido introducida y se cultiva extensamente como especie forrajera en todas las regiones áridas y semiáridas del oeste norteamericano desde Alaska, Canadá hasta California (Withman y Barker 1994). Existen variedades cultivadas de los tres niveles ploídicos, si bien las variedades diploides (como "Ruff' y "Parkway") y tetraploides (como "Ephraim") se cultivan más extensamente. Los programas de mejora de esta especie se centran principalmente en lograr una mejor distribución estacional del crecimiento, la selección de tolerancia a factores abióticos, como sequía y salinidad y mejora del rendimiento y la calidad del forraje (Dewey 1962; Rogler y Lorenz 1983; Robins et al. 2007). Un objetivo de mejora importante es el retraso de la fecha de floración ya que está relacionado directamente con la calidad del forraje (Asay 1986). Además, *A. cristatum* se está utilizado con éxito en el manejo de cuencas fluviales (Wang 2011), la estabilización de suelos afectados por erosión o por la contaminación de metales pesados (Miller y Dyer 2002; Meng et al. 2013; Guo et al. 2014).

A. cristatum como recurso genético para la mejora de trigo

Las mayores pérdidas de producción en el cultivo de trigo suelen ser debidas a enfermedades como las royas (*Puccinia striiformis, P. triticina y P. graminis*) y el oídio (*Blumeria graminis*). Aunque se han identificado un buen número de genes que confieren resistencia a esas enfermedades (McIntosh et al. 2016), muchos de esos genes introgresados en trigo se han vuelto inefectivos como consecuencia de la aparición de nuevas razas virulentas del patógeno. Por lo tanto, la búsqueda de nuevas fuentes de resistencia y la combinación de más de un gen de resistencia es un objetivo importante de cara a ampliar la base genética del trigo.

A.cristatum es considerada como un pariente silvestre del trigo cultivado y lo convierte en un donador potencial de genes para la mejora del trigo. Se han identificado un buen número de genes que controlan caracteres de interés agronómico en *Agropyron* tales como los genes que controlan la resistencia al virus del enanismo amarillo de la cebada (Shukle et al. 1987),

virus del mosaico estriado de trigo (Friebe et al. 1991), la roya amarilla, roya de la hoja y roya del tallo (Friebe et al. 1992); junto con la tolerancia al frío (Limin y Fowler 1987), a la salinidad (McGuire y Dvorak 1981) o a la sequía (Dewey 1962). Sin embargo, hasta la fecha son escasos los intentos sistemáticos para utilizar estos genes en la mejora de trigo. Esto contrasta con el éxito en la transferencia de genes de resistencia a enfermedades y plagas de trigo de otros miembros del género *Triticum*, como *T. urartu* y *T. monococcum* y de otros géneros incluyendo *Secale*, *Aegilops* y *Thinopyrum* (Feuillet et al. 2008).

A. cristatum se ha cruzado con trigo lo que ha permitido el desarrollo de líneas de adición de esta especie en trigo harinero (Chen et al. 1989; Jubault et al. 2006). Las líneas de adición constituyen un germoplasma de gran valor para determinar la localización cromosómica de genes de interés agronómico así como también para transferir estos genes al trigo. Por ejemplo, se han localizado genes que determinan un mayor número de espiguillas por espigas en el cromosoma 6P (Wu et al. 2006; Zhang et al. 2015), tolerancia a la sequía y al frío en el cromosoma 4P (Liu et al. 2010) y 7P (Lu et al. 2016) y resistencia la roya de la hoja en el cromosoma 1P (Ochoa et al. 2015), 2P (Li et al. 2016) y 6P (Song et al. 2016).

También se han obtenido híbridos y anfiploides fértiles entre especies del género *Triticum* y *A. cristatum* con el objetivo de utilizarlos como especies puente para transferir características de interés agronómico de *A. cristatum* al trigo (Martín et al. 1999; Soliman et al. 2001, 2007). Utilizando el anfiploide (*xAgroticum*, AABBDDPP) como especie puente mediante un programa de retrocruzamientos de ese octoploide con trigo harinero se obtuvo una línea fértil portadora de una translocación 1PS·1BL y que confiere resistencia a la roya de la hoja (*Puccinia triticina*) (Ochoa et al. 2015).

Dentro del marco del proyecto AGL2014-52445-R y en colaboración con el CIMMYT (Centro Internacional de Mejora de Maíz y Trigo) y la empresa Agrovegetal SA se ha llevado a cabo una evaluación para resistencia a enfermedades fúngicas de las líneas de adición de *A. cristatum* en trigo harinero (1P-6P) durante la campaña agrícola 2016/17. Los resultados de estas evaluaciones han permitido determinar que todas las líneas de adición (con excepción de la línea de adición del cromosoma 2P) muestran buenos niveles de resistencia a roya del tallo. Además, las líneas de adición de los cromosomas 1P y 6P, respectivamente,

presentan resistencia a la roya de la hoja y roya amarilla. Todo ello, hace de *A. cristatum* un donador potencial de genes de interés agronómico que permita ampliar la base genética del trigo.

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Objetivos

Objetivos

La presente Tesis plantea como objetivo general evaluar la variabilidad genética en *Agropyron cristatum* y utilizar esta especie como recurso genético del trigo harinero con el fin último de ampliar la base genética de este cultivo. Este objetivo se aborda desde los siguientes objetivos específicos:

1. Caracterizar una colección mundial de germoplasma de *A. cristatum.*

2. Localizar las regiones cromosómicas responsables de la resistencia a oídio (*Blumeria graminis*) en *A. cristatum*

3. Obtener un conjunto de marcadores moleculares capaces de detectar los cromosomas de *A. cristatum* en el fondo genético de trigo.

4. Desarrollar líneas de introgresión de *A.cristatum* en trigo.

5. Aislar y caracterizar un gen candidato (*VRN-P1*) de respuesta a la vernalización en *A. cristatum.*

Chapter I

Characterization of a world collection of *Agropyron cristatum* accessions

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Abstract

The genetic diversity was studied of 115 *Agropyron cristatum* accessions from 17 countries. Tetraploids were the most common (74.8%), followed by diploid (18.3%) and hexaploid (6.9%). We observed a relation between geographic distribution and ploidy level. The tetraploids, the most widespread, were found from Europe through Russia to East Asia. The diploids appeared over the same general range, except in Turkey, Iran and Georgia where no diploid accessions were found. Hexaploid accessions mainly came from a region comprising the east of Turkey, the north of Iran and Georgia. A selection of 71 accessions, including all three ploidy levels, were analyzed by capillary electrophoresis using six wheat simple sequence repeat (SSR) markers. All markers presented high levels of polymorphism, generating 166 different alleles ranging in size between 84 and 256 bp. Based on polymorphic information content values obtained (0.579 to 0.968), all the SSRs were classified as informative markers (values > 0.5). According to the dendrogram generated, all the *A. cristatum* accessions were distinctly classified. Diploid, tetraploid and hexaploid accessions are not clearly differentiated from each other on the basis of SSR markers. A field experiment was conducted to morphologically characterize 18 accessions including the three ploidy levels. Significant differences were found between the accessions in spike length, spike width and number of spikelets per spike. All the cytological, molecular, and morphological data demonstrate the high genetic diversity present in *A. cristatum*, making it a valuable resource for future breeding programs.

Keywords: *Agropyron cristatum,* crested wheatgrass*,* ploidy level, flow cytometry, genetic diversity, SSR

Introduction

The genus *Agropyron* includes the crested wheat-grass complex (cross-pollinated, caespitose, long-lived perennials), *A. cristatum* (L.) Gaertner being the most representative species. This species shows tolerance to cold, drought and high salinity and is one of the most important grasses in temperate regions (Asay and Jensen 1996). Although native to Europe and Asia, the species has been introduced and widely cultivated in North America as an excellent source of forage and is a component of the diet of beef and dairy cattle worldwide (Asay et al. 2003). It has also been valued for watershed management (Wang 2011) and stabilization of heavy metal-contaminated soils (Miller and Dyer 2002; Meng et al. 2013; Guo et al. 2014).

Agropyron cristatum is a Triticeae species and has diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) and hexaploid (2n = 6x = 42) forms, all based on the basic genome P (Asay and Jensen 1996). Breeding programs are used to improve the existing cultivars for both yield and quality (Asay et al. 2003; Gul et al. 2013), and *A. cristatum* (genome P) has been a source of genes for pest resistance and abiotic stress in wide hybridization programs to improve cereal crops (Asay 1992; Dong et al. 1992). Hybrids between *Triticum aestivum* L. and *A. cristatum* (Limin and Fowler 1990; Chen et al. 1989) have been obtained with the final aim of introducing traits from *A. cristatum* into *Triticum*. Both durum and common wheat—*A. cristatum* introgression lines have been developed and they could be a useful source of agronomic traits such as disease resistance (Soliman et al. 2001, 2007; Ochoa et al. 2015) and thousand-grain weight (Zhang et al. 2015).

Characterization of the genetic diversity of germplasm is fundamental for addressing questions about evolutionary processes, the development of conservation strategies and the efficient use of genetic resources in plant breeding programs. In *A. cristatum*, genetic variability studies have been performed based on morphological and agronomic traits (Knowles 1955; Dong et al. 1992; Ray et al. 1997; Mellish and Coulman 2002). Differences in DNA sequences detected by random amplified polymorphic DNA markers helped to characterize five diploid Spanish populations of *A. cristatum* (García et al. 2002). Markers with a higher discriminatory power and showing more repeatable patterns, such as amplified fragment length polymorphisms (AFLP) and simple sequence repeat (SSR) markers, were

used to study genetic diversity and to examine the relationships within and among populations from different *Agropyron* spp. (Mellish et al. 2002; Che et al. 2008, 2011). Mellish et al. (2002) examined the interpopulation relationships from *A. cristatum, A. desertorum* (Fisch. ex Link) Schult. *A. fragile* (Roth) Candargy and *A. mongolicum* Keng using AFLP markers. These authors found that majority (88%) of the AFLP variance was within populations. Che et al. (2008, 2011) used SSR to study genetic variation in different populations of *A. cristatum* originated from various regions of northern China and they found that 83% of the total variation was within regions. More recently, seed storage protein analysis was also introduced to assist analysis of phylogenetic relationships among accessions of crested wheatgrass species (Chen et al. 2013; Yousofi et al. 2013). Ploidy level information is also important for biodiversity and evolutionary studies as well as having a great practical importance for breeders. Karyotype analysis and determination of ploidy level have been carried out to study taxonomy and evolution in *A. cristatum* (Yang et al. 2014; Yousofi and Aryavand 2004).

The aims of this study were (1) to determine the ploidy levels of a world collection of *A. cristatum* accessions (2) to study the genetic variation employing SSR markers and (3) to evaluate morphological traits in a selected sample of accessions based on the results obtained in both (1) and (2).

Material and Methods

Plant material

A collection of 115 *A. cristatum* accessions were employed in this work. Seeds of 110 and 5 accessions were respectively supplied by the United States Department of Agriculture (USDA) and the Crop Research Institute in Prague, Czech Republic (Table 1). All entries were wild genotypes, except for four ("Ruff", "Parkway", "Farway" and "Ephraim") that were cultivars. The country of origin of all *A. cristatum* accessions and precise geographic source of 91 of these accessions were obtained from the Germplasm Resources Information Network (https://www.ars-grin.gov) and the Plant Genetic Resources Documentation in the Czech Republic (EVIGEZ) (http://genbank.vurv.cz/genetic/resources) databases. The accession number and the country in which each accession was collected are listed in the Table 1.

Table 1. Accessions of *A. cristatum* studied including code in the collection, accession number, origin, ploidy level and number of total alleles detected with six microsatellites.

Lab code	Accession number	Country of origin	Ploidy level	No. of alleles
Ac001	PI 109012 ("Ephraim")	United States	4x (28)[a]	23
Ac003	PI 172691	Turkey	4x (28)	27
Ac005	PI 173621	Turkey	4x (28)	26
Ac006	PI 173622	Turkey	6x (42)	26
Ac007	PI 173623	Turkey	6x (42)	-
Ac012	PI 206396	Turkey	4x (28)	-
Ac013	PI 222953	Iran	4x (28)	23
Ac016	PI 229521	Iran	4x (28)	26
Ac017	PI 229572	Iran	4x	24
Ac018	PI 229907	Iran	4x (28)	19
Ac020	PI 229909	Iran	4x (28)	-
Ac022	PI 235240	Spain	4x (28)	-
Ac025	PI 251096	Macedonia	4x (28)	24
Ac030	PI 277352	Russian Federation	2x (14)	18
Ac031	PI 279802 ("Farway")	Canada	2x	16
Ac033	PI 297869	Sweden	2x (14)	14
Ac035	PI 311004	Romania	4x (35)	-
Ac044	PI 314602	Russian Federation	4x (18)	-
Ac057	PI 368030	Turkey	4x (28)	29
Ac063	PI 380617	Iran	6x (42)	-
Ac064	PI 380618	Iran	6x (42)	29
Ac072	PI 392333	Uzbekistan	4x (28)	30
Ac074	PI 400999	Turkey	4x (28)	29
Ac075	PI 401000	Turkey	4x	25
Ac097	PI 401059	Iran	4x (28)	-
Ac098	PI 401060	Iran	4x (28)	25
Ac099	PI 401061	Iran	4x (28)	24
Ac106	PI 401068	Iran	4x (28)	-
Ac115	PI 401078	Iran	6x (42)	32
Ac121	PI 401084	Iran	6x (42)	27
Ac123	PI 401086	Iran	4x (42)	-
Ac130	PI 407559	Turkey	4x (28)	25
Ac131	PI 415799 ("Parkway")	Canada	2x (14)	-
Ac132	PI 429771	Kazakhstan	4x (28)	24
Ac141	PI 439914	Russian Federation	4x (28)	-
Ac142	PI 439915	Russian Federation	4x (28)	-
Ac149	PI 439923	Russian Federation	4x (28)	19
Ac154	PI 39929	Russian Federation	4x (14)	26
Ac155	PI 439931	Kazakhstan	2x (28)	27
Ac166	PI 439944	Kazakhstan	4x (28)	-
Ac168	PI 439946	Kazakhstan	4x	29
Ac177	PI 486163	Ukraine	4x	29
Ac180	PI 499381	China	4x (28)	27
Ac181	PI 499382	China	4x (28)	17
Ac182	PI 499383	China	4x (28)	29
Ac185	PI 499386	China	6x (28)	-
Ac189	PI 499390	China	4x (28)	20

Lab code	Accession number	Country of origin	Ploidy level	No. of alleles
Ac191	PI 499567	China	4x	26
Ac194	PI 516481	Morocco	4x	23
Ac195	PI 516482	Morocco	4x	25
Ac196	PI 531533	China	4x	27
Ac202	PI 531539	United States	4x (28)	-
Ac205	PI 547269	Kazakhstan	4x	34
Ac206	PI 547270	Kazakhstan	4x	-
Ac207	PI 547271	Kazakhstan	4x	21
Ac210	PI 547274	Kazakhstan	4x	-
Ac212	PI 547276	Kazakhstan	4x	33
Ac216	PI 547280	Kazakhstan	4x	-
Ac227	PI 547346	China	4x	-
Ac234	PI 564853	China	4x	27
Ac239	PI 564860	Russian Federation	4x	-
Ac242	PI 564862	Russian Federation	4x	26
Ac247	PI 564867	Russian Federation	2x	20
Ac248	PI 564868	Russian Federation	4x	25
Ac249	PI 564869	Russian Federation	4x	32
Ac250	PI 564870	Russian Federation	4x	25
Ac253	PI 564873	Russian Federation	4x	29
Ac254	PI 564874	Kazakhstan	4x	24
Ac255	PI 564875	Russian Federation	4x	30
Ac257	PI 564877	Kazakhstan	4x	25
Ac258	PI 564878	Russian Federation	4x	35
Ac262	PI 578518	Turkey	4x	32
Ac264	PI 598474	Russian Federation	4x	22
Ac265	PI 598483	China	4x	20
Ac266	PI 598488	China	4x	-
Ac269	PI 598550	China	4x	-
Ac272	PI 598632	Kazakhstan	2x	18
Ac273	PI 598633	Kazakhstan	2x	16
Ac274	PI 598634	Kazakhstan	2x	14
Ac281	PI 598639	Kazakhstan	4x	21
Ac289	PI 598716	Kazakhstan	2x	-
Ac290	PI 610837	Mongolia	4x	-
Ac293	PI 610842	Mongolia	2x	-
Ac294	PI 610846	Mongolia	4x	-
Ac289	PI 598716	Kazakhstan	2x	-
Ac298	PI 610871	Mongolia	4x	-
Ac299	PI 610874	Mongolia	4x	-
Ac300	PI 610878	Mongolia	4x	25
Ac302	PI 610894	Mongolia	4x	28
Ac304	PI 619440	China	4x	-
Ac308	PI 619556	Mongolia	4x	-
Ac310	PI 628671	Mongolia	2x	-
Ac311	PI 628672	Mongolia	4x	-
Ac312	PI 628678	Ukraine	4x	25
Ac304	PI 619440	China	4x	-
Ac313	PI 628683	Ukraine	2x	22
Ac314	PI 628689	Mongolia	4x	21

Lab code	Accession number	Country of origin	Ploidy level	No. of alleles
Ac315	PI 628690	Mongolia	2x	-
Ac317	PI 222957	Iran	4x (28)	-
Ac319	PI 632509	Turkey	4x	21
Ac320	PI 632533	Kazakhstan	4x	22
Ac324	PI 610836	Mongolia	4x	-
Ac326	PI 499388	China	2x (14+B)	13
Ac327	PI 499389	China	2x (14)	-
Ac328	PI 564857	Russian Federation	2x	23
Ac329	PI 578519 (“Ruff”)	United States	2x (14)	19
Ac330	W6 25117	Kazakhstan	4x	-
Ac331	PI 494615	Romania	2x	-
Ac332	W6 19302	Bulgaria	4x	-
Ac334	PI 598631	Kazakhstan	2x	-
Ac335	PI 499381	China	4x (28)	-
Ac336	01C2800010[b]	Georgia	4x	-
Ac337	01C2800011[b]	Georgia	4x	22
Ac338	01C2800012[b]	Georgia	6x	32
Ac341	01C2800018[b]	Turkey	4x	28
Ac344	01C2800021[b]	Russian Federation	4x	30
Ac350	PI 636511	Bulgaria	2x	-

[a]Number in parenthesis is the chromosome number given by the Germplasm Resources Information Network, within the USDA ("https://training.ars-grin.gov/gringlobal/search.aspx”)
[b]Accessions from the germplasm collection of the Crop Research Institute (Prague), Gene Bank Department, Czech Republic ("http://genbank.vurv.cz/genetic/resources/”) (EVIGEZ databases).

Ploidy analysis

The ploidy level of the 115 *A. cristatum* accessions was determined by estimating the relative DNA content using flow cytometry (Ploidy Analyser PA-I; Partec GmbH, Munster, Germany). For this analysis, 0.5 $cm^2$of young leaves was chopped with a razor blade for 30–60 s to release nuclei in a Petri dish containing 0.4 mL of nuclei isolation buffer (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50-µm nylon mesh (Partec 50-µm CellTrics disposable filter), and then, nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer). Finally, the samples were analyzed after 30 s of incubation. Barley (*Hordeum vulgare* ‘Betzes’, 2n = 2x= 14) was used as an internal reference. Approximately 0.25 $cm^2$of young barley leaves was chopped together with the *A. cristatum* samples. One plant per accession was evaluated and two measurements were obtained from each plant. The nuclear DNA content (2C value)

of diploid, tetraploid and hexaploid accessions was calculated relative to the value for *H. vulgare* as in Yousofi et al. (2004) and Döležel et al. (2007).

Chromosome counting

For somatic chromosome counting, 1-cm long root tips were collected from germinating seeds and pre-treated in ice-water for 24 h. They were fixed in a freshly prepared solution of ethanol-acetic acid (3:1) for 1 week and stained by the Feulgen technique.

SSR analysis and genetic variation

First, 29 primers pairs developed in wheat (*Triticum aestivum* L.) by Röder et al. (1998) and transferred to *A. cristatum* by Che et al. (2008) were screened for polymorphism in 15 diploid accessions of *A. cristatum* from different countries (unpublished results). Based on both polymorphism and accuracy, six SSR markers were selected and employed to evaluate genetic variation in 71 *A. cristatum* accessions with different ploidy levels, including both wild populations and cultivars (Table 1).

Total genomic DNA from each plant was extracted from young leaves using the protocol described by Murray and Thompson (1980). To prepare DNA samples for each accession, equal amounts of leaves from five individual plants of each accession were bulked. This population size (n=5) is be enough to detect (P=0.05) the most frequent alleles ($p \geq 0.5$) present in each accession (Hanson 1959). DNA samples were stored at -20ºC until amplifications by PCR. The DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

PCR amplification was performed in a 10-µl reaction mixture containing 20 ng of genomic DNA, 5X polymerase buffer, 2 mM $MgCl_2$, 0.8 mM of dNTP, 0.125 µM of forward primer, 0.25 µM of reverse primer, 0.25 µM of fluorochromes (6-HEX, FAM or NED) and 1 U of Taq polymerase (Promega). Forward primers were synthesized with a 19 bp long 5´ M13 tail (5′-CACGACGTTGTAAAACGAC-3′). Amplification cycles were performed in a Perkin Elmer Cetus DNA Thermal Cycler (9600) programmed as follows: (A) 1 min denaturation at 94ºC, (B) 35 cycles of denaturation at 94ºC for 1 min, annealing at 53ºC for 1 min and polymerization at 72ºC for 1.5 min; (C) followed by a 10 min final extension at 72ºC.

The PCR products were separated using an automated capillary sequencer ABI3130 Genetic Analyzer (Applied Biosystems/HITACHI, Madrid, Spain) in the Genomics Unit of the Central Research Support Service at the University of Córdoba. The size of the amplified fragments was calculated based on an internal DNA standard (400HDROX) with GeneScan software (version 3.x) and the results were analyzed using Genotyper software (Version 3.7) all from Applied Biosystems. The number of different alleles and polymorphic information content (PIC) were also computed (Botstein et al. 1980). The Dice coefficient was calculated as a measure of similarity between accessions. Cluster analysis was performed using the unweighted pair group method with arithmetic means (UPGMA), a hierarchic agglomerative method equivalent to the average linkage between groups method. A dendrogram was calculated using the NTSYS (from Numerical Taxonomy System) software, Version 2.0 (Applied Biostatistics, Setauket, USA).

Morphological characteristics

We selected 24 *A. cristatum* accessions for field trails. These accessions were selected based on ploidy level (diploid, tetraploid and hexaploid) and grouped in different clusters in the dendrogram. The seedlings were vernalized at 4ºC for four weeks and 8 h photoperiod in a growth chamber. After this, they were moved to the field under natural conditions. Field experiments were conducted at the University of Córdoba (Spain) in the 2015/16 season in a Entisol loam soil (Typical Xerufluvent) following a randomized complete block design with two replications. The distance between plants and rows were 0.2 m and 1 m, respectively. During the 2015/16 growing season the rainfall was 354.8 mm and the average of the temperature was 16.2ºC. At maturity stage, five plants from each genotype were used for phenotypic evaluation. Six out of the 24 *A. cristatum* accessions selected did not flower and hence could not be evaluated. We assessed the following characteristics: spike length, spike width and spikelets per spike. All data were collected from the main tillers. Spike length was measured from the base of the rachis to the tip of the terminal spikelet and spike width in the middle part of the spike. An analysis of the variance was carried out and mean values were compared using the least significant differences method ($P<0.05$). Statistical analysis was performed with Statistix 8.0.

Results

Ploidy level

The ploidy level of the 115 *A. cristatum* accessions is given in Table 1. Overall, we found 21 diploid (2n=2x=14), 86 tetraploid (2n=4x=28) and 8 hexaploid (2n=6x=42) accessions. Figure 1 shows flow cytometry fluorescence histograms of representative examples of each ploidy level. The mean value of 2C DNA content was 13.64 pg in diploid accessions, 27.06 pg in tetraploid accessions and 39.31 pg in hexaploid accessions (Table 2). The ploidy levels obtained herein were validated by chromosome counts from root tip cells of two accessions in each ploidy level. Chromosome counting confirmed that diploid accessions (Ac272 and Ac155) had 14 chromosomes, tetraploid accessions (Ac205 and Ac302) had 28 chromosomes and hexaploid accessions (Ac064 and Ac121) had 42 chromosomes, respectively. Figure 2 shows mitotic metaphase cells of three *A. cristatum* accessions with different ploidy levels. The results obtained in the present study concerning the ploidy level of the accessions agree with those given by USDA, except for six accessions (Table 1).

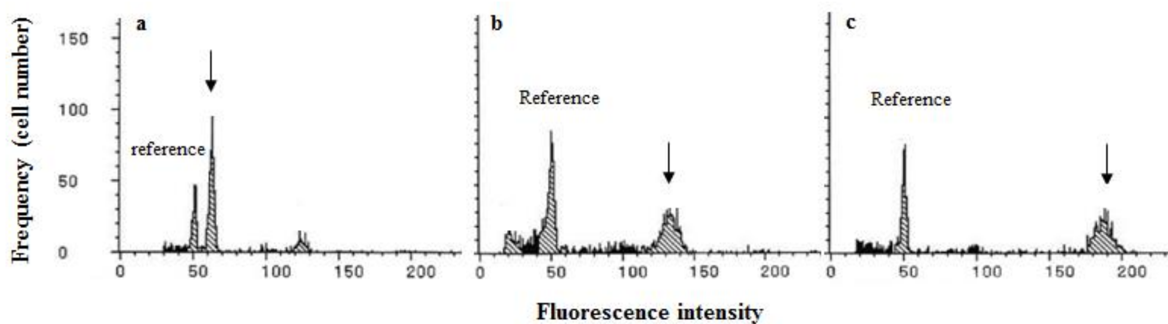

Figure 1. Flow cytometric histogram patterns of *A. cristatum* accessions with different ploidy levels (a) Ac272 diploid; (b) Ac302 tetraploid and (c) Ac064 Hexaploid. *Hordeum vulgare* was used as the internal reference.

Table 2.Ploidy level and nuclear 2C DNA content (pg) of the *A. cristatum* accessions.

Ploidy level	Number of accessions	2C DNA content (Mean ± SD)
Diploid (2x)	21 (18.3 %)	13.64 ± 1.21
Tetraploid (4x)	86 (74.8 %)	27.06 ± 1.34
Hexaploid (6x)	8 (6.9 %)	39.31 ± 2.12

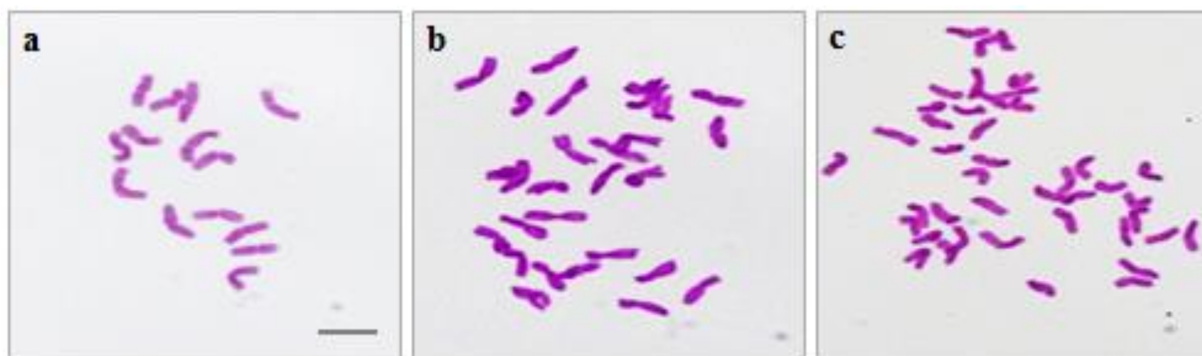

Figure 1 Micrographs showing mitotic metaphase cells of three *A. cristatum* accessions with different ploidy levels (a) Ac272 diploid; (b) Ac302 tetraploids and (c) Ac064 hexaploid. Bar = 10 µm

Table 3 shows the ploidy level of the 115 accessions evaluated in this study grouped by their origin. All these were wild genotypes collected in their respective countries of origin except in five cases: the diploid cultivar “Ruff”, the tetraploid cultivar “Ephraim” and a tetraploid accession (Ac202) obtained from a colchicine-treated diploid, all of them from the USA, and the two diploid cultivars “Farway” and “Parkway”, from Canada. The tetraploids were the most widely distributed in all countries, followed by diploids. Hexaploid accessions were restricted to Georgia, Iran and Turkey, except for one accession found in China. The geographic distribution of the 91 *A. cristatum* accessions studied including diploid, tetraploid and hexaploid is given in Figure 3.

Table 3. Ploidy level in 115 accessions of *A. cristatum* from 17 countries.

Country	No. of accessions	Ploidy level
Bulgaria	2	2x, 4x
Canada	2	2x
China	16	2x, 4x, 6x
Georgia	3	4x, 6x
Iran	15	4x, 6x
Kazakhstan	20	2x, 4x
Macedonia	1	4x
Mongolia	13	2x, 4x
Morocco	2	4x
Romania	2	2x, 4x
Russian Federation	18	2x, 4x
Spain	1	4x
Sweden	1	2x
Turkey	12	4x, 6x
Ukraine	3	2x, 4x
United States	3	2x, 4x
Uzbekistan	1	4x

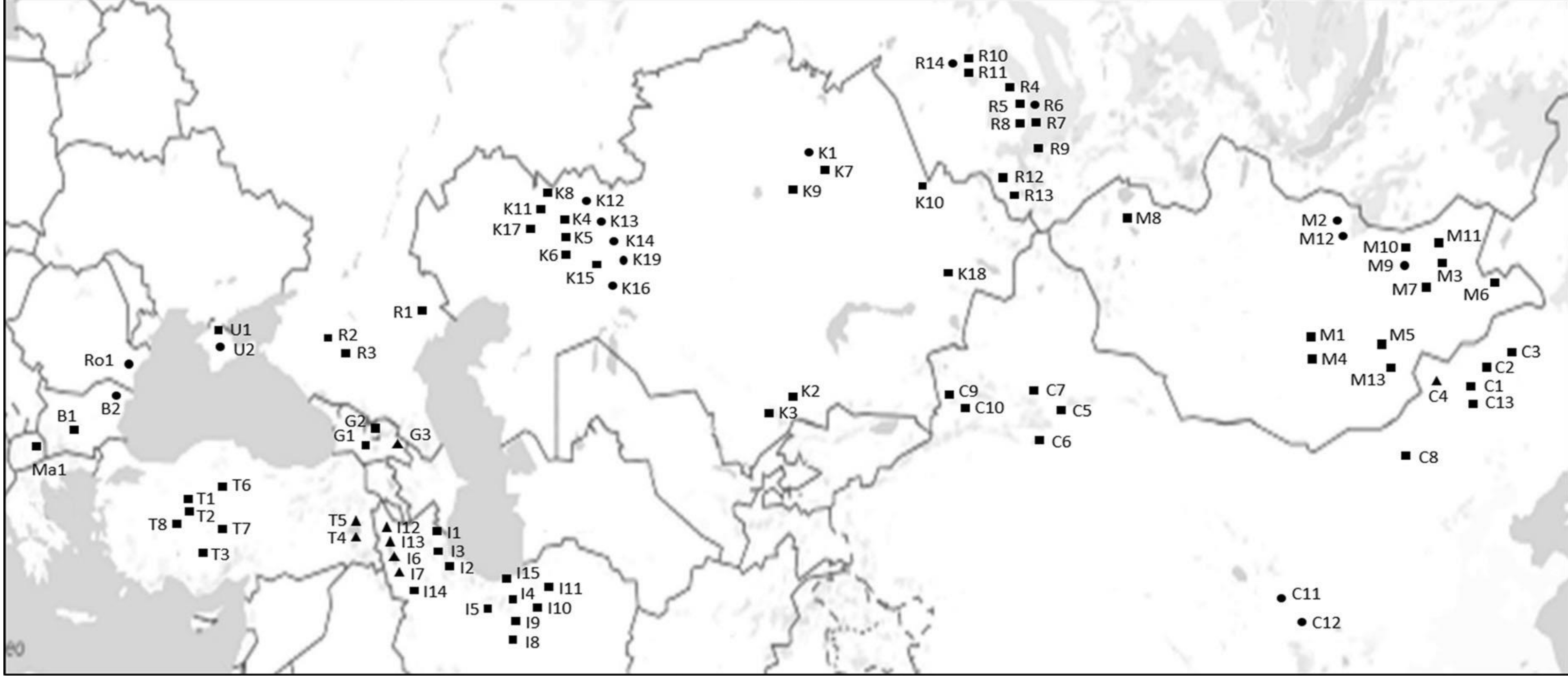

Figure 2. The geographic location of 91 *Agropyron cristatum* accessions. Bulgaria: Ac332^{B1}, Ac350^{B2}; China: Ac180^{C1}, Ac181^{C2}, Ac182^{C3}, Ac185^{C4}, Ac189^{C5}, Ac191^{C6}, Ac265^{C7}, Ac266^{C8}, Ac269^{C9}, Ac304^{C10}, Ac326^{C11}, Ac327^{C12}, Ac335^{C13}; Georgia: Ac336^{G1}, Ac337^{G2}, Ac338^{G3}; Iran: Ac013^{I1}, Ac016^{I2}, Ac017^{I3}, Ac018^{I4}, Ac020^{I5}, Ac063^{I6}, Ac64^{I7}, Ac097^{I8}, Ac098^{I9}, Ac099^{I10}, Ac106^{I11}, Ac115^{I12}, Ac121^{I13}, Ac123^{I14}, Ac317^{I15}; Kazakhstan: Ac155^{K1}, Ac166^{K2}, Ac168^{K3}, Ac205^{K4}, Ac206^{K5}, Ac207^{K6}, Ac210^{K7}, Ac212^{K8}, Ac216^{K9}, Ac254^{K10}, Ac257^{K11}, Ac272^{K12}, Ac273^{K13}, Ac274^{K14}, Ac281^{K15}, Ac289^{K16}, Ac320^{K17}, Ac330^{K18}, Ac334^{K19}; Macedonia: Ac025^{Ma1}; Mongolia: Ac290 ^{M1}, Ac293^{M2}, Ac294^{M3}, Ac298^{M4}, Ac299^{M5}, Ac300^{M6}, Ac302^{M7},Ac308^{M8}, Ac310^{M9}, Ac311^{M10}, Ac314^{M11}, Ac315^{M12}, Ac324^{M13}; Romania: Ac331^{Ro1}; Russia: Ac044^{R1}, Ac149^{R2}, Ac154^{R3}, Ac239^{R4}, Ac242^{R5}, Ac247^{R6}, Ac248^{R7}, Ac249^{R8}, Ac250^{R9}, Ac253^{R10}, Ac255^{R11}, Ac258^{R12}, Ac264^{R13}, Ac328^{R14}; Turkey: Ac001^{T1}, Ac003^{T2}, Ac005^{T3}, Ac006^{T4}, Ac007^{T5}, Ac012^{T6}, Ac130^{T7}, Ac319^{T8}; Ukraine: Ac312^{U1}, Ac313^{U2}. Diploid, tetraploid and hexaploid accessions are represented by filled circles, squares and triangles, respectively.

SSR and cluster analysis

Six out of the 29 SSR markers analyzed, with clear amplification patterns, were selected to evaluate 71 accessions of the *A. cristatum* collection differing in ploidy level (12 diploid, 54 tetraploid and 5 hexaploid accessions) (Table 1). The parameters of variation analyzed for SSR markers are presented in Table 4. All SSRs presented high levels of polymorphism, generating a total of 166 different alleles with fragment sizes between 84 and 256 bp. The average value of alleles/SSRs was 27.7, ranging from 8 (Xgwm382) to 63 (Xgwm271). Based on PIC values obtained (from 0.579 to 0.968), all the SSR were classified as informative markers (PIC > 0.5).

Table 4. Marker, size range, number of alleles and polymorphic information content (PIC) observed in 71 accessions of *A. cristatum* studied with six microsatellites markers.

SSR Marker	Size range (bp)	Number of alleles							**PIC**
		Diploid (n = 12)[a]		Tetraploid (n = 54)[a]		Hexaploid (n = 5)[a]		Total (n = 71)	
		N[b]	Mean[c]	N[b]	Mean[c]	N[b]	Mean[c]	N[b]	
Xgwm135	126-178	9	1.6	18	2.5	2	1.8	18	0.881
Xgwm205	122-203	28	6.3	56	8.7	20	9.4	56	0.961
Xgwm257	190-233	5	1.8	11	1.7	5	2.2	11	0.728
Xgwm271	142-256	31	6.4	61	8.8	30	11.4	63	0.968
Xgwm314	130-142	4	1.6	9	2.3	8	3.6	10	0.742
Xgwm382	84-107	1	1	8	1.8	1	1	8	0.579
Total		*78*	-	*163*	-	*66*	-	*166*	
Mean		*13*	*3.1*	*27.1*	*4.3*	*11*	*4.9*	*27.7*	*0.809*

[a]Number of accessions evaluated
[b]Number of different alleles
[c]Mean number of alleles per accession

The total number of different alleles was: 78 in diploid accessions, varying from 1 (Xgwm382) to 31 (Xgwm271); 163 in tetraploid accessions, varying from 8 (Xgwm382) to 61 (Xgwm271); and 66 in hexaploid accessions, varying from 1 (Xgwm382), to 30 (Xgwm271), respectively. The mean number of alleles per accession was 3.1 in diploids, 4.3 in tetraploids and 4.9 in hexaploids, respectively. The number of different alleles was higher in tetraploids than diploids or hexaploids, probably due to the large number of tetraploid accessions evaluated compared with diploid and hexaploid accessions (Table 4).

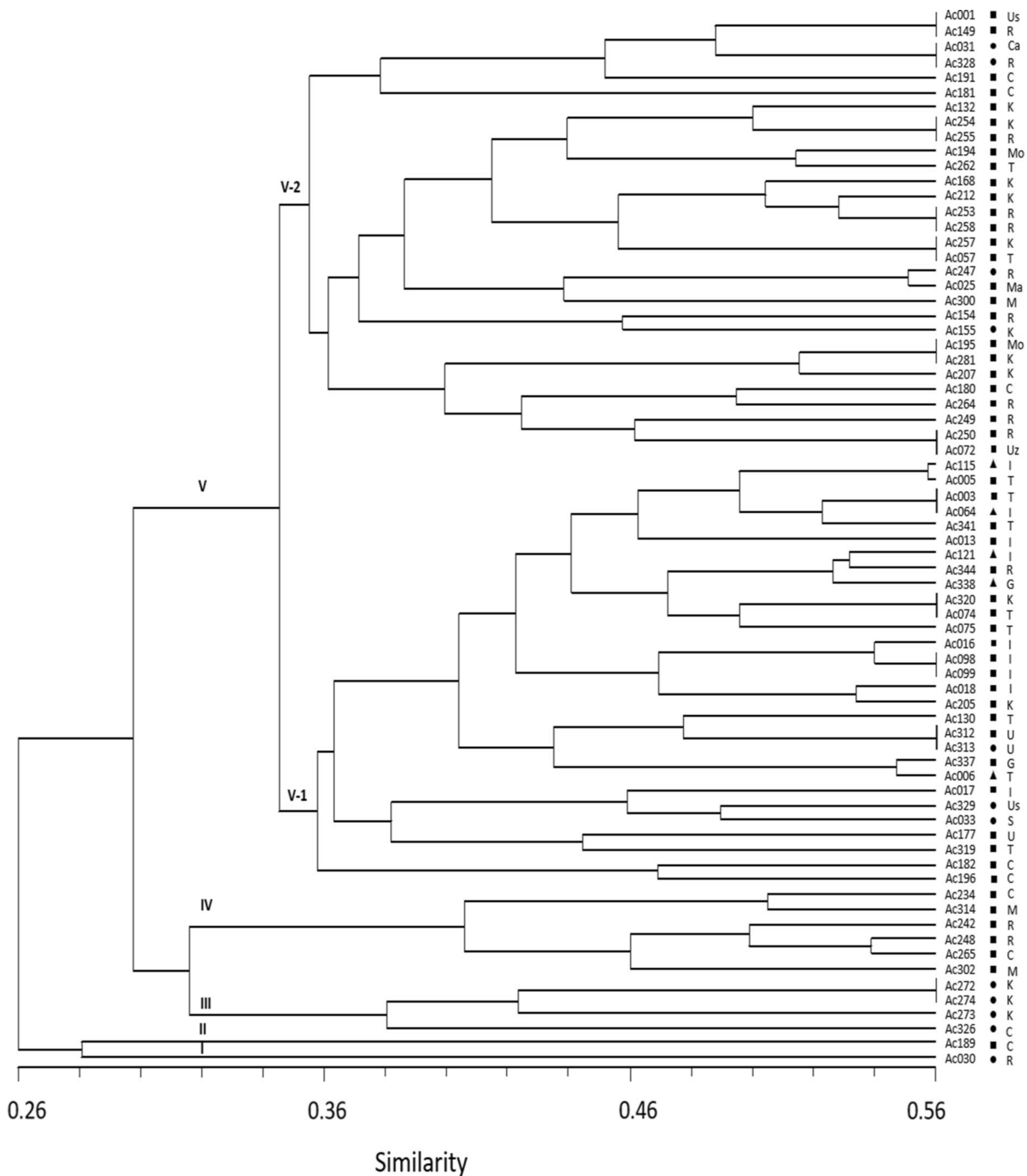

Figure 3. UPGMA dendrogram obtained from cluster analysis of 71 *A. cristatum* accessions based on the Dice similarity coefficient using six SSR markers. Symbols to the right are: B (Bulgaria), Ca (Canada), C (China), G (Georgia), I (Iran), K (Kazakhstan), Ma (Macedonia), M (Mongolia), Mo (Morocco), Ro (Romania), R (Russian Federation), S (Sweden), T (Turkey), U (Ukraine) and Uz (Uzbekistan). Diploid, tetraploid and hexaploid accessions are represented by filled circles, squares and triangles, respectively.

SSR data were also used to establish genetic relationships among these accessions (see Table 1). A dendrogram was obtained from the UPGMA analysis based on the Dice similarity coefficient (Figure 4), and from this, the accessions could be classified into five main groups. Group I included one diploid accession, which was from Russia (Ac030), but its precise geographic origin is not specified by the USDA, and Group II is also formed by one member, the tetraploid accession Ac189, which was collected in northwest of China. Group III comprises four diploid accessions: one of them collected in the center of China and the other three in a region in the west of Kazakhstan. Group IV contains six tetraploid accessions collected from different regions of Central and East Asia specifically Mongolia and China as well as Russia. The fifth cluster is divided into two subgroups. Group V-1 is composed of 29 accessions including two diploids, 22 tetraploids and all the 5 hexaploids. The accessions of this cluster are mainly distributed in a region comprising Turkey, Iran, Georgia and the south of Ukraine. Finally, Group V-2 is composed of 30 accessions including 4 diploid and 26 tetraploid accessions showing a wider geographical distribution than those of Group V-1. They were mainly found from eastern to western Asia. Accessions collected in Russia, Kazakhstan, Turkey and China are included in this cluster, as well as two from Morocco and one from Macedonia.

The Dice similarity coefficient showed that the greatest genetic distance found was between an Russian accession (Ac030) and an accession from Kazakhstan (Ac168), with a coefficient of 0.044, whereas the smallest genetic distance found was between two accessions from Iran (Ac098 and Ac099), with a coefficient of 0.653. Among the three crested wheatgrass cultivars included in the SSR analysis, ‘Fairway’ (Ac031) and “Ephraim” (Ac001) were closely related in Group V-2 of the dendrogram whereas “Ruff”(Ac329) appears in Group V-1.

Morphological evaluation

Mean and standard errors of the studied traits are presented in Table 5. Statistically significant differences were found between the accessions for the characters evaluated. Spike length ranged from 3.3 cm for diploid accession Ac273 to 7.8 cm for tetraploid accession Ac057. Spike width ranged from 1.0 cm for tetraploid accession Ac205 to 2.7 cm for hexaploid

accession Ac115. The number of spikelets per spike ranged from 25.0 for tetraploid accession Ac168 to 50.2 for diploid accession Ac313. Compared with spike length and spike width, the number of spikelets per spike was highly variable both between and among accessions as indicated by the large standard errors. Spikes morphologies of representative diploid, tetraploid and hexaploid accessions are shown in Figure 5. All accessions seed set and were fertile, except for Ac247 that was sterile.

Table 5.Spike length, spike width, and number of spikelets per spike in 18 accessions of *A. cristatum* with different ploidy levels.

Accession	Spike length (cm)	Spike width (cm)	Spikelet number
Ac006 (6x)	6.4 ± 1.0 bc	1.7 ± 0.0 bcd	46.5 ± 7.7 ab
Ac030 (2x)	4.6 ± 0.2 def	1.6 ± 0.1 bcde	43.7 ± 2.1 abc
Ac031 (2x)	6.3 ± 0.5 bcd	2.0 ± 0.4 bc	37.5 ± 5.5 abcd
Ac033 (2x)	4.3 ± 0.3 ef	1.3 ± 0.1 cde	32.2 ± 4.9 bcd
Ac057 (4x)	7.9 ± 0.8 a	1.2 ± 0.1 de	34.6 ± 3.1 bcd
Ac115 (6x)	4.0 ± 1.1 efg	2.7 ± 0.5 a	36.2 ± 11.0 abcd
Ac131 (2x)	4.4 ± 0.8 ef	1.4 ± 0.0 bcde	35.7 ± 8.3 bcd
Ac155 (2x)	3.7 ± 0.5 efg	1.3 ± 0.3 cde	35.7 ± 2.4 bcd
Ac168 (4x)	5.01±1.0 cdef	1.6 ± 0.5 bcde	25.0 ± 2.2 d
Ac177 (4x)	5.9 ± 0.0 cde	1.6 ± 0.2 bcde	39.9 ± 5.5 abcd
Ac205 (4x)	7.6 ± 0.7 a	1.0 ± 0.1 e	31.9 ± 2.6 bcd
Ac247 (2x)	4.1 ± 0.5 ef	1.8 ± 0.2 bcd	31.9 ± 8.2 bcd
Ac262 (4x)	5.8 ± 0.2 cde	1.8 ± 0.3 bcd	36.3 ± 1.5 abcd
Ac273 (2x)	3.3 ± 0.4 fg	1.4 ± 0.2 bcde	30.0 ± 3.0 bcd
Ac274 (2x)	3.4 ± 0.1 efg	1.7 ± 0.1 bcd	25.0 ± 4.6 d
Ac313 (2x)	5.1 ± 0.2 cde	1.7 ± 0.1 bcd	50.2 ± 3.0 a
Ac329 (2x)	5.3 ± 0.3 cde	2.0 ± 0.2 bc	42.0 ± 5.8 abc
Ac341 (4x)	4.6 ± 0.6 cdef	2.0 ± 0.2 ab	28.8 ± 5.2 cd

Means (± SE) followed by different letters in the same column indicate significant differences (*least significant difference test*, *P* = 0.05)

Comparison of the spike length, spike width and number of spikelets per spike of accessions with different ploidy levels (Table 6) showed that the diploid accessions had significantly ($P<0.05$) shorter spikes than tetraploid accessions with the hexaploid showing an intermediate spike length compared to both diploids and tetraploids. No significant differences were found for the spike width between diploid and tetraploid accessions. Hexaploids showed a significantly larger mean of spike width than diploid and tetraploid accessions ($P<0.05$).

Nevertheless, due to the small number of hexaploid accessions evaluated, we cannot conclude that these accessions have wider spikes compared with diploids and tetraploids. A

larger number of hexaploid accessions should be evaluated to test differences for this character between accessions with different ploidy levels.

Figure 4. Spike morphology of *A. cristatum* accessions with different ploidy levels. From left to right: diploid Ac274, Ac313 and Ac329; tetraploid Ac168, Ac262 and Ac205 and hexaploid Ac006 and Ac115.

Table 6. Mean of the spike length, spike width and number of spikelets per spike of accessions with different ploidy levels.

No. of accessions	Spike length (cm)	Spike width (cm)	Spikelet number
10 (2x)	4.4 ± 0.3 a	1.6 ± 0.1 a	32.7 ± 2.3 a
6 (4x)	6.1 ± 0.5 b	1.5 ± 0.1 a	36.3 ± 2.1 a
2 (6x)	5.2 ± 1.2 ab	2.2 ± 0.5 b	41.3 ± 5.1 a

Means (± SE) followed by different letters in the same column indicate significant differences (*least significant difference test*, P = 0.05)

Discussion

A. cristatum contains three levels of ploidy, namely diploid (2n = 14), tetraploid (2n = 28) and hexaploid (2n = 42) (Dewey 1984). The three expected ploidy levels were detected among the accessions examined in the present work. Flow cytometry is an efficient method to estimate DNA content in plants (Döležel et al. 2007) and the results were validated by chromosome counting in some accessions included in the three ploidy levels. In a total of 115 accessions evaluated, tetraploid accessions were by far the most common (74.8 %),

followed by diploid (16.3 %) and hexaploid (6.9 %). The mean of nuclear DNA content (2C value) obtained for diploid, tetraploid and hexaploid accessions in this work agree with those previously obtained for diploid accessions (Vogel et al. 1999) and both tetraploid and hexaploid accessions (Yousofi et al. 2004).

From the 115 accessions included in this study, the ploidy level had not been previously reported for 72 accessions. Ploidy levels of the other accessions were the same as those previously found by the USDA (Table 1), except in six cases. Specifically, we found that Ac035, Ac044, Ac123 and Ac154 were tetraploid, whereas the chromosome numbers given by the USDA for these accessions were 35, 18, 42 and 14, respectively. Similarly, Ac155 and Ac185 were diploid and hexaploid, respectively, whereas they were classified as tetraploids by the USDA.

Our results support the view that there is a relationship between geographic distribution and ploidy (Asay and Dewey 1979; Dewey and Asay 1982; Dewey 1984). The tetraploids were the most widely distributed and they were found throughout the entire area of distribution, ranging from Europe, Morocco and the Middle East across Central Asia to Siberia, China, and Mongolia. The diploids appeared in small disseminated areas distributed over the same general range as the tetraploids, except for Turkey, Iran and Georgia, where no diploids accessions were found. Dewey and Asay (1982) found that hexaploids were rare in Turkey and Iran. In contrast, in the present study, hexaploid accessions were mainly found in a region comprising the west of Turkey, the north of Iran and Georgia, with one also found in the north of China. Hexaploid accessions have previously been reported in northern China by Che et al. (2008).

Polyploidization seems to be frequent in *A. cristatum*, which is favored by its perennial condition. The ploidy variation in *A. cristatum* might be explained by the formation of unreduced gametes 4x; the union of a gamete 4x with other normal 2x would give rise to a hexaploid plant. All hexaploid accessions included in the SSR analysis were in Group V-1 of the dendrogram (Figure 4) and all of them were geographically localized to a small region comprising the north of Iran, the east of Turkey and Georgia (Figure 3). The close genetic relationships found between tetraploid and hexaploid accessions in that region support the hypothesis that hexaploid forms might have originated from the tetraploids through the

formation of unreduced gametes. In the *Agropyron* genus, Sadasivaiah and Weijer (1981) found that hexaploid *A. dasystachyum* (Hook.) Scribn. originated as a result of fertilization between unreduced gametes from the tetraploid form of this species.

Wheat and barley molecular markers have been a valuable source of polymorphic markers for variation studies and mapping of related wild Triticeae species, i.e., *Hordeum* species (Said and Cabrera 2009; Said et al. 2012; Mattera et al. 2015). In crested wheatgrass, both wheat SSR (Han et al. 2014) and conserved orthologous sequence (COS) molecular markers (Chapter II and III) have been used for physical mapping of specific *A. cristatum* chromosomes. The results of our study confirm that available wheat SSR markers are powerful for evaluating genetic diversity of 2x, 4x and 6x *A. cristatum* accessions. The discriminatory power of SSRs studied here was revealed by the UPGMA dendrogram (Figure 4). Accessions analyzed included a wide pool of genotypes, and in line with this, we observed a high degree of variation.

The SSR markers used in this research were previously employed by Che et al. (2008) to study genetic variation in 69 different populations of *A. cristatum* originating from various regions in northern China. In this study, we used SSR markers and capillary electrophoresis as tools to assess the genetic variation and determine the relationships among 71 *A. cristatum* accessions from different parts of the world. A high level of polymorphism was found in these materials (PIC >0.5). The most informative marker was Xgwm271 with a total number of 63 alleles and a PIC value of 0.968 (Table 4). Che et al. (2008) also considered this marker one of the most informative to fingerprint *A. cristatum* populations.

The high level of polymorphism within the accessions of *A. cristatum* reveals the wide genetic variation in this species. Our results show that diploid, tetraploid and hexaploid accessions are not clearly differentiated from each other on the basis of SSR markers. It has been postulated that the P genomes exhibit segmental autosomy in the tetraploid *A. cristatum* accessions and that the two P genomes present in the tetraploid accessions should be considered different, one of them being derived from the other by reciprocal translocations (Hsiao et al. 1989; Martín et al. 1999). Rearrangement of P-genome chromosomes present in tetraploid *A. cristatum* may also contribute to the high genetic variation found between these tetraploid accessions.

Breeding work on crested wheatgrass has been carried out and improved cultivars in all three ploidy levels have been released (Asay et al. 1995, 2003; Asay and Jensen, 1996). The potential of combining the genetic resources of diploid, tetraploid, and hexaploid through interploidy hybridization has also been exploited by crested wheatgrass breeders. Hybridization schemes involving 6x-2x, 6x-4x, and 4x-2x have shown potential for expanding the genetic resources of both 4x and 6x breeding populations (Dewey 1969, 1973; Asay and Dewey 1979; Asay et al. 1995; Jensen et al. 2005). Genetic introgression between the three ploidy levels suggest that the 2x, 4x and 6x accessions of crested wheatgrass should be treated as a common gene pool.

The high genetic variation obtained is related to the high morphological diversity observed in the field. *A. cristatum* is a cross-pollinated species resulting in wide variability within the species in the characters assessed (Table 5). Significative differences between diploid and tetraploid accessions for spike length have been found in the present work. Ploidy increases are also known to affect tiller characteristics in crested wheatgrass. Studies on the effect of increased ploidy have found that tetraploids have reduced the number of tillers (Tai and Dewey, 1966) and were taller (Mellish and Coulman, 2002) than diploids. Variation in spike length and number of spikelets per spike found in the accessions evaluated in this work could be of interest to increase seed yield potential. Differences were also found in the number of tillers, growth habit (erect and prostrate) and fertility of the accessions (data not shown).

The high quality and easily reproducible data presented here could be useful for maintaining the genetic variation in *A. cristatum* germplasm, as well as helping in the selection of parental lines for developing mapping populations. Finally, the results also indicate that the *A. cristatum* germplasm collection has a high level of genetic diversity and, as a consequence, represents valuable resource for future breeding programs.

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Chapter II

Chromosomal location of genes for resistance to powdery mildew in *Agropyron cristatum* and mapping of Conserved Orthologous Set (COS) molecular markers

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Abstract

Agropyron cristatum exhibits resistance to *Blumeria graminis* f. sp. *tritici*. Disomic and ditelosomic chromosome addition lines of *A. cristatum* in wheat 'Chinese Spring' were utilized to determine which *A. cristatum* chromosomes carry resistance gene(s). Resistance is conferred by gene(s) on 2PL and 6PL chromosome arms. The availability of molecular markers capable of detecting these chromosome arms in a wheat background would be very useful for marker-assisted introgression of 2PL and 6PL chromatin into common wheat. With this aim, the applicability of 170 wheat COS (Conserved Orthologous Set) markers (92 and 78 from wheat homoeologous group 2 and 6 respectively) were assessed for their utility in *A. cristatum.* A total of 116 (68.2 %) COS markers successfully amplified product in *A. cristatum* and 46 (40.0 %) of these markers were polymorphic between *A. cristatum* and common wheat. From marker loci mapping on wheat homoeologous group 2 chromosomes, 23 markers (34.9%) were polymorphic between *A. cristatum* and common wheat and from them 13 markers were assigned to chromosome arm 2PL and six markers were mapped to chromosome 4P of *A. cristatum* showing that this chromosome is related to wheat homoeologous group 2. From marker loci mapping on wheat homoeologous group 6 chromosomes, 23 (46.0%) markers were polymorphic between *A. cristatum* and common wheat and from them 17 markers were located on chromosome 6P, six of them were mapped to chromosome arm 6PS and five to chromosome arm 6PL, respectively. The specific COS markers allocated on the long arms of chromosomes 2P and 6P may have a role in marker assisted screening in wheat breeding for powdery mildew disease resistance.

Key words: *Agropyron cristatum*, COS markers, powdery mildew, wheat-*A. cristatum* addition lines.

Introduction

The genus *Agropyron* (Gaertn.) belongs to the tribe Triticeae and includes the crested-grass complex (cross-pollinated and long-lived perennials). Dewey (1984) proposed that *Agropyron* be restricted to species of the crested wheatgrass complex, a polyploid series based on the P genome. The most representative species is *Agropyron cristatum* (L.) Gaertner, which has diploid (2n = 2x = 14), tetraploid (2n = 4x =28) and hexaploid (2n = 6x =42) forms. *A. cristatum* has economic importance as forage and has also been considered a useful genetic resource for wheat genetic improvement. It has been found to display numerous traits beneficial to wheat, such as tolerance to drought (Asay and Johnson 1990; Dong et al. 1992) and low temperatures (Limin and Fowler 1990), as well as resistance to diseases including powdery mildew (Sharma and Knott 1966; Dewey 1984; Knott 1989).

The interspecific and intergeneric hybridization is an effective tool for introgression of genes from one species to others. The Triticeae tribe, which includes wheat, is the group in which this strategy has been applied most successfully. The production of the fertile amphiploid *xAgroticum* (DDPP, 2n = 4x = 28) (Martín et al. 1999), between the diploid wheat *Aegilops tauschii* Coss. and *A. cristatum*, allowed the development of new lines resistant to diseases using this amphiploid as a bridge for backcrossing to durum wheat (*Triticum turgidum* conv. durum Desf.) (Soliman et al. 2001, 2007). Furthermore, a compensating common wheat-*A. cristatum* Robertsonian translocation line conferring resistance to leaf rust (*Puccinia triticina* Erikss.) has also been developed using the *xAgroticum* amphiploid as a bridge for backcrossing to common wheat (Ochoa et al. 2015).

Powdery mildew caused by *Blumeria graminis* f. sp. *tritici* is a devastating disease that causes severe yield losses in most wheat production areas. Breeding resistant varieties is the most economical and effective way to control this disease. Although a number of *Pm* genes for resistance to powdery mildew have been identified (McIntosh et al. 2013), many of them tend to become ineffective when new pathogen variants emerge due to co-evolution of the host and pathogen. Therefore, there is a need to screen new potential sources of genetic resistance and combine more than one resistance gene to widen the gene pool of wheat.

Wide hybridization has great potential for enhancing resistance to diseases by introducing useful alien genes into wheat. Powdery mildew resistance (*Pm*) genes/alleles have been identified and introgressed from distant genera such as *Secale* (Friebe et al. 1994), *Haynaldia* (Chen et al. 1995) and *Elytrigia* (Luo et al. 2009). In *Agropyron*, an allele of *Pm2* for wheat powdery mildew resistance was identified in a putative *A. cristatum*-derived line conferring high resistance to powdery mildew at both the seedling and adult stages (Ma et al. 2015).

The availability of molecular markers capable of detecting *A. cristatum* chromatin in a wheat background would be very useful for marker-assisted introgression of *A. cristatum* into common wheat. The development of the conserved orthologous set (COS) (Quraishi et al. 2009) has provided a source of potentially useful markers for *A. cristatum*. COS markers also allow comparative studies among Triticeae species and thus their transference to *A. cristatum* is an important goal.

The aims of this research were to identify the *A. cristatum* chromosome(s) responsible for the observed resistance to powdery mildew and to obtain a set of COS molecular markers capable of detecting chromatin segments from these chromosome(s) in common wheat background.

Materials and methods

Plant material

The plant material in this study included the common wheat ‘Chinese Spring’ (CS) (2n = 6x = 42, AABBDD), *A. cristatum* accession PI222957 (2n = 4x = 28; PPPP) provided by the United States Department of Agriculture, and the available CS/*A. cristatum* disomic addition lines 1P, 2P, 3P, 4P, 5P and 6P and ditelosomic addition lines 2PS, 2PL, 4PS, 6PS and 6PL (Chen et al. 1992, 1994; Jubault et al. 2006).

Reaction to powdery mildew

Ten plants of each line were grown under greenhouse conditions surrounded by the susceptible wheat cv. ‘Meridiano’ that served as spreader plants. Mildew infection occurred naturally and after the first symptom of infection the plants were transferred to a growth

chamber under controlled environmental conditions (20 °C and 70% of relative humidity with a 12 h/12 h light/dark cycle). The severity of the powdery mildew was determined by estimating the percentage of leaf area covered by lesions using a modified Cobb's scale (Peterson et al. 1948), with scores ranging from 0 (indicating that the plant was completely resistant) to 8 (indicating that the plant was highly susceptible). The maximum area covered with pustules in the Cobbs's scale was assigned to 8 score. Plants were assessed for the percentage leaf area covered with lesions four times during the growing season (at 20, 40, 60 and 80 days) and these four disease severity ratings were used to calculate the area under the disease progress curve (AUDPC) according to the formula described by Campbell and Madden (1990). Mean values were compared with Fisher´s least significant difference test at $P = 0.05$ using Statistix 9 software.

Molecular markers

A total of 170 COS markers (92 and 78 from wheat homoeologous groups 2 and 6, respectively) (Quraishi et al. 2009) were assessed for their utility in *A. cristatum* (Supplementary file 1). Total genomic DNA was extracted from common wheat (CS), *A. cristatum* accession PI222957, the *T. aestivum* (CS)/*A. cristatum* disomic addition and the ditelosomic addition lines. For DNA extraction, about 0.1 g of young leaf tissue was excised. DNA was isolated using the CTAB method (Murray and Thompson 1980). The concentration of each sample was estimated using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Amplifications were performed using a TGradient thermocycler (Biometra, Go¨ttingen, Germany) with 50 ng of template DNA in a 25 µl volume reaction mixture containing 5 µl of 1x PCR Buffer, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs and 0.625 U of Taq DNA Polymerase (Promega, Madison, WI, USA). PCR conditions for the COS markers were as follows: 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 50 s at 58 °C, and 50 s at 72 °C. The PCR products were analyzed in polyacrylamide gels (10% w/v, C: 2.67%) stained with ethidium bromide.

Results

Chromosome location of resistance to powdery mildew

The accession of *A. cristatum* used in this work was immune to infection by powdery mildew, while the wheat CS parent was susceptible. To determine which *A. cristatum* chromosome(s) was the source of this resistance, the AUDPC was calculated for the wheat CS/*A. cristatum* disomic addition lines. The AUDPC analysis showed significant differences in degree of susceptibility between the addition lines. Disomic addition lines for chromosomes 1P, 3P and 5P were susceptible to powdery mildew and obtained the highest AUDPC values.

Table 1. Mean values of the area under the disease progress curve (AUDPC) for powdery mildew of *A. cristatum,* common wheat (CS) and CS/*A. cristatum* disomic and ditelosomic addition lines.

Line	AUDPC
A.cristatum	0
T. aestivum (CS)	46.0 ab
CS + 1P	41.0 bc
CS + 2P	25.5 d
CS + 2PS	53.9 a
CS + 2PL	30.5 d
CS + 3P	46.7 ab
CS + 4P	-[1]
CS + 4PS	41.3 bc
CS + 5P	52.5 a
CS + 5PL	52.1 a
CS + 6P	28.5 d
CS + 6PS	51.0 ab
CS + 6PL	30.0 d

Non tested

Letters in common within a column indicate that differences are not statistically significant at p = 0.05

The percentages of AUDPC obtained for these three addition lines were not significantly different from those for the susceptible wheat parent CS (Table 1). In contrast, disomic addition lines for chromosomes 2P and 6P had significantly lower AUDPC values than the wheat parent CS. Indeed, the AUDPC values obtained for these two lines were significantly lower than those for all the other addition lines as well as the wheat parent. These results indicated that chromosomes 2P and 6P contain genes for resistance to powdery mildew.

Unfortunately, all the 4P disomic addition line plants died and hence, they could not be assessed.

To determine the arm location of the gene(s) conferring resistance to powdery mildew, ditelosomic addition lines for the short and long arm of both chromosomes 2P and 6P were evaluated for their reaction to powdery mildew. The two ditelosomic addition lines carrying the long arm of chromosome 2P and 6P showed significantly lower AUDPC values than the two ditelosomic addition lines carrying the short arm of these chromosomes (Table 1). These results indicated that chromosome arms 2PL and 6PL contained the resistance genes to powdery mildew. Differences in AUDPC values between the ditelosomic addition lines for the 2PL and 6PL chromosome arms were not significant.

Transferability and chromosome location of COS markers in A. cristatum

A total of 92 COS markers from wheat homoeologous group 2 and 78 from homoeologous group 6 were studied for their transferability to *A. cristatum*. All markers were first screened for polymorphisms (presence/absence or size polymorphisms) between common wheat and *A. cristatum*. Out the 92 markers from homoeologous group 2, 66 (71.7%) consistently amplified *A. cristatum* products and of these 23 (25.0% of the total) were polymorphic between *A. cristatum* and wheat (Table 2). Nineteen out these 23 polymorphic markers were mapped using the *A. cristatum*/wheat addition lines. Thirteen COS markers were assigned to chromosome 2P and six markers amplified products on chromosome 4P (Table 3). The remaining four markers did not amplify products in any of the available *A. cristatum*/wheat addition lines, and hence, we were unable to map them. All the markers mapped on chromosome 2P were located on the long arm of this chromosome as demonstrated by their presence on 2PL and their absence on 2PS ditelosomic lines, respectively (Table 4). Examples of amplification of homoeologous group 2 COS markers are given in Fig. 1.

Table 2. Number and frequency of wheat COS markers amplified in *A. cristatum*

Wheat Group	Polymorphic	Non polymorphic	Total number
2	23 (34,9)[a]	43 (65.1)	92
6	23 (46,0)	27 (54.0)	78
Total	46 (39,6)	70 (60.4)	170

[a]Number in parenthesis are the percentages of wheat markers relative to the total number of amplified markers

Table 3. Number and frequency of polymorphic COS markers localized in *A. cristatum*

Wheat group	Chromosome location			Non localized	Total number
	2P	4P	6P		
2	13 (56,5)[a]	6 (26,1)	-	4 (17,4)	23
6	-	-	17 (73,9)	6 (26,1)	23
Total	13 (28,3)	6 (13,1)	17 (36,9)	10 (21,7)	46

[a] Number in parenthesis are the percentages of wheat located markers relative to the total number of tested markers

Out the 78 markers from homoeologous group 6, 50 (64.1%) were successfully amplified in *A. cristatum*, and, of these, 23 (29.5% of the total) were polymorphic between *A. cristatum* and wheat. Seventeen of these markers were located on chromosome 6P as demonstrated by their presence in the wheat 6P addition line (Table 3). The other six markers did not amplify products in any of the available CS/*A. cristatum* addition lines, and hence, we were unable to map them. Examples of amplification of homoeologous group 6 COS markers are given in Fig. 2. Six COS markers which amplified product in the 6P disomic addition line did not amplify products in any 6PS or 6PL ditelosomic addition lines (Table 4), and hence, we were unable to determine the arm location of these markers. A total of 36 polymorphic markers were assigned to *A. cristatum* chromosomes: 13 in 2P, 6 in 4P and 17 in 6P. Characterization and chromosome arm locations of wheat COS markers on chromosomes of *A. cristatum* are summarised in Table 4.

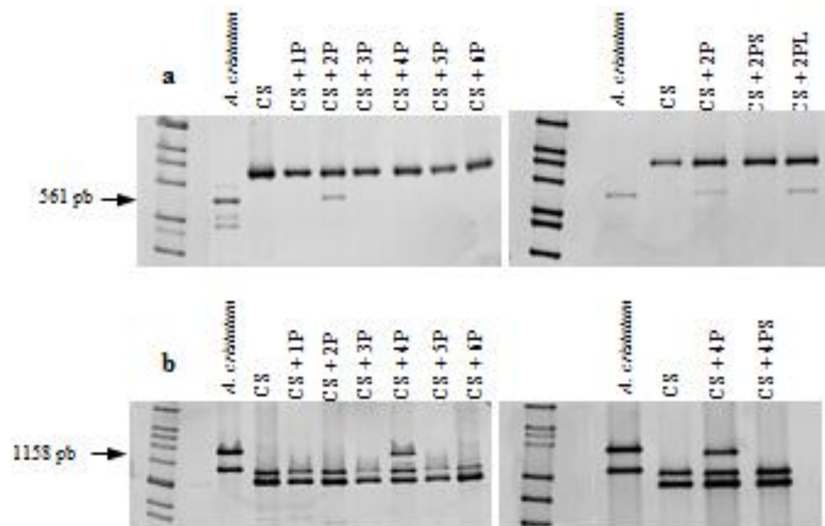

Figure 1. PCR amplification profiles used for location of DNA molecular markers on chromosome 2P and 4P. **a** COS746 mapped on the long arm of chromosome 2P. **b** COS680 mapped on chromosome 4P

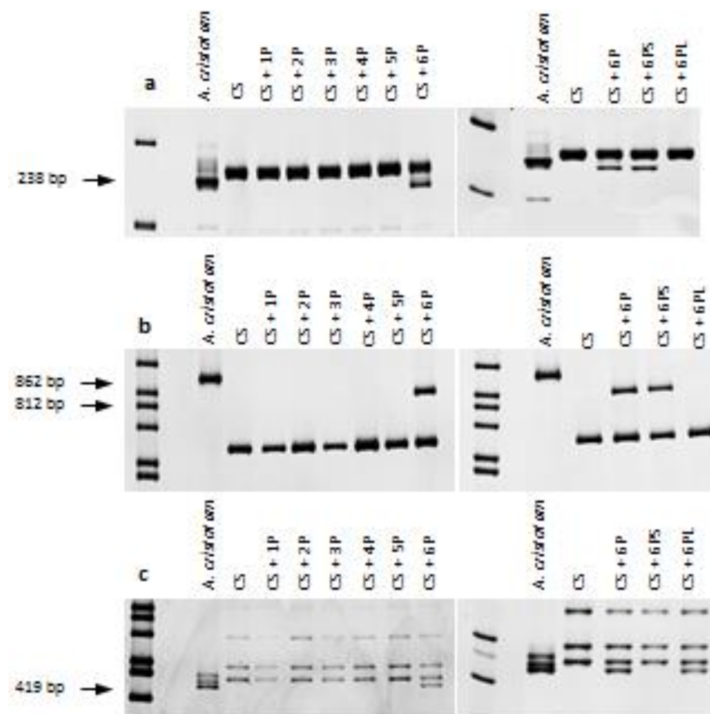

Figure 2. PCR amplification profiles used for location of DNA molecular markers on chromosome 6P. **a** COS508 and **b** COS440 mapped on the short arm of chromosome 6P. **c** COS476 mapped on the long arm of chromosome 6P

Table 4. Characterization and chromosome localization of COS markers on *A. cristatum*.

Marker	Product size in *A.cristatum*	Product size in *T. aestivum*	Chromosome location in *A. cristatum*	Short arm	Long arm	Chromosome location in wheat[a]	Location in wheat (cM)[a]
COS670[b]	431	463	4P	-	-[n.a.]	2AS-2BS-2DS	19,4
COS672	1043	986	4P	-	-[n.a.]	2AS-2BS-2DS	24,8
COS676	720	708	4P	-	-[n.a.]	2AS-2BS-2DS	30,6
COS677	569	631	4P	-	-[n.a.]	2AS-2BS-2DS	31,7
COS680	1158	974	4P	-	-[n.a.]	2AS-2BS-2DS	35,9
COS682	1016	851	4P	-	-[n.a.]	2AS-2BS-2DS	38,9
COS697	925	1012	2P	-	+	2AL-2BL-2DL	136,4
COS716	811	897	2P	-	+	2AL-2BL-2DL	152,0
COS725	456	496	2P	-	+	2AL-2DL	162,4
COS732	573	548	2P	-	+	2AL-2DL	168,3
COS735	553	533	2P	-	+	2AL-2BL-2DL	173,2
COS736	294	277	2P	-	+	2DL	173,5
COS746	561	700	2P	-	+	2AL-2BL-2DL	186,9
COS748	665	775	2P	-	+	2AL-2BL-2DL	189,5
COS749	428	466	2P	-	+	2AL-2BL-2DL	190,2
COS751	725	534	2P	-	+	2BL-2DL	192,6
COS752	409	426	2P	-	+	2AL-2BL-2DL	192,9
COS755	530	510	2P	-	+	2AL-2BL-2DL	194,9
COS758	618	595	2P	-	+	2AL-2BL-2DL	196,8
COS440[c]	862	560	6P	+	-	6AS-6BS	4,0
COS444	345	366	6P	+	-	6AS-6BS-6DS	12,5
COS447	766	695	6P	+	-	6AS	46,1
COS450	714	681	6P	+	-	6AS	50,8
COS453	722	768	6P	+	-	6AS	57,1
COS457	660	687	6P	-	+	6AL-6BL-6DL	119,0
COS463	255	240	6P	-	-	6AL-6BL-6DL	130,7
COS465	420	393	6P	-	-	6DL	132,2
COS466	689	800	6P	-	+	6BL-6DL	134,1
COS468	464	563	6P	-	-	6AL-6BL-6DL	135,2
COS471	1054	1002	6P	-	-	6B-6DL	137,5
COS476	419	471	6P	-	+	6DL	141,7
COS480	387	350	6P	-	+	6BL	144,9
COS501	1003	880	6P	-	-	6AL-6BL-6DL	181,0
COS503	456	378	6P	-	-	6AL-6BL-6DL	181,2
COS507	254	300	6P	-	+	6AL-6BL-6DL	186,4
COS508	238	265	6P	+	-	6DL	187,0

[a]Quraishi et al. (2009)
[b]COS670 to COS758 loci markers are from wheat homoeologous group 2
[c]COS440 to COS508 loci markers are from wheat homoeologous group 6
[n.a.] data not available due to the lack of ditelosomic 4PL addition line in CS

Discussion

Broadening the genetic base of cultivated wheat by the introgression of resistance genes from related species may increase our chances of achieving adequate resistance against powdery mildew. The *A. cristatum* accession used in this work was immune to the infection with powdery mildew under controlled conditions. This agrees with our previous results showing that this accession was immune to the existing powdery mildew races in the region both under controlled and field conditions (Soliman et al. 2007).

Comparison of the powdery mildew resistance of the susceptible wheat CS and the alien donor *A. cristatum* indicated that both 2P and 6P chromosomes carried genes conferring resistance to powdery mildew. Unfortunately, all the 4P disomic addition line plants died, and hence, could not be assessed, and no addition line of chromosome 7P of *A. cristatum* in wheat CS has been obtained. Hence, we are unable to rule out the presence of additional resistance genes on chromosomes 4P and 7P. Our results agree with those previously obtained by Song et al. (2013), which showed that powdery mildew resistance was carried on chromosome 6P from a different *A. cristatum* accession to that used in the present study. In addition, a new powdery mildew resistance gene has been recently mapped on chromosome 2P of *A. cristatum* (Li et al. 2017).

In the present study, we found that chromosome arms 2PL and 6PL from *A. cristatum* seem to harbour powdery mildew resistance gene(s). A reduction in the level of infections was evident both in seedlings and adult plants of the two 2PL and 6PL ditelosomic lines. Powdery mildew resistance genes/alleles have been located on the long arm of both homoeologous group 2 and 6 of wheat and related species. Genes conferring resistance to powdery mildew have been located on 2AL (Xu et al. 2011) and 2BL chromosome arms of wheat (Zhao et al. 2013). Quantitative trait loci with major effects were consistently detected on wheat chromosomes 2BL and 6BL (Hao et al. 2015). In addition, powdery mildew resistance gene(s) from wild species closely related to common wheat have been located on chromosome 2BL (Mohler et al. 2005) and 6BL (Xie et al. 2012) of wild emmer *T. dicoccoides.* These results agree with those found in comparative mapping between wheat and *A. cristatum* (see below), showing that synteny is conserved between both the long arms

of wheat homoeologous groups 2 and 6 and 2PL and 6PL chromosome arms of *A. cristatum*, respectively.

Comparative mapping has shown that gene content and order are highly conserved between related plant species (Gale and Devos 1998). The conservation of the content and order of genes among different species has improved the effectiveness and predictive value of information transfer. In this study, we utilized the conserved synteny between Triticeae species to transfer wheat COS markers to *A. cristatum*. The marker analysis showed that 116 (68.2%) COS markers successfully amplified product in *A. cristatum* and 46 (40.0%) of these markers were polymorphic between *A. cristatum* and wheat CS. The high transference rate of COS markers to *A. cristatum* was expected since these markers were intended for comparative studies among grasses (Quraishi et al. 2009). COS markers have been transferred to other related species of wheat, such as *Hordeum chilense* (Mattera et al. 2015) but, as far as we know, this is the first study transferring wheat COS markers to *A. cristatum.*

The location of COS markers on chromosomes 2P and 6P were determined based on the COS locations mapped in wheat (see Supplementary file 1). From marker loci mapping on wheat group 2 chromosomes, 13 markers (56.5%) were assigned to chromosome 2P and 6 markers (26.1%) were assigned to chromosome 4P of *A. cristatum*. These 13 wheat marker loci assigned to chromosome 2P were mapped to the long arm of group 2 chromosomes (Quraishi et al. 2009) and they were assigned to the same arm location in *A. cristatum* indicating that synteny is conserved between the long arm of wheat homoeologous group 2 and the long arm of chromosome 2P from *A. cristatum* (Table 4). On the other hand, the marker loci mapping to the short arm of wheat group 2 chromosomes were assigned to chromosome 4P showing that this chromosome is related to wheat homoeologous group 2. None of the marker loci mapped to chromosome 4P amplified product in the ditelosomic 4PS addition line, suggesting that these markers may be located on the long arm of chromosome 4P. Unfortunately, we were unable to confirm this result due to the lack of the ditelosomic line for the 4PL arm. Most (10) of the COS loci mapping to group 6 chromosomes have the same arm locations in *A. cristatum* indicating a homoeologous relationship between chromosome 6P and wheat group 6 (Table 4). These results agree with those previously found by Han et al. (2014) using wheat SSR molecular markers.

Ten (21.7%) COS markers polymorphic between *A. cristatum* and common wheat (4 from homoeologous group 2 and 6 from homoeologous group 6) did not amplify product in any of the available CS/*A. cristatum* disomic addition lines and, hence, could not be mapped. One explanation for the lack of amplification of these markers in the *A. cristatum* chromosome present in the addition lines would be genetic differences between the *A. cristatum* accession used in this study and the accession used to obtain the addition lines. The *A. cristatum* accession (PI222957) used here was a tetraploid ecotype from Iran, whereas the chromosome addition lines were isolated from a backcross of an intergeneric hybrid of wheat CS with a tetraploid ecotype of *A. cristatum* from Inner Mongolia, China (Chen et al. 1989, 1992).

Studies of genetic diversity have shown that there is large allelic variation in microsatellite loci between different populations of *A. cristatum* collected from similar ecogeographical regions (Che et al. 2008, 2011). We have found differences in the size of the amplified COS fragment in the *A. cristatum* genome used as a control and the *A. cristatum* chromosomes present in the addition lines for some markers. For example, the marker COS440 showed differences in the length of the amplified fragment in the *A. cristatum* accession used as a control and the amplified fragment in the 6P disomic addition line (Fig. 2b). The presence of polymorphic markers (both presence or absence and differences in the size of the amplified fragment) between the two accessions of *A. cristatum* also indicated that there is genetic variation within this species. Furthermore, it has been postulated that the P genomes exhibit segmental autosomy in the tetraploid *A. cristatum* and that the two P genomes present in the tetraploid accessions should be considered different, one of them being derived from the other by reciprocal translocations (Hsiao et al. 1989; Martín et al. 1999). We cannot rule out the possibility that rearrangement of P-genome chromosomes present in tetraploid *A. cristatum* is also the cause of the genetic variation found between these two tetraploid accessions.

In conclusion, the wheat CS-*A. cristatum* 2PL and 6PL ditelosomic addition lines have high resistance to powdery mildew compared to that of CS wheat. These lines will provide excellent germplasm resources for wheat breeding. The mapped COS markers specific to both 2PL and 6PL chromosome arms will facilitate marker-assisted screening in wheat breeding for powdery mildew.

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Chapter III

Development and characterization of wheat-*Agropyron cristatum* introgression lines and mapping of conserved orthologous set molecular markers

In preparation

Copete A, Cabrera A. Development and characterization of wheat-*Agropyron cristatum* introgression lines and mapping of conserved orthologous set molecular markers

Abstract

The P genome of *Agropyron* Gaertn. contains many desirable genes that can be utilized as genetic resources to improve wheat. In this study, wheat – *Aegilops cylindrica* Host gametocidal chromosome 2C^{c} addition lines were crossed with wheat –*Agropyron cristatum* (L.) Gaertn. disomic addition line for chromosomes 4P, 5P and 6P, respectively. We successfully induced chromosomal translocations between wheat and these three P genome chromosomes. The frequency of translocation in the progeny was 19.0 % for chromosome 4P, 9.9 % for chromosome 5P and 5.4 % to chromosome 6P. The wheat-*A. cristatum* introgressions were detected by genomic *in situ* hybridization (GISH) using P genomic DNA and repetitive pAs1 sequence as probes. Different types of translocations were identified including whole arm and terminal translocations as well as dicentric chromosomes. For marker-assisted selection of P chromatin into common wheat background it would be very useful the availability of molecular markers capable of detecting these P genome introgressions. With this aim, 157 wheat conserved orthologous set (COS) markers (105 and 52 from wheat homoeologous groups 4 and 5, respectively) were assessed for their utility in *A.cristatum*. A total of 135 (85.9 %) COS markers successfully amplified product in *A. cristatum* and 47 (34.8 %) of these markers were polymorphic between *A. cristatum* and common wheat. From marker loci mapping on wheat homoeologous group 4 chromosomes, 29 markers (30.5 %) were polymorphic between *A. cristatum* and common wheat and from them 12 markers were located on chromosome 4P (seven of them were mapped to chromosome arm 4PS and four to chromosome arm 4PL, respectively) and one was located on chromosome 6P. From marker loci mapping on wheat homoeologous group 5, 18 (45.0 %) were polymorphic between *A. cristatum* and common wheat and from them 11 markers were located on chromosome 5P (six of them were mapped to chromosome arm 5PS and five to chromosome arm 5PL, respectively). All of the wheat - *A. cristatum* alien translocation lines obtained would be valuable for identifying *A. cristatum* chromosome 4P, 5P and 6P related genes and providing genetic resources and new germplasm accessions for the genetic improvement of wheat.

Keywords: *Agropyron cristatum***,** COS markers, GISH*,* gametocidal chromosome, wheat-*A. cristatum* introgression lines.

Introduction

Agropyron is a genus of the Triticeae tribe composed of no more than 10 species which constitute what is known as the "crested wheatgrass complex" containing only those species with P genome (Dewey 1984, Asay and Jensen 1996). *A. cristatum* L. Gaertn is the most representative species of the genus with diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) forms. It carries many useful traits, including resistance to barley yellow dwarf, wheat streak mosaic virus (Sharma et al. 1984), leaf rust (Ochoa et al. 2015) and powdery mildew (Li et al. 2016).

The development of introgression lines of *A. cristatum* into wheat has potential for wheat breeding. Chromosome addition lines of *A. cristatum* in common wheat were developed (Chen et al. 1992, 1994; Han et al. 2014) and these lines have been successfully used to determine the chromosome location of genes coding for important traits including tolerance to drought and cold on chromosome 4P (Liu et al. 2010), high numbers of spikelets, florets and kernels per spike on chromosome 6P (Wu et al. 2006) and resistance to stem rust on chromosome 5P (data not shown). Wheat-*A. cristatum* addition lines have been also used to transfer alien chromosome segments carrying genes of interest such as enhanced thousand-grain weight and spike length from *A. cristatum* to wheat (Zhang et al. 2015). Also, fertile amphiploids between *A. cristatum* and wheat (Soliman et al. 2001; Soliman et al. 2007) have been used successfully as bridge to introduce resistance to leaf rust (*Puccinia triticina* Erikss.) from *A. cristatum* to wheat (Ochoa et al. 2015).

Endo (1988) found a unique genetic system in common wheat that induces frequent chromosome breakage in the gametes. When gametocidal chromosomes (*Gc*) from certain wild species belonging to the genus *Aegilops* were introduced into common wheat in monosomic condition, chromosome rearrangements occur in the gametes lacking *Gc* chromosomes. Such *Gc*-induced chromosomal rearrangements are either lethal to gametes or semi-lethal, and in the latter case the gametes are fertilized to develop into viable zygotes carrying rearranged chromosomes (Endo, 1990). The *Gc* system proved to be effective in inducing structural rearrangements in barley chromosomes added to common wheat (Shi and Endo 1999), rye (Friebe et al. 2000; Shi et al. 2005), *Haynaldia villosa* L. (Chen et al. 2008), *Leymus racemosus* (Lam.) Tzvelev (Chen et al. 2005; Yuan et al. 2003) and *H. chilense* (Said

and Cabrera, 2009; Cherif-Mouaki et al. 2011; Said et al. 2012). Alien chromosomal translocations between wheat and *Agropyron* induced by gametocidal chromosomes have also been previously reported by Liu et al. (2010) and Li et al. (2016).

The availability of molecular markers capable of distinguishing between *A. cristatum* chromatin in wheat background constitutes an important tool for selection. The development of Conserved Orthologous Set (COS) (Quraishi et al. 2009) signifies an important source of markers useful for *A.cristatum.* COS markers allow comparative studies with wheat and thus their transference to *A. cristatum* is an important goal. The aims of this research were a) to produce introgression lines between wheat and chromosomes 4P, 5P and 6P from *A.cristatum*; b) to obtain a set of COS molecular markers capable of detecting chromatin segments from the chromosomes 4P, 5P and 6P in common wheat background, and use them to locate the *A. cristatum* fragments in the introgression lines.

Materials and methods

Plant material

The plant material used in this study included the common wheat (*Triticum aestivum* L.) 'Chinese Spring' (CS),*A. cristatum*accession PI222957 (2n=4x=28; PPPP) provided by USDA (United States Department of Agriculture) and the available CS/*A. cristatum* disomic addition lines 1P, 2P, 3P, 4P, 5P and 6P and the ditelosomic 4PS, 5PL, 6PS and 6PL addition lines (Chen et al. 1992, 1994; Jubault et al. 2006). Disomic addition lines for chromosomes 4P, 5P and 6P were crossed with a wheat disomic addition line carrying the gametocidal chromosome $2C^c$ from *Aegilops cylindrica* Host. The F1 plants monosomic for both the *A.cristatum* chromosome (4P, 5P or 6P) and the gametocidal chromosome were selfed to obtain F2 plants.

Fluorescence in situ hybridization (FISH)

The somatic chromosome count were obtained using 1 cm of root tips from the germinating seeds and pretreated in ice water for 24 h. The root tips were fixed in a freshly prepared solution of ethanol-acetic acid (3: 1) for 1 week. The FISH protocol was carried out as a modified protocol used by Cabrera et al. (2002). The probe pAs1 (1Kb) isolated from

Aegilops tauschii Coss. (Rayburn and Gill, 1986) was used to determine the D genome of wheat. Genomic DNA from *A. cristatum* was used as probe to identify P genome introgressions. The pAs1 probe was labeled with biotin-16-dUTP (corporate Roche) and the total DNA of *A. cristatum* with digoxigenin-11-dUTP (corporate Roche) using nick translation. All chromosome preparations were hybridized simultaneously with both pAs1 and *A. cristatum* genomic DNA probes. Biotin- and digoxigenin-labelled probes were detected with streptavidin-Cy3 conjugates (Sigma, St. Louis, MO, USA) and anti-digoxigenin-FITC antibodies (Roche Corporate), respectively. The chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield (vector Laboratories, Inc.). The signals were visualized using a Leica DMRB epifluorescence microscope and the images were captured with a Leica DFC7000T camera equipped with an exposimeter spot Leica Wild MPS 52 and were processed with LEICA application suite v4.0 software (Leica, Germany).

Molecular Marker analysis

A set of 157 COS markers (Quraishi et al. 2009) (105 from wheat homoeologous group 4 and 52 from homoeologous group 5) (Supplementary file 1) were assessed for their utility in *A. cristatum.* Total genomic DNA was isolated from young frozen leaf tissue using CTAB method (Murray and Thompson 1980). Samples were stored at -20ºC until PCR amplification was carried out. The concentration of each sample was estimated using a Nano-Drop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Amplifications were made using a TGradient thermocycler (Biometra, Göttingen, Germany) and performed with 20 ng of template DNA, in a 25 µl volume reaction including 5µl of 1x PCR Buffer, 0.5 µM of each primer, 1.5 mM MgCl2, 0.3mM dNTPs and 0.625 U of Taq DNA Polymerase (Promega, Madison, WI, USA). PCR conditions of COS markers were as follows: 4 min at 94 ºC, followed by 35 cycles of 45 s at 94 ºC,50 s at 58°C, and 50 s at 72 ºC. The PCR products were analysed in polyacrylamide gels (10% w/v, C: 2.67%) stained in ethidium bromide.

Specific molecular marker (AcOPX11-817) from *A. cristatum* developed by Wu et al. (2010) was used to detect P genome introgressions in wheat background. PCR conditions for the

AcOPX11-817 marker were as follows: 5 min at 95 ° C, followed by 34 cycles of 1 min at 94 ° C, 1 min at 53 ° C, and 1:30 sec at 72 ° C. Amplifications products were analyzed in 2 % agarose gels in 1 X TBE buffer and visualized by ethidium bromide staining.

Results

Cytogenetic and molecular characterization of F2 wheat-A. cristatum introgression plants involving chromosome 4P, 5P and 6P

A total of 86, 90 and 97 F2 plants were established from the crosses between the CS/*Ae. cylindrica* disomic addition line for the 2C^c gametocidal chromosome and the CS/*A. cristatum* disomic addition line for chromosomes 4P, 5P and 6P, respectively (Table 1). All these 273 F2 plants were screened for the presence of P genome chromosomes using the specific AcOPx11-817 molecular marker and from them, 91 (33.4 %) amplified product indicating that all these plants contained introgressions from *A. cristatum* (Figure. 1). The number of F2 plants containing P genome introgressions varied in the offspring of the three different crosses: 21 (24.4%), 32 (35.6%) and 38 (39.1%) F2 plants for chromosome 4P, 5P and 6P, respectively (Table 1) were found.

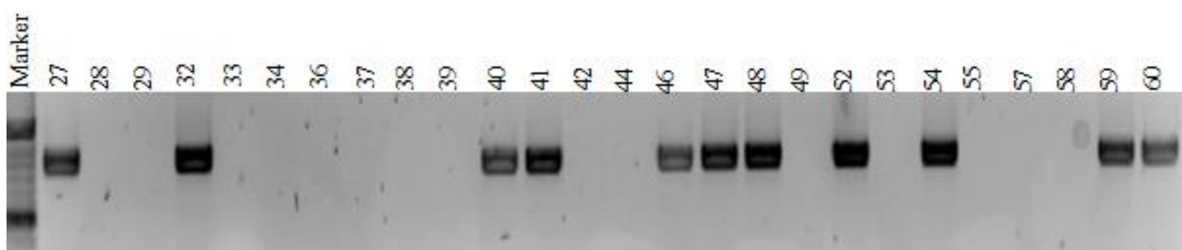

Figure 1. PCR amplification results with AcOPx11-817 marker in 26 F2 plants from the T220 x CS+5P cross.

Table 1. Number of wheat F2 plants containing 4P, 5P and 6P genome Introgressions

Cross	N° of germinated seeds	N° of established plants	N° of plants with P genome
CS + $2C^c$ x CS + 4P	100	86 (86.0 %)	21 (24.4%)
CS + $2C^c$ x CS + 5P	100	90 (90.0 %)	32 (35.6%)
CS + $2C^c$ x CS + 6P	133	97 (72.9 %)	38 (39.1%)
Total	333	273 (81.9 %)	91 (33.4 %)

Cytogenetic analysis using FISH with both the pAs1 repetitive and *A. cristatum* genomic DNA as probes were carried out to identify the P genome introgression of the 91 wheat F2 plants which were positive for the AcOPx11-817 molecular marker (Table 2). Monosomic plants were the most frequently obtained in the F2 progeny from all the three crosses. A total of 9 plants contained wheat-*A. cristatum* translocations: 4 involving chromosome 4P (%), 3 involving chromosome 5P (%) and 2 (%) involving chromosome 6P. The pAs1 specially hybridize on D-genome chromosomes of wheat and was useful to identify the wheat genome implicated in these translocations. Most of these wheat-*A-cristatum* translocations were centromeric translocations involving both A or B genome chromosomes from wheat, except one terminal translocation between chromosome 6P and one D-genome chromosome. Figure 2 shows FISH analysis on mitotic metaphase cells of different *A.cristatum* introgressions in wheat background. The identification of the chromosome arm involved in both the wheat-*A. cristatum* translocations and telosomic plants was carried out using chromosome specific molecular markers transferred from wheat to *A. cristatum* in the present work (see belong). COS0068 and COS0021 markers were used to identify the 4PS and 4PL chromosome arm, respectively. COS108 and COS150 markers were used to identify the 5PS and 5PL chromosome arm, respectively. For the identification of the 6PS and 6PL chromosome arms we used COS440 and COS476, respectively (see Chapter II). All *A. cristatum* chromosome arms involved in the wheat-*A.cristatum* translocations were identified (Table 2) except that involved in the terminal 6P-D genome translocation, probably due to the small fragment from 6P chromosome translocated to wheat (Figure 2 e,f). Examples of amplification of COS specific molecular markers used for the identification of chromosome arms involved in both the wheat-*A.cristatum* translocations and telosomes are given in Figure 3.

Table 2. Wheat-*A cristatum* introgressions obtained involving chromosomes 4P, 5P and 6P.

Chromosome	P genome introgression	N° of plants
4P	Monosomic	14
	Translocation 4PL·A/B	1
	Translocation 4PS·A/B	3
	Monotelosomic 4PL	1
	Ditelosomic 4PS	1
	Monosomic + telosome	1
5P	Monosomic	26
	Translocation 5PL·A/B	1
	Translocation 5PS·A/B	1
	Monosomic + telosome	2
	Monosomic + one deletion	1
	Dicentric A/B-5PS	1
6P	Monosomic	34
	Translocation 6PL·A/B	1
	Translocation 6P/D terminal	1
	Monotelosomic 6PL	1
	Ditelosomic 6PS	1

Transferability and chromosome location of COS markers in A.cristatum

A total of 105 COS markers from wheat homoeologous group 4 and 52 from homoeologous group 5 were studied for their transferability to *A. cristatum*. All markers were first screened for polymorphisms (presence/absence or size polymorphisms) between common wheat and *A. cristatum.* Out the 105 markers from homoeologous group 4, 95 (90.5%) consistently amplified products in *A.cristatum,* and of these 29 (30.5%) were polymorphic between *A. cristatum* and wheat (Table 3). Thirteen out these 29 polymorphic markers were mapped using the CS/*A. cristatum* addition lines. Twelve COS markers were assigned to chromosome 4P and one marker amplified product on chromosome 6P (Table 4). The remaining sixteen markers did not amplified in any addition line and hence, they could not be mapped. From the twelve COS markers assigned to chromosome 4P, seven were allocated on the short arm, as demonstrated by their presence in 4PS ditelosomic line (Table 5). Due to the lack of the

4PL ditelosomic addition line we used the wheat-*A.cristatum* translocation lines involving 4PL chromosome arm obtained in this work to confirm the allocation of the remaining five markers on 4PL chromosome arm. Examples of amplification of homoeologous group 4 COS markers are given in Figure 3.

Out the 52 markers from homoeologous group 5, 40 (76.9%) consistently amplified in *A. cristatum* products and from them, 18 (45%) were polymorphic between *A. cristatum* and wheat. Eleven COS markers amplified on the 5P chromosome and seven did not amplified products in any of the available CS/*A. cristatum* addition lines and hence, they could not be mapped. Of the eleven markers located on the 5P chromosome, 5 were amplified on the long arm of the chromosome 5P as demonstrated by their presence in 5PL ditelosomic addition line. Due to the lack of the 5PS ditelosomic addition line, we used the translocation lines involving 5PS chromosome arm obtained in this work to confirm the allocation of 6 markers on the short arm of chromosome 5P. Examples of amplification of homoeologous group 5 COS markers are given in Figure 3.

Table 3. Number and frequency of wheat COS markers from wheat homoeologous group amplified in *A. cristatum*

Wheat group	Polymorphic	Non polymorphic	Total number
4	29 (30,5) [a]	66 (69,5)	105
5	18 (45,0)	22 (55,0)	52
Total	47 (34,8)	88 (65,2)	157

[a]Number in parenthesis are the percentages of wheat markers relative to the total number of amplified markers

Table 4. Number and frequency of polymorphic COS markers from wheat homoeologous 4 and 5 localized in *A. cristatum*

Wheat group	Chromosome location			Non localized	Total number
	4P	5P	6P		
4	12 (41,4) [a]	-	1 (3,4)	16 (55,2)	29
5	-	11 (61,1)	-	7 (38,9)	18
Total	12 (25,5)	11 (23,4)	1 (2,12)	23 (48,9)	47

[a]Number in parenthesis are the percentages of wheat markers relative to the total number of amplified markers

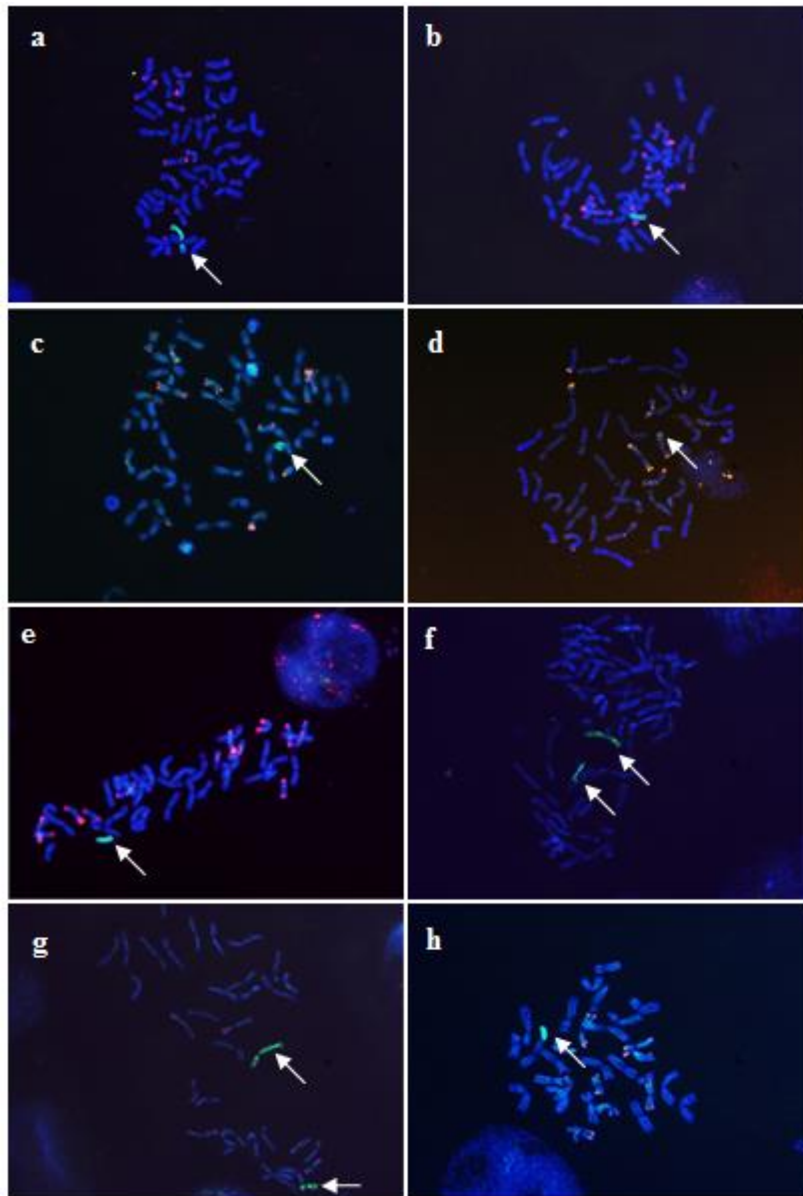

Figure 2. Analysis by FISH of wheat F2 plants containing introgressions from chromosomes 4P, 5P or 6P from *A. cristatum*. (**a**) Monosomic 5P; (**b**) Centromeric translocation 4PL·A/B; (**c**) Centromeric translocation 4PS·A/B; (**d**) Terminal translocation 6P/D;(e) Telosomic 4PL; (**f**) Monosomic 5P plus telosome; (**g**) Monosomic 5P and a deleted chromosome; (**h**) Dicentric translocation 5P·A/B. Genomic DNA from *A. cristatum* was visualised in green and pAs1 repetitive probe was visualized in red. Chromosomes were counterstained with DAPI.

Table 5. Characterization and chromosome localization of COS markers from wheat homoeologous groups 4 and 5 on *A. cristatum*.

Marker	Product size in *A.cristatum*	Product size in *T. aestivum*	Chromosome location in *A. cristatum*	Short arm	Long arm	Chromosome location in wheat[a]	Location in wheat (cM)[a]
COS0018	210	275	4P	-	+	4BS-4DS	25,8
COS0080	908	781	4P	-	+	4AL-4BS-4DS	60,8
COS0025	295	385	4P	-	+	4BS-4DS	63,2
COS001 [b]	320	262	4P	-	+	4AL-4BS-4DS	79,7
COS0021	228	251	4P	-	+	4AL-4DS	84,7
COS0017	560	439	4P	+	-	4AS-4BL-4DL	86,9
COS0023	950	871	4P	+	-	4AL-4B-4D	105,9
COS0094	350	258	4P	+	-	4AS-4BL-4DL	108,8
COS0068	610	592	4P	+	-	4AS-4DL-4BL	109,5
COS0043	800	744	4P	+	-	4AL-4BL-4DL	114
COS0064	198	251	4P	+	-	4AL-4DL	134,9
COS0087	698	877	4P	+	-	4DL	155
COS0105	400	474	6P	-	-	4AS-4BL-4DL	169,3
COS108 [c]	735	624	5P	+	-	5AS-5BS-5DS	22
COS113	451	528	5P	+	-	5AS-5BS	33,4
COS119	375	338	5P	+	-	5AS-5BS	24,6
COS126	306	295	5P	+	-	5AS-5BS-5DS	18,6
COS128	489	461	5P	+	-	5AS-5BS	28,8
COS134	695	644	5P	-	+	5AL-5B-5DL	181,1
COS135	212	266	5P	-	+	5AL-5BL-5DL	172,8
COS139	330	277	5P	-	+	5AL-5BL-5DL	174,5
COS144	282	301	5P	+	-	5AL-5BL-5DL	174,7
COS147	796	735	5P	-	+	5BL-5DL	183,6
COS150	383	389	5P	-	+	5BL	178,7

[a]Quraishi et al. (2009)
[b]COS001 to COS0105 loci markers are from wheat homoeologous group 4
[c]COS108 to COS150 loci markers are from wheat homoeologous group 5

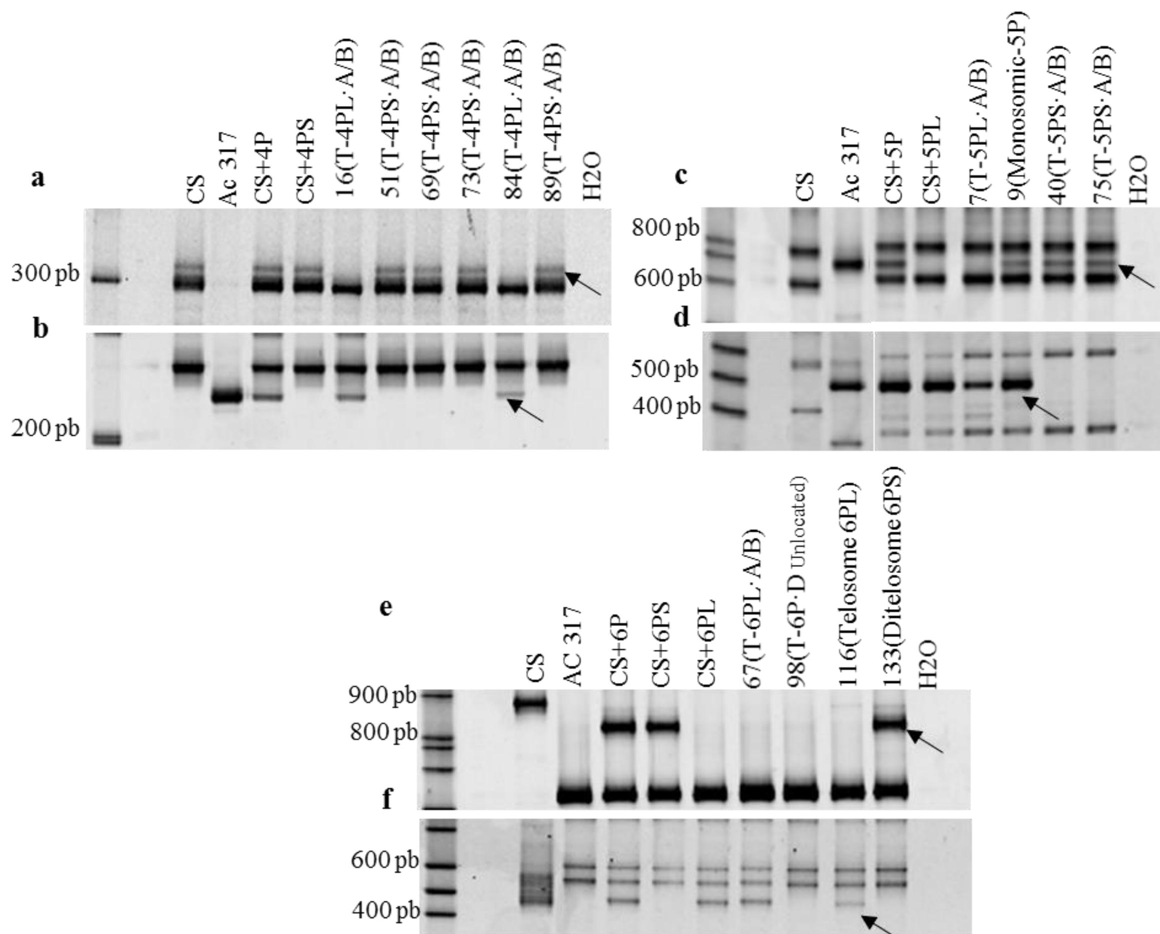

Figure 3. Molecular characterization of wheat-*A.cristatum* translocation lines with chromosome specific COS markers. **a** COS 0068-4PS; **b** COS 0021-4PL; **c** COS 108-5PS; **d** COS 150-5PL; **e** COS 440-6PS; **f** COS 476-6PL

Discussion

Endo (1988) demonstrated that chromosome $2C^c$ from *Ae. cylindrica* induces chromosomes breaks in CS wheat and some of its relatives (Shi and Endo 1999; Friebe et al. 2000; Said and Cabrera 2009, 2012; Cherif-Mouaki et al. 2011, Mattera et al. 2015). In the present study, alien translocation lines were successfully obtained utilizing the wheat-*A. cristatum* 4P, 5P and 6P addition lines. Chromosome breakages were found in the interstitial regions of the chromosome arms as well as in the centromeric regions of the chromosomes. Breaks in the interstitial regions led to deletion and terminal translocations whereas those on centromeric regions resulted in *A.cristatum*-wheat whole-arm translocations and telosomes.

Overall, the frequency of plants carrying translocations generated by gametocidal chromosome was 9.9%. The frequency of translocation obtained varied depending on the *A. cristatum* chromosome involved in the translocation, ranging from 19.0 % for chromosome 4P, 9.9 % for chromosome 5P and 5.4% to chromosome 6P. Most of the translocation lines were whole-arm translocations, which indicated that both the *A. cristatum* and wheat chromosomes were more easily broken at the site of the centromere. In addition to the proximal centromere, breakpoints were also found in the interstitial regions of the chromosome arms as demonstrated by wheat-*A. cristatum* dicentric translocation and terminal translocation detected in the progeny of CS+2C/wheat-*A. cristatum* 5P addition line and CS+2C/wheat-*A. cristatum* 6P addition line, respectively.

Chromosome translocations between wheat and the P genome have not been widely reported.Luan et al. (2010) used the wheat-*A. cristatum* disomic addition line for chromosome 6P as bridge materials to produce wheat-*A. cristatum* 6P translocation lines induced by gametocidal chromosomes. These authors found that the frequency of translocation induced by gametocidal chromosome was 5.08%, which was similar to the frequency of plants (5.8 %) carrying wheat-6P chromosome translocations generated by the gametocidal chromosome in the present work. Liu et al. (2010) obtained a frequency of 3.75% translocations between wheat an *A. cristatum* chromosome 4P which was lower than those (19.0 %) obtained in the present work.

Previous studies have yielded a limited set of molecular markers useful for *A. cristatum.* Chen et al. (1994) used a set of 14 wheat RFLP (Restriction Fragment Length Polymorphim) probes identifying each homoeologous chromosome arm set of wheat-*A.cristatum* addition lines. Luan et al. (2010) used seven STS (Sequence Tagged Set) markers and one SCAR (Sequence Characterized Amplified Region) specific for chromosome 6P. Han et al. (2014) amplified 6P genome-specific bands using 21 polymorphic SSR (Simple Sequence Repeat) markers from wheat homoeologous group 6 and four SSR 4P-genome specific markers were used by Liu et al. (2010). To date, this is the first reports transferring wheat COS markers to homoeologous group 4P and 5P from *A. cristatum*. The high transference rate of COS markers to *A. cristatum* (85,9 %) was expected since these markers were intended for comparative studies among grasses (Quraishi et al. 2009). This rate is similar than that

obtained by Mattera et al. (2015) transferring COS markers to other Triticeae specie such as *H. chilense.* The transferability of COS markers reported in this work clearly outperforms both STSs and SSRs. Besides, these markers show a good collinearity among different grasses and they are therefore expected to be highly informative for comparative mapping between *A. cristatum* and wheat.

A high proportion (83%) of wheat group 4 and 5 COS markers mapped to chromosomes 4P and 5P, respectively, indicating that synteny is conserved between wheat and *A. cristatum* for these two chromosomes. There was, however, one exception. One marker (COS105) from wheat homoeologous group 4 did not amplified the PCR product in the 4P addition line in wheat but did amplified product in the 6P addition line indicating that this marker was located in chromosome 6P.

The arm location of wheat group 5 COS markers in *A. cristatum* showed a good correspondence with wheat, with all loci mapping on the same arm location in both wheat and *A. cristatum*. Only COS144 mapped on the long arm of wheat homoeologous group 5 was located on the short arm of chromosome 5P. However, the most notorious change refers to marker loci on group 4 since nearly half of the markers located on this group were found in the opposite arms in *A. cristatum* (Table 5). These results might indicate that some rearrangement have occurred on chromosome 4P. Nevertheless, further studies would be required to confirm this hypothesis.

Twenty-three (48.9%) (16 from homoeologous group 4 and 7 from homoeologous group 5) polymorphic COS markers between *A. cristatum* and wheat did not amplified product in any of the available CS/*A. cristatum.* One explanation for the lack of amplification of these markers in the *A. cristatum* chromosome present in the addition lines would be genetic differences between the *A. cristatum* accession used in this study and the accession used to obtain the addition lines. The *A. cristatum* accession (PI222957) used here was a tetraploid ecotype from Iran, whereas the chromosome addition lines were isolated from a backcross of an intergeneric hybrid of wheat CS with a tetraploid ecotype of *A. cristatum* from Inner Mongolia, China (Chen et al. 1989, 1992). Studies of genetic diversity have shown that there is large allelic variation in microsatellite loci between different populations of *A. cristatum*

collected from similar (Che et al. 2008, 2011) or different ecogeographical regions (see chapter I)

In conclusion, the genetic stocks characterized here include new wheat-*A. cristatum* recombinations useful for genetic studies and with a potential for breeding. Furthermore, these lines allowed the identification of new molecular markers useful for selecting new introgressions involving both chromosomes 4P and 5P from *A. cristatum*.

Acknowledgements

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Chapter IV

Cloning and characterization of a putative orthologue of the wheat vernalization (*VRN1*) gene in perennial wheatgrass (*Agropyron cristatum*)

In preparation

Copete A, Cabrera A. Cloning and characterization of a putative orthologue of the wheat vernalization (*VRN1*) gene in perennial wheatgrass (*Agropyron cristatum)*

Abstract

Flowering time is important in the adaption of crop plants to different environments and has considerable impacts on economic value in agricultural crops. Wheatgrass (*Agropyron cristatum*) is a perennial Triticeae species and one of the most important grasses for pasture and forage in the temperate regions of the world. Late heading is of major interest in breeding programs of forage crops and the possibility to increase the time to heading by molecular means may be facilitated by the identification and characterization of floral genes. Besides, characterization of orthologues genes of the wheat is useful to stablish genetic relationships between grasses. In this study, the sequence of an orthologue gene of *VRN-A1* of hexaploid wheat (*Triticum aestivum*) was characterized in wheatgrass (*A. cristatum).* This gene, designated as *VRN-P1*, is expected to be involved in vernalization response. The high sequence similarity of *VRN-P1* to both *VRN-A1* and *VRN-H1* and their localization on the 5PL chromosome arm in *A. cristatum* indicated that *VRN-P1* is orthologous to *VRN1* from wheat and barley. The isolated gene maintain the exon/intron organization of *VRN1*gene and encoded a MADS box transcription factor. Phylogenetic and amino acid analysis confirmed that the isolated sequence belongs to the *VRN1*. Furthermore, phylogenetic analysis of the deduce protein revealed an intimate genetic relationship between *VRN-P1* and *VRN1* from Triticeae species.

Keywords: *Agropyron cristatum,* crested wheatgrass*, Triticum aestivum, VRN1,* vernalization

Introduction

Perennial Triticeae species such as wheatgrasses (*Agropyron* spp.) are one of the most important grasses for pasture and forage worldwide (Asay and Jensen 1996). *A. cristatum* (L.) Gaertner is the most common species of crested wheatgrass, with diploid (2n=2x=14, PP), tetraploid (2n=4x=28, PPPP) and hexaploid (2n=6x=42, PPPPPP) cultivars grown. These crops play an important role in forage and livestock systems, forming the plant basis for beef and milk production worldwide (Hart et al. 1983). In addition to its resistance to drought and cold and its moderate tolerance of salinity, *A. cristatum* has also been valued for watershed management (Wang 2011) and stabilization of soils affected by metal contamination (Miller and Dyer 2002; Meng et al. 2013; Guo et al. 2014) as well as tertiary pool for wheat improvement (Ochoa et al. 2015).

Time of flowering plays a key role in the adaptation of plant species to different environments and has considerable impacts on economic value in agricultural crops. Flowering in higher plants is a complex process involving activation and repression of numerous genes engaged in different pathways (Cockram et al. 2007). In wheatgrasses, flowering time has major impact on the production of forage quality and persistence of these grassland crop. The stem and inflorescence production comprise a significant reduction in the digestibility, nutritional value and productivity of the crop (Minson 1990). Thus, the development of late maturing cultivars is a goal for crested wheatgrass breeding programs and the possibility to increase the time to heading by molecular means may be facilitated by the identification and characterization of floral genes.

Both environmental and endogenous signals influence the switch from vegetative to reproductive growth in plants. In *Arabidopsis* many genes have been characterized involved in theses pathways (Amansino 2010). One of these pathways, vernalization pathway, is controlled by prolonged exposure to cold. In *Arabidopsis,* vernalization reduces the expression of the main repressor of flowering, *FLOWERING LOCUSC* (*FLC*), allowing the plant to flower through the activation of *FLOWERING LOCUS T(FT*) (Andres and Coupland 2012). In cereals, three major genes have been characterized to be involved in this pathway: *VRN1, VRN2, VRN3* (Trevaskis et al. 2007). In hexaploid wheat, homoeologous *VRN1* genes are located on the long arms of the group 5 chromosomes, and named *VRN-A1*, *VRN-B1*, and

VRN-D1 (Law et al. 1976; Iwaki et al. 2002; Yan et al. 2003). The wheat *VRN1* homoeologues encode MADS box transcription factors with homology to the *Arabidopsis* meristem identity genes *APETALA1*, *CAULIFLOWER*, and *FRUITFULL* (Yan et al. 2003; Danyluk et al. 2003; Trevaskis et al. 2003; Kinjo et al. 2012). These genes regulate the transition between vegetative and reproductive phase (Ferrándiz et al. 2000; Shitsukawa et al. 2007).

Large effort has been made to unravel and control vernalization in annual grass species like wheat and barley, but less work has concentrated on genes regulating flowering control in forage crops. *VRN1* has been found specifically involved in the vernalization response in perennial ryegrass (Jensen et al. 2005; Andersen et al. 2006) and attempt to delay the flowering in a commercial grass species by expressing a floral repressor isolated from perennial ryegrass have been carried out (Jensen et al. 2001). As a cool-season grass, crested wheatgrass requires vernalization to induce floral initiation (Hu et al. 2001). The aims of the current work were to clone and to characterize a candidate gene for vernalization response, designated as *VRN-P1*, in wheatgrass (*A.cristatum*), to determine their chromosome location and also provide information on the phylogeny of the Poaceae family based on protein sequences.

Materials and methods

Plant material

Two diploid cultivars of *A. cristatum*, "Ruff" (PI 578519) and 'Parway' (PI 415799) (2n=2x=14, PP) with the same flowering time during 2015 and 2016 seasons were used for marker testing. These cultivars were supplied from the Agriculture Research Service of the USDA (United States Department of Agriculture). The common wheat (*Triticum aestivum* L.) cv. 'Chinese Spring' (CS) was used as positive control in all PCR analysis. A set of available wheat CS/*A. cristatum* disomic and ditelosomic addition lines (Chen et al. 1994; Li et al. 1997, 1998) were used to determine the chromosome location of the vernalization gene in *A. cristatum*.

Markers amplification

Genomic DNA was isolated from young leaf tissue of all the plant material mentioned above using CTAB (Cetyl Trimethyl Ammonium Bromide) method (Murray and Thompson 1980). Primers were designed in the conserved regions of the *VRN-A1* gene from *T. aestivum* using publicly available sequences (GenBank AY747599). Three *VRN-A1* gene-specific primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000) (Table 1, Figure 1). PCR reactions were carried out in 10µl reaction volume containing 20 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of forward and reverse primer and 0.25 U DNA polymerase (Promega Madison, WI, USA). For the amplification, genomic DNA was subjected to an initial denaturation step at 95ºC for 5 min. The amplification conditions were for 35 cycles at the following temperatures: 95 ºC for 30 s in denaturing stage, the annealing and elongation characteristics were shown in Table 2, followed by a final extension at 72 ºC for 5 min. Amplification product were analyzed in 10% (w/v, C: 2.67%) of polyacrylamide gels and visualized by ethidium bromide staining.

Table 1. Primer sequences, amplified region and fragments size (bp) from *VRN-P1* gene in *A. cristatum* and *T. aestivum*

Primer	Sequence	Amplified region	Length in *A. cristatum*	Length in *T. aestivum*
VRN1_fw1 VRN1_rv1	5'GGTGGGGCATCGTGTGGCTG3' 5'CCATGACTCGGTGGAGAACTCGT3'	First exon (I)	497	510
VRN1_fw2 VRN1_rv2	5'GGACAAAATTCTTGAACGGTATGAGCG3' 5'GTCTCAACCTTCGCCTTCAGTTTCC3'	Second to the third exon (II)	1213	1300
VRN1_fw3 VRN1_rv3	5'GGAAACTGAAGGCGAAGGTTGAGAC3' 5'CATCCTCTGCCCTCTCGCCTG3'	Third to the eighth exon (III)	972	1100

Table 2. Specific annealing temperature and elongation time of the PCR of the markers used to sequence *VRN-P1* gene in *A. cristatum.*

Markers	Annealing		Elongation	
	Time	Temperature	Time	Temperature
VRN1_fw1/ VRN1_rv1	60 s	62°C	30s	72°C
VRN1_fw2/ VRN1_rv2	60 s	58°C	2:40s	72°C
VRN1_fw3/ VRN1_rv3	60 s	60°C	2:40s	72°C

Cloning and sequencing

Amplicons obtained from *A. cristatum* cv 'Roof' were cloned into pGEMT vector (Promega, Madison, WI, USA). Three clones were sequenced on both strands starting with the M13-forward and reverse primers located on the vector. The sequence reads obtained were assembled into a single sequence using Geneious version 5.0.4 (Kearse et al. 2012).

The genomic sequences were aligned with both the complete *VRN-A1* from *T. aestivum* (AY747599) and *VRN-H1* from *Hordeum vulgare* (AY750993) and coding DNA sequences (CDSs) of the *A.cristatum VRN-P1* gene were determined using BLASTN or BLASTX (Zhang and Madden 1997) for nucleotide and amino acid sequences respectively. For this purpose, a sequence alignment of multiple proteins was generated using Clustal W (Thomson et al. 1994) implemented in the Geneious package with a Gap Open Penalty established in 12 and the Gap Extension Penalty of 0.3.

The nucleotide and deduced amino acid sequence of the *VRN-P1* genomic DNA were used for BLAST searches on the GenBank/EMBL/DDBJ databases in order to characterize them. The putative deduced amino acid sequences of the protein were aligned using the multiple sequence alignment program ClustalW (Thompson et al. 1994).

Phylogenetic relationships of the amino acid sequence

The evolutionary relationship of the amino acid sequence obtained in *A. cristatum* with other related genes available from the GenBank was assessed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm (Sokal and Michener 1958) based on the deduced amino acid sequences. The confidence in the different nodes was calculated using 1,000 bootstrap replications. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965). All positions containing gaps were eliminated from the dataset (complete deletion option). A phylogenetic tree was constructed using the MEGA4 software (Tamura et al. 2007).

Results

Cloning and sequencing of VRN-P1 gene.

Using primer designed on conserved regions of *VRN-A1* gene from wheat, three fragments were amplified in the cultivars “Ruff” and “Parkway” of *A. cristatum* (Table 1, Figure 1). Electrophoretic analysis of these three amplicons revealed that they had the same size in both “Ruff” and “Parkway” but variation in the *A. cristatum* amplicons were different from the common wheat CS used as standard (Table 1). The amplified fragment 1 (497 bp) included 5-untranslated region (UTR) and the first exon. The amplified fragment 2 (1213 bp) included exon two to exon three and the amplified fragment 3 (972 bp) included exon three to exon eight (Figure 1). We failed to amplify the first intron probably due to the big size of the fragment. The three amplified fragments were sequenced and compared by BLAST analysis with the sequences of the *VRN-A1* from *T. aestivum* (AY747599) and *VRN-H1* from *H. vulgare* (AY750993) available in GeneBank database. The sequence had not been previously described and was designated as *VRN-P1* (Gene Bank accession number MG596801).

The analysis of the sequence at nucleotide level revealed that *VRN-P1* shares an identity of 74% with *T. aestivum* and 53% with *H. vulgare*. The VRN1_fw2 / VRN1_rv2 and VRN1_fw3 / VRN1_rv3 primers pairs overlap between them facilitating sequence assembly. The size of the *VRN-P1* gene in *A. cristatum* was 2368 bp and conserved the same structure, consisting of eight exons and seven introns, as previously reported for the *VRN-A1* gene of common wheat and *VRN-H1*gene of barley (Figure 1). Table 3 showed the size of the eight exons and seven introns of the *VRN-P1* gene. All exons were the same sizethan those of wheat and barley, respectively. However, the introns in *A. cristaum* showed differences in size and in general they were smaller than those of wheat and barley, respectively.

The *A. cristatumVRN-P1* homoeologues encode MADS box transcription factors with homology to the vernalization-responsive MADS box *VRN-A1* from wheat (Figure 2). According to the coding sequences (CDS) alignment of *A. cristatum* with *T. aestivum* 30 SNPs were identified: 18 synonymous and 12 non-synonymous. Among the synonymous SNPs, 3 were located in the MADS domain, 3 in the intervening, 5 in the K-box and 7 in the C-terminal regions, respectively. On the other hand, from the non-synonymou SNPs, 1 was

located in the intervening, 2 SNPs in the K-box and 9 in the C-terminal regions, respectively (Figure 2).

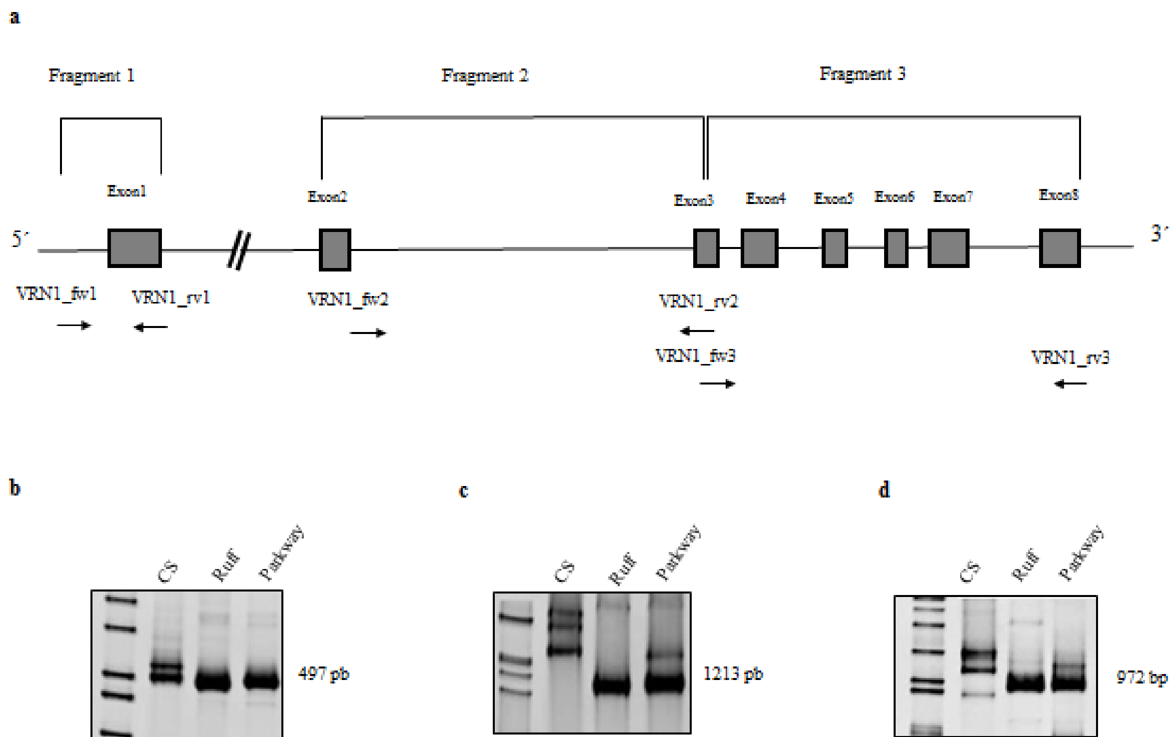

Figure 1. (A) Squematic representation of the wheat *VRN1* gene and the three amplified fragments in *A. cristatum*. PCR amplification in acrylamide gels of *VRN1* gene in two commercial varieties of *A. cristatum* ("Ruff" and "Parkway") using (B) primer pair VRN1_fw1/VRN1_rv1 designed to amplified the first fragment (C) primer pair VRN1_fw2/VRN1_rv2 designed to amplified the second fragment, and (D) primer pair VRN1_fw3/VRN1_rv3 designed to amplified the third fragment.

Table 3. Size in bp of the different exons and introns of the *VRN* sequences evaluated.

	Species		
	A. cristatum	*T. aestivum*[1]	*H. vulgare*[2]
	VRN-P1	*VRN-A1*	*VRN-H1*
Exon 1	185	185	185
Exon 2	79	79	79
Exon 3	65	65	65
Exon 4	100	100	100
Exon 5	42	42	42
Exon 6	42	42	42
Exon 7	113	113	113
Exon 8	109	109	109
Intron 1	-	3014	10789
Intron 2	1067	1475	2487
Intron 3	89	89	88
Intron 4	185	144	195
Intron 5	105	151	144
Intron 6	89	91	89
Intron 7	118	155	250
Total	2388	5854	14777

[1]*T. aestivum* [NBCI ID: *VRN-A1*AY747599], Fu et al. (2005)

[2] *H. vulgare* [NBCI ID: *VRN-H1*AY750993], von Zitzewitz et al. (2005)

Aminoacid deduced sequence analysis

The comparison of the 735 bp CDS revealed that the *VRN-P1* gene showed 95% identity to *T. aestivumVRN-A1* gene (Genebank AY747599) and 96% identity to *H. vulgareVRN-H1* gene (Genebank AY750993) (Figure 2). Protein from *VRN-P1* gene from *A. cristatum* contained 244 amino acids with molecular weight of 27.8 kDa and an isoelectric point of 9.55. The amino acid chain shared 95% and 94% similarity between *T. aestivum* and *H. vulgare*, respectively. Table 4 shows amino acid comparison between *A. cristatum*, *T. aestivum* and *H. vulgare*. A total of 11 aminoacid changes were found comparing *A. cristatum* with *T. aestivum.* These changes were one in the intervening domain, two in the K-box domain and the remain in the C-terminal domain, this last was the region were more aminoacid changes were found.

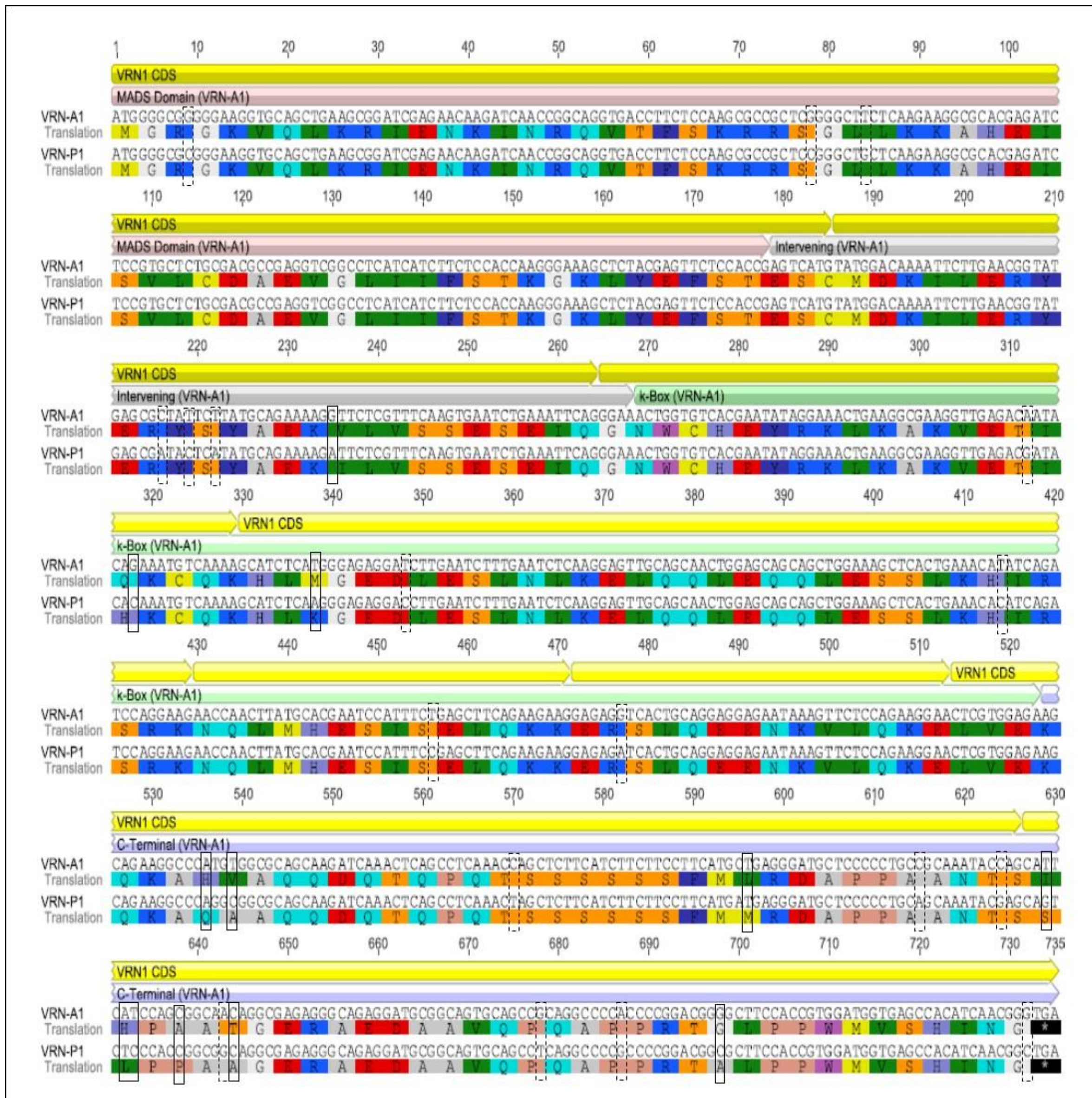

Figure 2. Comparison of the coding DNA sequence of the *VRN-A1* gene from *T. aestivum* and *VRN-P1* gene from *A. cristatum* and the deduced amino acid sequences. The yellow regions represent the 8 different exons, and the pink, grey, green and purple regions represent the structure of the MADS box protein in wheat. The synonymous SNPs are boxed in dotted rectangles. The non-synonymous SNPs are boxed in underline boxes.

Table 4. Amino acid comparison between *VRN1* translated gene of *A. cristatum* and *T. aestivum.*

Position	Domain	*A. cristatum*	*T. aestivum*	Exon
79	Intervening	Ile	Val	2
106	K-box	His	Glu	3
113	K-box	Lys	Met	4
179	C-terminal	Glu	His	4
180	C-terminal	Ala	Val	7
199	C-terminal	Met	Leu	7
210	C-terminal	Ser	Ile	7
211	C-terminal	Leu	His	8
213	C-terminal	Pro	Ala	8
215	C-terminal	Ala	Thr	8
233	C-terminal	Ile	Gly	8

NBCI ID common wheat*VRN-A1*: [AAW73220] (Fu et al. 2005)

Chromosome location of VRN-P1

The chromosome location of *VRN-P1* gene in *A. cristatum* was determined by PCR amplification in a set of available common wheat CS/*A. cristatum* disomic addition lines using the three primers pairs designed on the conserved regions of the wheat *VRN-A1* gene (Figure 3). Electrophoretic analysis demonstrated that the three markers amplified products of 497 bp, 1213 bp and 972 bp, respectively in the CS+5P chromosome addition line indicating that *VRN-P1* gene was located on chromosome 5P of *A. cristatum.* The same amplicons were obtained for the three markers in the CS+5PL ditelosomic addition line indicating that *VRN-P1* gene was located on the long arm of chromosome 5P.

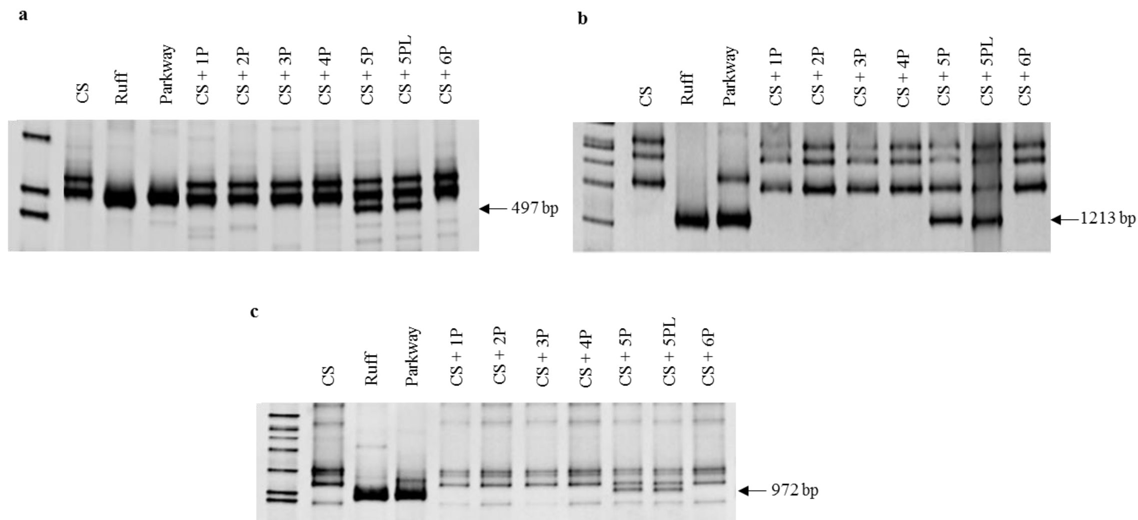

Figure 3. PCR amplification on *A. cristatum* (“Ruff” and “Parkway”) in acrylamide gels showing the localization of *VRN-P1* on the long arm of chromosome 5P. (A)VRN1_fw1/VRN1_rv1 (fragment 1); (B) VRN1_fw2/VRN1_rv2 (fragment 2) and (C) VRN1_fw3/VRN1_rv3 (fragment 3) in the disomic addition lines of *A. cristatum* in wheat CS and ditelosomic for the 5PL chromosome arm.

Phylogenetic analysis

The amino acid sequence of the *VRN-P1* obtained in this study, together with other *VRN1* amino acid sequence present in the databases, were used to construct a dendogram of the family Poaceae based on the maximum composite likelihood method (Figure 4). All sequences were arranged in six groups. *VRN-P1* sequence from *A. cristatum* was clustered together with those from *Triticum* spp, *H. vulgare* and *Secale cereale*, all of them Triticeae species included in the first group. The second group was formed by the protein sequences from both perennial species *Festuca arundinacea* and *Lolium perenne*, which displayed a closed relationship between them. The third group was formed by *Brachypodium distachyon* and the fourth group by *Avena strigose*. The fifth and sixth groups were composed by *Oryza sativa* and *Zea mays*, respectively, which are Poaceae species more phylogenetic distant from Triticeae tribe..

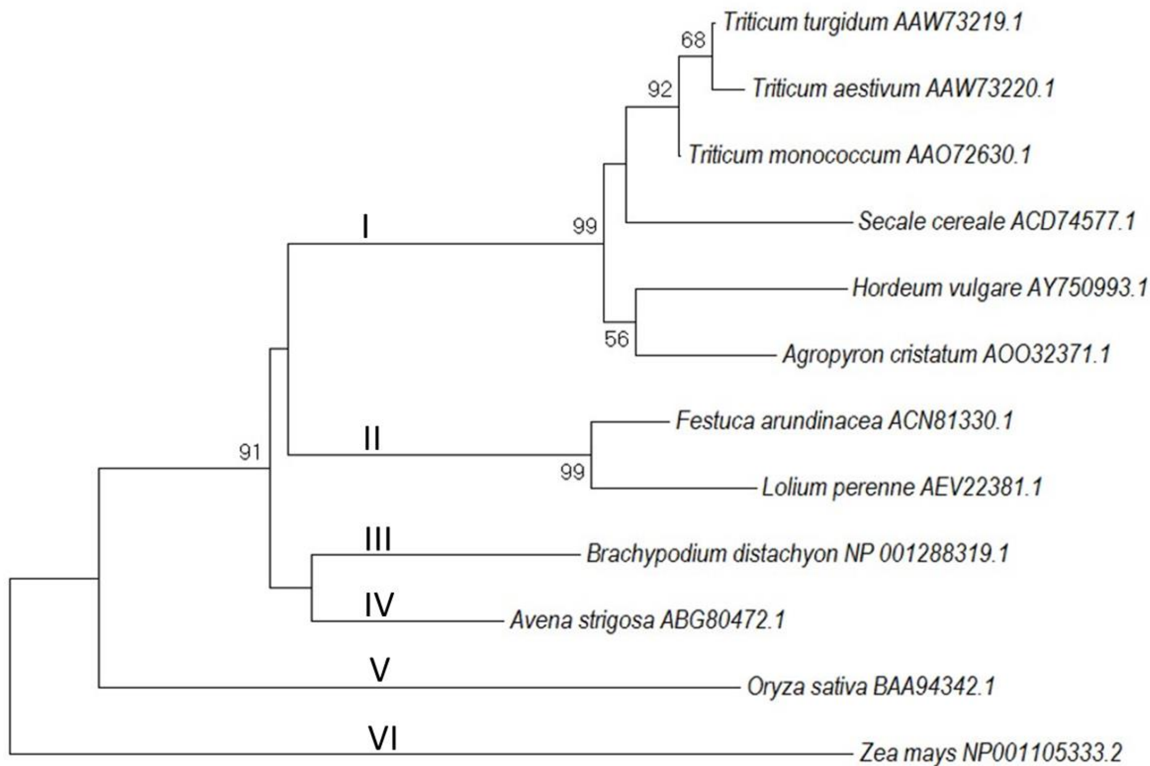

Figure 4. Phylogenetic tree showing the relationship of *A. cristatum VRN-P1* with *VRN1* gene from other species belonging to the Poaceae family. Protein sequences were retrieved from public databases (their accession number appear at the end of the sequence names). Number at branch nodes represent bootstrap values of 1000 replicates for neighbor joining (maximum likelihood distances). The bootstrap values less than 50% are not shown. The scale bar represents 0.01 average changes.

Discussion

Flowering time has major impact on the production of forage quality and seed production, being one of the most important trait to be considered in breeding. The identification and characterization of the genes controlling flowering time in cereal species like wheat (Galiba et al. 1995; Snape et al. 1985; Trevaskis et al 2003), rye (Plaschke et al. 1993) and barley (Börner 2002) have been studied and were useful to stablish genetic relationships between grasses (Laurie 1997; Hay and Ellis 1998).

Comparative mapping in the grasses showed that the same loci in wheat, barley and rye had been arranged in exactly the same order along large stretches of their chromosomes (Devos et al. 1993; Moore et al. 1995). Using this approach, the function of most genes could be

predicted by comparing different genomes, transferring functional annotation from the most studied organism proteins to orthologous proteins in less characterized organisms (Wei et al. 2002). In addition, this extensive conservation is useful to isolate and characterize genes in species with poor information of its genomic resources, as *A. cristatum*. Taking advantage of this conservation and using the sequence information of *VRN-A1* gene of wheat, the DNA sequence of the putative orthologue gene in *A. cristatum* was characterized for the first time.

The analysis at nucleotide level showed that the structure of *VRN-P1* gene was the same that those of both *VRN-A1* of wheat and *VRN-H1* of barley. The initiation codon, ATG and the termination codon TGA for translation of *VRN-P1* were in homologous positions to those in *VRN-A1* and *VRN-H1* genes from wheat and barley, respectively.

Most dissimilarities were found on the non-coding regions and differences in the sizes (bp) of six of the seven intronic regions were observed in the three species (Table 3). Unfortunately, we failed to sequence intron 1 of *VRN-P1*gene. In wheat, the intron 1 of *VRN-A1* contain 3.014 bp and in *VRN-H1* of barley this intron has 10.789 bp. It is expected that intron 1 of *VRN-P1* gene will be of such as large size as in both *VRN-A1* and *VRN-H1* and this may be the cause of failure to sequence it. Intronic regulatory elements have been identified as enhancers, repressors and promoters of gene transcription (Busch et al. 1999; Deyholos and Sieburth 2000; Fiume et al. 2004). In wheat the intron 1, together with the promoter of the *VRN-A1* gene, contain the regulatory sites for different protein interactions and mutations in either one of these two regulatory sites would be sufficient to eliminate or reduce the vernalization response (Fu et al.2005). So, further effort will be carried out to sequence intron 1 of *VRN-P1* gene.

All featured displayed by the deduced amino acid sequence for *VRN-P1* were found to be highly conserved with other species. The deduce amino acid sequence obtained by the CDS of the *VRN-P1* gene of *A. cristatum* is classified in the MADS superfamily proteins. *VRN-A1* is one of the MADS box proteins, and act as a floral switch for the transition of vegetative to reproductive development in plants (Ng and Yanofsky, 2001). The analysis of the interaction of the loci that are part of the process of vernalization in the tribe Triticeae have similar interactions (Cockram et al. 2007) and numerous studies coincide that the gene *VRN-A1* determine the winter/spring growth habit in wheat, depending on insertions or deletions in

the promoter of the first intron (Yan et al. 2003; Fu et al. 2005). Moreover Li et al. (2013) showed that in winter wheat the vernalization requirement duration is controlled by *VRN-A1* at protein level. Changes in only one amino acid of the protein in winter wheat could delay flowering during 3 week (Li et al. 2013) demonstrating that a single gene may evolve different mechanisms to allow adaptation to multiple environmental conditions.

The results of this work showed 30 SNPs between wheat and *A. cristatum* rising in 11 amino acid changes. Most of these amino acid changes were located in the C-terminal domain, the region less conserved in MADS box proteins. Moreover, the C terminal domain is a region that does not seem contribute to the functional specificity indicating that this region can be swapped among different MADS box genes without affecting the specific functions of the resulting hybrid proteins (Krizek and Meyerowitz, 1996). The characterization of the *VRN-P1* expression profile will be of interest in order to study the functional specificity of this gene as well as to introduce different alleles variants in new cultivars.

The complete amino acid sequences of the *VRN-P1* obtained in this study together with other homoeologous *VRN1* protein sequences present in the databases were used to construct a phenogram for aminoacid sequences (Figure 4). Six main clusters were observed, being higher the relation between the *A. cristatum* sequences and the common barley sequences used.

The *VRN-P1* gene was physically located on the long arm of chromosome 5P (Figure. 3). The localization of *VRN-P1* gene is collinear with *VRN-A1* in wheat, located on the long arm of chromosome 5 in each of the A, B, and D genomes, with barley *VRN-H1*gene, located in the 5HL chromosome arm and rye *VRN-R1* gene located on the 5RL chromosome arm (Dubcovsky et al. 1998; Galiba et al. 1995; Iwaki et al. 2002). This conservation of synteny in homoeologous group 5 suggests that the *VRN-P1* gene isolated in the present study is orthologue to the *VRN1*gene.

Recent studies suggest that the *Lolium perenne VRN1* gene is orthologue to wheat *VRN1-A1* and that the function of the gene is also conserved, although the *L. perenne VRN1* promoter region is not conserved with wheat promotor region. These results showed different regulatory proteins in *VRN1* transcription in wheat and perennial ryegrass (Andersen et al.

2006). Recently, RNA-Seq analysis has been conducted of the flowering transcriptome in tetraploid crested wheatgrass and next-generation deep-sequencing technologies have provided new approach to study the transcriptome of different floral developmental stages in *A. cristatum* (Zeng et al. 2017). These studies will allow to investigate gene expression for floral initiation and development in crested wheatgrass.

In summary, the *A. cristatum VRN-P1* homoeologues encode MADS box transcription factors with homology to the vernalization-responsive MADS box *VRN-A1* from wheat and *VRN-H1* from barley. The localization of *VRN-P1* genes on the 5PL chromosome arm and the high sequence similarity of *VRN-P1* to both *VRN-A1* and *VRN-H1* indicated that *VRN-P1* is orthologous to *VRN1* from wheat and barley.

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Discusión general

Discusión general

Agropyron cristatum (L.) Gaertner, es una de las especies de mayor importancia económica del género *Agropyron* debido a su cultivo como planta forrajera. Se considera además un recurso genético del trigo puesto que en su genoma se han descrito genes de resistencia o tolerancia a diversos estreses bióticos o abióticos que pueden ser de interés para ampliar la base genética del trigo (Dewey 1984; Knott 1989; Asay y Johnson 1990; Limin y Fowler 1990; Dong et al. 1992). *A. cristatum* es una especie que presenta una amplia distribución geográfica que abarca desde el Este de Europa hasta gran parte de Asia. Actualmente existen en distintos bancos de germoplasma más de 500 accesiones de esta especie, pero la información básica para su posible uso en mejora, como el nivel ploídico, no aparece descrito para la mayoría de ellas.

En el capítulo I, se efectuaron diferentes estudios sobre una colección de 115 accesiones con el objetivo de que una mejor caracterización de éstas pueda facilitar su uso en mejora genética. Mediante el empleo de la citometría de flujo, se estimó el nivel ploídico de esta colección de accesiones. Este análisis, junto con la ubicación de las accesiones en un mapa, permitió estimar la frecuencia y distribución de los distintos niveles ploídicos (2x, 4x y 6x) descritos en la especie. Al respecto, se observó que las accesiones tetraploides resultaron ser más frecuentes (74.8%) que las accesiones diploides y hexaploides. Así mismo, se observó que las accesiones tetraploides se encuentran distribuidas por todo el rango de distribución propuesto en la especie. Las accesiones diploides fueron menos frecuentes (18.3%) que las tetraploides y aparecen distribuidas en el mismo rango de distribución que las tetraploides, excepto en la región que comprendería la actual Turquía e Irán. Las accesiones hexaploides resultaron ser menos frecuentes (6.9%) que las diploides concentrándose mayormente en el Noroeste de Irán y el Este de Turquía. La distribución de las poblaciones hexaploides obtenidas en este trabajo procedentes de Irán, coincide con los resultados obtenidos por Dewey y Asay (1975) que analizan el nivel ploídico de 200 poblaciones de este país. Estos autores destacaron también la baja frecuencia de las poblaciones diploides en Irán dado que solo detectan una población diploide. De los resultados del estudio realizado por Dewey y Asay (1975) así como los obtenidos en el presente trabajo se observa que, dentro del rango de distribución propuesto en la especie, existe al menos una región que incluiría la actual

Turquía e Irán en la que las poblaciones diploides son menos frecuentes en comparación con el resto así como también se observa un mayor número de poblaciones hexaploides. Este hecho pudiera indicar la existencia en esta especie de diferentes patrones evolutivos.

La investigación de la diversidad genética de una especie es un requisito previo para trazar una estrategia eficaz de conservación y utilización (Govindaraj et al. 2015). En *A. cristatum* este tipo de trabajos han sido escasos y en general han empleado un reducido número de accesiones. Otro de los estudios desarrollados en el capítulo I fue una evaluación de la diversidad genética de esta colección de accesiones analizada mediante su contenido de ADN, los alelos obtenidos mediante el empleo de marcadores SSR y caracteres agro-morfológicos. En el estudio llevado a cabo con marcadores SSR, se observó un elevado polimorfismo dentro de cada accesión indicando una alta variabilidad genética en la especie. Esta hipótesis se apoya también en el hecho de que los índices de similitud entre accesiones obtenidos por el Análisis de Grupos han sido bajos. Esta alta variabilidad podría explicarse por la hipótesis de que el genoma P presente en las accesiones tetraploides, está compuesto en realidad por dos genomas, uno de los cuales se deriva del otro mediante translocaciones recíprocas (Hsiao et al. 1989; Martín et al. 1999). Por otro lado, el hecho de que en el Análisis de Grupos todas las muestras hexaploides se encuentran agrupadas en el mismo subgrupo (V-1) y de que se observe una estrecha relación genética entre las muestras tetraploides y hexaploides, sugiere que éstas últimas pudieran proceder de las accesiones tetraploides generándose mediante la formación de gametos no reducidos. En este sentido el hecho de que se hayan detectado gametos no reducidos en otra especie de este género (*A. dasystachyum*) apoya esta hipótesis (Sadasivaiah and Weijer 1981).

En este trabajo se ha llevado a cabo además un ensayo para evaluar características agromorfológicas empleando 24 de las 71 accesiones incluidas en el estudio de variabilidad genética. Se detectaron diferencias significativas entre accesiones para longitud y anchura de la espiga, así como para el número de espiguillas. Al evaluar estos caracteres entre los diferentes niveles ploídicos se ha observado que las accesiones tetraploides presentaron una mayor longitud de la espiga que las accesiones diploides. Por otro lado, las espigas de las accesiones hexaploides fueron más anchas que las espigas de las accesiones diploides y tetraploides. No se observaron diferencias entre los tres niveles ploídicos para el número de

espiguillas por espiga. En este sentido sería aconsejable incluir un número mayor de accesiones hexaploides en un futuro ensayo con el objetivo de evaluar la posible influencia que el nivel de ploidía puede tener en los caracteres agromorfológicos de esta planta forrajera. En el ensayo se observó además, una alta variabilidad para otros caracteres, como fertilidad, fecha de floración, porte de la planta y el número de tallos. Esta alta variabilidad para caracteres agromorfológicos puede ser explicada por la alta variabilidad genética presente en estas accesiones como así ha sido puesta de manifiesto mediante el estudio realizado con marcadores moleculares.

Los resultados obtenidos permitieron obtener información de aquellas accesiones que *a priori* podrían ser más interesantes para ser empleadas en mejora de este cultivo en el desarrollo de nuevas variedades más adaptadas al Valle del Guadalquivir si bien sería conveniente realizar nuevos ensayos en otros ambientes para poder evaluar el potencial mostrado por algunas de estas accesiones. Por otra parte, las diferencias observadas entre plantas para algunos caracteres de interés agronómico como porte, número de tallos o fecha de floración, entre otros, sugiere que estos materiales podrían ser empleados en el desarrollo del mapa genético de la especie.

Como se ha comentado anteriormente en el genoma P del género *Agropyron* se han descrito genes que son de interés para la mejora de los trigos cultivados. Se han obtenido híbridos interpecíficos entre distintas especies del género *Agropyron* y trigo blando, pero el apareamiento meiótico observado entre los genomas de ambas especies ha sido muy bajo (Jubault, et al. 2006). Este hecho sugiere que las líneas de translocación estables pudieran ser una alternativa para introgresar genes de *A. cristatum* en el trigo. Este es uno de los objetivos de un programa de mejora que se está llevando a cabo en el Departamento de Genética de la Universidad de Córdoba. Para ello inicialmente se empleó como puente genético el anfiploide *xAgroticum* (DDPP) obtenido en el cruzamiento entre *A. tauschii* (2n=4x=28, DDDD) y una accesión tetraploide de *A. cristatum* (2n=4x=28, PPPP) (Martín et al 1999). Una planta octoploide obtenida en este cruzamiento se ha retrocruzado hacia los trigos cultivados con el objetivo de introgresar genes de interés agronómico en el cultivo del trigo. El primer resultado ha sido la incorporación de resistencia a roya de la hoja en trigo harinero mediante la introgresión de la translocación 1PS•1BL procedente de *A.cristatum* (Ochoa et al. 2015). En este sentido, el empleo de las líneas de adición disómicas y ditelosómicas de *A. cristatum*

en trigo blando desarrolladas por Chen et al. (1992) pueden ser de gran utilidad en la obtención de nuevas líneas de translocación puesto que pueden permitir concentrar los esfuerzos en mejora en el desarrollo de nuevas líneas de introgresión en aquellos cromosomas que contienen genes de interés para el cultivo del trigo. Actualmente se conoce muy poco acerca de las regiones específicas de cromosomas de *A. cristatum* que puedan contribuir a mejorar las características agronómicas en trigo. Sin embargo, con el uso de las líneas de adición se han atribuido diversos caracteres favorables a genes localizados en cromosomas específicos de *A. cristatum*, como por ejemplo: resistencia a la roya de la hoja en los cromosomas 1P (Ochoa et al. 2015), 2P (Jiang et al. 2018) y 6P (Song et al. 2016), resistencia a la roya de tallo en el cromosoma 5P (datos no publicados), tolerancia a la sequía y al frío en los cromosomas 4P (Liu et al. 2010) y 7P (Lu et al. 2016) o genes que aumentan el número de espiguillas por espiga en el cromosoma 6P (Wu et al. 2006; Zhang et al. 2015).

La identificación de cromosomas de *A. cristatum* que poseen genes de resistencia o tolerancia a oídio, enfermedad causada por *B. graminis,* fue uno de los objetivos abordados en el capítulo II. Como resultado se observó que los brazos largos de los cromosomas 2P y 6P de *A. cristatum* portan genes de interés para el control de esta enfermedad. Song et al (2013) y Li et al (2016) detectaron también genes de resistencia a oídio en los cromosomas 6P y 2P, respectivamente, pero el presente trabajo supone un avance ya que se localizan en el brazo de ambos cromosomas.

Otro de los trabajos de esta tesis desarrollado en el capítulo III ha sido la de intentar generar nuevo germoplasma de trigo blando con introgresiones de *A. cristatum*. Para ello, las líneas de adición disómicas de los cromosomas 4P, 5P y 6P de *A. cristatum* en trigo blando fueron cruzadas con otras portadoras del cromosoma gametocida $2C^c$ de *Ae. cylindrica,* con el propósito de promover roturas en los gametos del trigo e introgresar fragmentos de estos cromosomas de *A. cristatum* en trigo. La evaluación y caracterización de las plantas F2 obtenidas permitió la detección de translocaciones entre el genoma del trigo y los cromosomas 4P, 5P y 6P. La mayoría de las translocaciones obtenidas son centroméricas, si bien se ha obtenido una translocación terminal entre trigo y el cromosoma 6P. La mayoría de las translocaciones obtenidas están en monosomía por lo que sería necesario obtener nuevas generaciones F3 por autofecundación de las plantas F2 con el objetivo de intentar generar

líneas de translocación disómicas. Es de destacar las translocaciones obtenidas con el cromosoma 6P ya que en este cromosoma se han localizado genes de interés agronómico como tolerancias a roya y oídio (Song et al. 2013; Song et al. 2016), mayor número de espiguillas por espiga (Wu et al. 2006), mayor peso de mil semillas (Zhang et al. 2015) y múltiples vástagos fértiles (Ye et al. 2015).

Así mismo, se han obtenido plantas telosómicas para el brazo largo del cromosoma 4P y plantas telosómicas para el brazo corto del cromosoma 5P. La autofecundación de estas plantas podría permitir la obtención de las correspondientes líneas de adición ditelosómicas para estos brazos cromosómicos. La obtención de este nuevo germoplasma es importante, ya que no se han obtenido previamente, y pueden ser empleadas en un futuro para la localización de nuevos genes de interés para la mejora del trigo así como en el desarrollo de mapas físicos.

El desarrollo de marcadores moleculares estrechamente ligados a los genes de interés es una herramienta deseable en mejora genética. El mapeo físico de una colección de marcadores localizados en sus respectivos cromosomas o brazos cromosómicos puede facilitar a su vez la detección de marcadores ligados a los genes de interés. El mapeo físico en el genoma P de *A.cristatum* de una colección de marcadores COS desarrollados en trigo ha sido uno de los objetivos propuestos en los capítulos II y III. Previamente se evaluó la transferencia de estos marcadores a *A. cristatum*. En total, de los 327 marcadores COS analizados, 251 de ellos (76.7 %) fueron transferidos a *A.cristatum*. De los 251 marcadores, 93 de ellos (37.1 %) fueron polimórficos con el trigo de los cuales 60 de ellos (64.51 %) se pudieron asignar a cromosomas de *A. cristatum* (Tabla 1).

Tabla 1. Número y frecuencia de marcadores COS polimórficos localizados en los cromosomas 2P, 4P, 5P y 6P de *A. cristatum*

Grupo de trigo	Localización cromosómica				No localizados	Número total
	2P	4P	5P	6P		
2	13 (56.5) [a]	6 (26.1)	-	-	4 (17.4)	23
4	-	12 (41.4)	-	1 (3.4)	16 (55.2)	29
5	-	-	11 (61.1)	-	7 (38.9)	18
6	-	-		17 (73.9)	6 (26.1)	23
Total	13 (13.97)	18 (19.35)	11 (11.85)	18 (19.35)	33 (35.48)	93

[a]El número en paréntesis indica los porcentajes de marcadores de trigo localizados en *A.cristatum,* en relación con el número total de marcadores probados.

En total se han conseguido mapear físicamente un total de 60 marcadores en diferentes brazos cromosómicos: 2PL, 4PS, 4PL, 5PS, 5PL, 6PS y 6PL. El uso de estos marcadores es de interés para el desarrollo de mapas comparativos entre trigo-*A.cristatum*, así como puede permitir el seguimiento de las introgresiones procedentes del genoma P de *A. cristatum* en trigo en los programas de introgresión. En la figura 1 se observa un diagrama que muestra la ortología entre trigo-*A. cristatum* para los marcadores COS transferidos. La elaboración de este mapa permite contrastar la localización en cada cromosoma de los marcadores en *A.cristatum* y trigo observándose, en general, una alta conservación de la sintenia, particularmente para el cromosoma 5P, mientras que fue menor en los cromosomas 2P, 4P y 6P.

Algunos marcadores específicos del brazo largo de los cromosomas de trigo, se mapearon en el brazo corto del mismo cromosoma homeólogo en *Agropyron*. Esto se debe posiblemente a inversiones intracromosómicas o duplicaciones intragenómicas, producto de cambios estructurales que son relativamente frecuentes en la evolución de las especies de la tribu Triticeae (Liu et al 1992; Hu et al 2012).

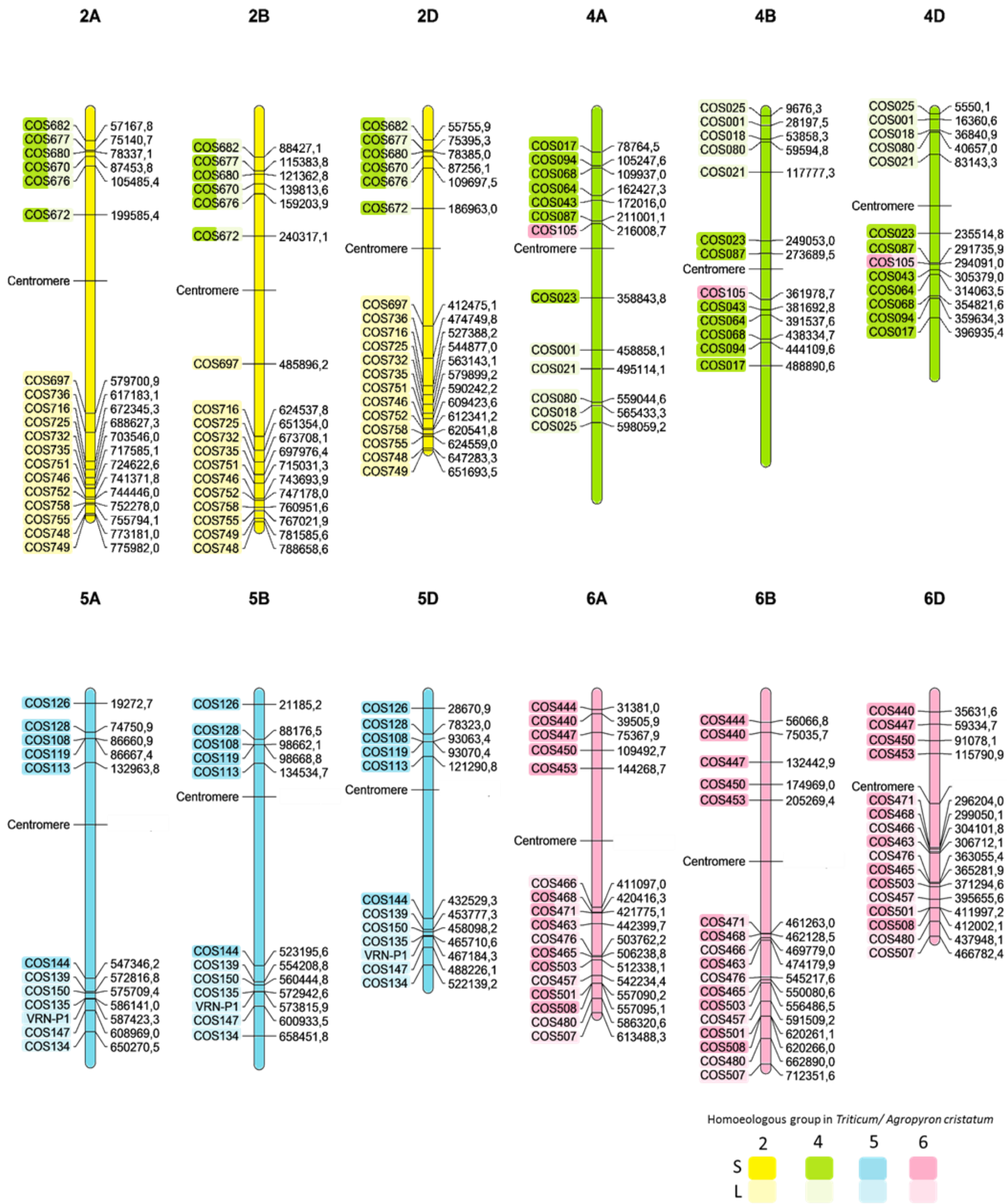

Figura 1. Visualización de la ortología entre los cromosomas de trigo- *A.cristatum*, desde la perspectiva de los grupos homeólogos 2, 4, 5, y 6 de trigo. La posición de los marcadores se encuentra al lado izquierdo y la posición genómica en trigo (Kb) al lado derecho. Los marcadores señalados con dos colores no han sido ubicados en los brazos del cromosoma en el cual se localizan.

De igual forma, es pertinente mencionar que los marcadores localizados en el cromosoma 4P de *A. cristatum* presentaron mayor colinealidad con el genomio 4A de trigo, que con el B y el D. En este sentido, en un estudio previo se menciona que las regiones pericéntricas del

cromosoma 4P están invertidas en comparación con las homeólogas de los cromosomas 4B y 4D, aunque concuerdan con el orden que tiene la región homeóloga 4A, indicando que este cromosoma de *A. cristaum* ha sufrido una inversión paracéntrica similar a la del cromosoma 4A de trigo (Miftahudin et al. 2012; Danilova et al. 2014).

Por otra parte, se pudo observar que el brazo corto del grupo homeólogo 2 de trigo presenta homeología con el cromosoma 4P, así como el grupo homeólogo 4 lo hace con el cromosoma 6P de *A. cristatum*. En consecuencia con lo anterior, Said et al. (2018) empleando sondas específicas de cromosomas mapeadas físicamente mediante FISH en cromosomas metafásicos, observaron una distorsión de la colinealidad en los cromosomas 2P, 4P, 6P y 7P de *A. cristatum*, señalando translocaciones reciprocas entre los cromosomas 2PS y 4PL.

En base a los resultados obtenidos en el capítulo III de esta tesis, la evaluación mediante FISH de las plantas de trigo que contenían algún tipo de introgresión del genoma P, detectada por mostrar amplificación para el marcador AcOPX11-817 (Wu et al. 2010), reveló que la mayoría de las plantas de trigo eran portadoras de un cromosoma (4P, 5P o 6P). En este sentido, el empleo de marcadores COS de los dos brazos cromosómicos de un mismo cromosoma obtenidos en los capítulos II y III puede ser de utilidad en estudios futuros que se lleven a cabo siguiendo la misma metodología descrita en el capítulo III de cara a reducir la población de plantas a caracterizar mediante FISH. A priori y teniendo en cuenta los resultados obtenidos en este trabajo el empleo de marcadores específicos de brazos cromosómicos reduciría considerablemente la población de plantas que se necesitaría analizar mediante técnicas de hibridación *in situ*, facilitando de esa manera la detección de los genotipos de interés y acelerando la selección de los genotipos deseados.

Una estrategia empleada en mejora genética para el control de enfermedades que afectan a las plantas es la búsqueda de genes de resistencia. Otra estrategia en la lucha contra las enfermedades es evitar que coincidan la fase patogénica del agente causante de la enfermedad con la fase susceptible de la planta, manejando para ello cambios en las fechas de siembra o por ejemplo desarrollando nuevas variedades capaces de acelerar tiempos de floración para reducir la severidad y/o incidencia del agente causante de la enfermedad. Por tal razón el estudio de factores como la respuesta al ataque de patógenos en sus diferentes procesos

reproductivos, así como el estudio de los genes implicados en el proceso de floración pueden ser de utilidad en la mejora genética.

En el capítulo IV de esta tesis se ha estudiado en *A. cristatum* el gen de vernalización *VRN-P1* implicado en el proceso de floración. Un mayor conocimiento del proceso de vernalización en *A. cristatum* podría ser de ayuda en la mejora genética de este cultivo con el objetivo de promover un retraso en el proceso de iniciación de la floración pudiendo de esta manera incrementar el valor nutricional y productivo del cultivo. En este trabajo, las líneas de adición de trigo-*A. cristatum* se emplearon para localizar un gen de vernalización en *A. cristatum* (*VRN-P1).* El gen se localizó en el brazo largo del cromosoma 5P y manteniéndose la sintenia con el gen *VRN1* de trigo, cebada y centeno localizados en el brazo largo del grupo homeólogo 5 (Dubcovsky et al. 1998; Galiba et al. 1995; Iwaki et al. 2002).

En resumen, los análisis del nivel ploídico, variabilidad genética y el ensayo agromorfológico realizados a una colección de accesiones proporciona un mayor conocimiento sobre las accesiones estudiadas y de la especie en general que pueden ser de ayuda en mejora genética de esta especie así como en el diseño de programas de conservación de los recursos genéticos de *A. cristatum*. Los marcadores COS que han sido mapeados físicamente en distintos brazos cromosómicos pueden ser de utilidad en el futuro para el desarrollo de nuevas líneas de introgresión, así como ser de ayuda en la búsqueda de marcadores moleculares ligados a los genes de interés que se puedan encontrar en los brazos cromosómicos de diferentes cromosomas del genoma P de *A. cristatum*. Estos marcadores podrían ser de utilidad como información adicional para estudios de evolución del genoma de *A. cristatum,* el desarrollo de mapas comparativos entre trigo y *A.cristatum* así como permiten el seguimiento de las introgresiones procedentes del genoma P de *A. cristatum* en trigo en un programa de mejora encaminado a introgresar genes de esta especie en el trigo.

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Conclusiones

1. The genetic diversity was evaluated in an *Agropyron cristatum* collection consisting of 115 accessions from 17 countries. All the cytological, molecular, and morphological data demonstrate the high genetic diversity present in *A.cristatum*, making it a valuable resource for breeding programs.

2. Flow cytometry analysis in the *A. cristatum* collection revealed that the tetraploid level (74.8%) was the most frequent in this species followed by the diploid (18.3%) and the hexaploid (6.9%). A relationship between geographic distribution and ploidy level was observed.

3. All the wheat simple sequence repeat (SSR) markers used in this study presented high levels of polymorphism, generating 166 different alleles. Based on polymorphic information content (PIC) values obtained (0.579–0.968), all the SSRs were classified as informative markers.

4. *Agropyron cristatum* exhibits resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). Resistance is conferred by gene(s) located on chromosome arms 2PL and 6PL.

5. A total of 60 wheat COS markers have been mapped to *A. cristatum* chromosomes: 13 localized in chromosome 2P, 18 in chromosome 4P, 11 in chromosome 5P and 18 in chromosome 6P, respectively. The mapped COS markers specific to both 2PL and 6PL chromosome arms will facilitate marker-assisted screening in wheat breeding for powdery mildew.

6. A candidate gene for vernalization response, designated as *VRN-P1* in *A.cristatum* was identified based on DNA sequence homology to *VRN-A1* of hexaploid wheat (*T. aestivum*). Coding sequences of *VRN-P1* showed high similarity with *VRN-A1* from *T. aestivum* (95%). The localization of *VRN-P1* on the 5PL chromosome arm in *A. cristatum* indicated that *VRN-P1* is orthologous to *VRN1* from wheat, barley and rye.

7. Wheat-*A. cristatum* translocation lines involving chromosomes 4P, 5P and 6P have been obtained. From these lines, translocation involving 6PL chromosome arm may be a valuable germplasm for introgression of powdery mildew resistance into wheat.

8. Mapping of wheat COS markers and *VRN-P1* gene in *A. cristatum* revealed that macrosynteny between wheat and *A. cristatum* chromosomes is generally well conserved indicating that introgression from P genome into wheat is feasible.

Supplementary files

Supplementary file 1. COS markers used in this work. For simplicity a Lab code (COS Lab) was assigned to each COS markers designed by Quraishi et al. (2009).

Information provided by Quraishi et al. (2009). Genomics in cereals: From genome-wide conserved orthologous set (COS) sequences to candidate genes for trait dissection. Func Integr Genomics 9:473-484

COS Lab code	**Polimorphic *A. cristatum* / wheat**	**Wheat accession**	**Wheat position**	**Wheat chromosome**	**Chromosome arm**	**Primer Forward**	**Primer Reverse**	**Amplicon size**
COS669	No	GPI:C:746665	18,5	2	2AS|2BS|2DS	TAGTTGTGTCAGCAGCAGCC	CATTGGTGGAGTAGACCTGC	350
COS670	Yes	GPI:C:746848	19,4	2	2AS|2BS|2DS	TAACAATGGACTTGGTTGGG	ACTTGTAGCAGCGATGAAGC	860
COS671	No	GPI:C:750922	22,3	2	2AS|2BS|2DS	AGGGAACTGCCTCACTCC	GTAGTACTCGTAGTCGGCGG	374
COS672	Yes	BE494907	24,8	2	2AS|2BS|2DS	GGATGTTTTGGCAAGTGACC	TTCCTATCAGCATTCCTTGG	707
COS673	No	GPI:C:723421	28,1	2	2BS	GAAGCTAATTGACCTGTGTGG	TTCTTCTCCTTGGCTATCCC	258
COS674	No	GPI:C:750791	28,3	2	2BS|2DS	GATCTAGAGGCGGTCAAGC	ATGACGGCGTACTTCTTAGC	350
COS675	No	GPI:C:741406	29,7	2	2BS	TAGTTTTGTTTCCCCTTCCC	CGCTGAAGGATTTGTATTCC	710
COS676	Yes	GPI:C:758799	30,6	2	2AS|2BS|2DS	CGCAGAAAAACAAAGCCAC	TCTGAAAAAGTTCCCGTCAG	711
COS677	Yes	GPI:C:754613	31,7	2	2AS|2BS|2DS	GTTCAACACCAATTGAAGGG	GAGCAGATGTTCCAGCCG	705
COS678	No	GPI:C:745448	32,1	2	2BS	CTCCTGCTTTCTCTCGTCG	GCACTTGGCGTAGTTGGG	562
COS679	No	GPI:C:767104	33,5	2	2AS|2BS|2DS	ATGCTGTCTTGCTCTGAACC	TCGTAGAAGACAGGTGGTGG	425
COS680	Yes	GPI:C:770951	35,9	2	2AS|2BS|2DS	CGGAGTGGCTCTCAACTTC	ATGGCACCAACAGCTAAAAG	884
COS681	No	GPI:C:748390	36	2	2BS|2DS	ACAGACAGAGAGGAAATCGC	TAGCCGCCGACGGAGTAG	327
COS682	Yes	GPI:C:815421	38,9	2	2AS|2BS|2DS	GTGGTCAAGTTATTGTCGCC	AAATGCACAAGAGAAGACCG	887
COS683	No	GPI:C:771296	44,4	2	2DS	TCCAGAGGCTGATATTGAGG	GGCAGTCTTTTCCTGTTCC	268
COS684	No	GPI:C:726890	44,6	2	2BS|2DS	TCTTCCATGCCTTCTACTGG	ATTAATGCTCCCAAATGTCG	514
COS685	No	GPI:C:741602	45,8	2	2AS|2BS	CGAAGCTAAGTTCCTTGTGC	CCTCGACACATCCTTCTACC	865
COS686	No	GPI:C:748201	46,4	2	2BS|2DS	TGTCACTGTTGTCATCTGCC	TCATGTGAAATGACTCACGG	751
COS687	No	GPI:C:752652	48,6	2	2BS|2DS	GCAGATCCCGAACAGCTTC	GATCTTTGTCCATGTCCAGG	424
COS688	No	GPI:C:719382	52,3	2	2BS|2DS	GACTCACGACCATACATGCC	TCACTTGGGAAAAGATCAGG	344
COS689	No	BE604760	54,2	2	2DS	CTACGTTCGAGGCTTTTACG	AAGGTCAGGTCGTAGTTCAGC	898
COS690	No	GPI:C:737274	54,4	2	2AS|2BS|2DS	AGGCGGTAAGCAGAAAGTGG	CAGAAATTCTCTTGATGCTCG	622
COS691	No	GPI:C:751379	56,3	2	2AS|2DS	CAGATGATCCGATAACCAGC	GAAAGAAACCCTCCTTGAGC	736
COS692	No	GPI:C:748718	57,4	2	2AS|2BS	CCTTCCTCCTCACTCAGGC	GAGGAGCACGGGGGAGTC	280
COS693	No	GPI:C:764204	60,1	2	2AS|2DS	GAGCTTCAAGGAGTTCAACG	ATCCATGAAATCCAGGTTCC	460
COS694	Yes	GPI:C:733078	62,8	2	2BS|2DS	AAGAAGAAGAGCACCCATCC	TGTTGAAGACATTGACGACG	441
COS695	No	GPI:C:756234	63,9	2	2AS|2DS	GTGCTACCCTCCTCCACC	GACAGAGTTGAGCATGAGGC	504
COS696	No	GPI:C:749557	69	2	2AS|2DS	TGCTGAAATCAATAGCTCTGT	TCCCTTCTCACTTTCTCCCT	259
COS697	Yes	GPI:C:734048	136,4	2	2AL|2BL|2DL	ATGTTGGAAGCATTACAGCC	CTCTTTGCAAGGTCTTCTGC	666
COS698	No	GPI:C:723204	136,7	2	2BL	TCCAGTTAAATTCGAAACCG	CTCTTCCAGGGTATTCTGCC	537
COS699	No	GPI:C:753726	136,7	2	2BL|2DL	AGTAGTATTGCCATGGCTGC	TTGGTAGCAGAACTGTGTGG	379
COS700	No	GPI:C:740050	138,9	2	2AL|2BL	CTCCAGTCCTTCTTCGAGC	AAACTTGTGGTAAATTGCCG	389
COS701	No	GPI:C:740970	140,2	2	2AL|2BL|2DL	AAGTCAAGCTCGCCTTCC	GTAGAAGACGATGAGGTCGC	439
COS702	No	GPI:C:741637	141,3	2	2AL|2BL|2DL	GTCATGGCCTGAAGTCTACC	ATGTAAGCAAAGGCAAAAGG	894
COS703	No	GPI:C:754616	141,5	2	2AL|2DL	ACCCACATGCTACACATCG	CTTGTAGTAGATGCCCACCC	469
COS704	No	GPI:C:749924	143,4	2	2AL|2BL	CATCCTCCACACCACACC	GTTCAGAGAGATGGGAACCG	573

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS705	No	GPI:C:744070	143,7	2	2AL|2BL|2DL	CACCTACCTCGTCAACGG	GCCGAGAGCTCGATCAGG	758
COS706	No	GPI:C:752286	144,2	2	2BL	GCTTCAGGTCGCTGGAGG	AAGATATGCCTGCGTTTGC	780
COS707	No	GPI:C:729382	145,5	2	2BL|2DL	GTGCAGATGTCATTGATTGC	CCTGCATTCCTGTATCTTGG	376
COS708	No	GPI:C:751189	145,8	2	2AL	CCAGTGATATTTCTGGACGG	CATATTTTCAACTCACCACGG	773
COS709	No	GPI:C:753541	147,5	2	2AL|2BL	GCTCTCCTACAACCCAATCC	ATCTGATAAAGCCCTGGTGG	448
COS710	No	GPI:C:769917	148,6	2	2BL	CTCTCCGTCCTCCTCTCCT	CTTCGGTGACCGTCGACAT	283
COS711	No	GPI:C:739382	148,8	2	2BL|2DL	CGGATGTTGTTTTTGATGTCG	TTCTTTTCTGCTATGGTGGC	309
COS712	No	GPI:C:749297	149,3	2	2AL|2BL|2DL	CGAAAGACAAAGTGGTGTCC	ACTCTGCGCTGTTCTTGAGG	258
COS713	No	GPI:C:781058	149,3	2	2AL|2BL|2DL	AACTGCGATGAACTCTCTGC	AAAGTCTAGCACAAAGGGAGC	278
COS714	Yes	GPI:C:735057	150,8	2	2AL|2BL|2DL	CACGAGCTCAACTCTTCTCC	AAAGACTTGGATCCGGGC	281
COS715	No	GPI:C:752156	151,3	2	2AL|2BL|2DL	CATTTGAGAGAACTGCAATGG	GGGTAATAAAAGTGGGACCG	292
COS716	Yes	BF291656	152	2	2AL|2BL|2DL	GCAGTTGGCACATCAACTAA	GGCAGAGGTCAAAACAAGAA	724
COS717	No	GPI:C:761117	152,1	2	2AL|2BL|2DL	GGTACTTGCCCATCTTAAACC	GTGCTCACCATGAAGAAAGG	332
COS720	No	GPI:C:751906	155,7	2	2BL|2DL	TTGTTGTTGTTCTTGTTGCC	TCGGAGAAGATTCAGAGACG	879
COS721	Yes	GPI:C:756600	155,7	2	2AL|2BL|2DL	CTCAGCAGCCTCAGATACG	GCACAATCCACTAATCTGGC	606
COS722	No	GPI:C:758903	158,7	2	2AL|2BL|2DL	CTACCAGTACAACCCCAAGG	GCTGTAGTAGTCGTAGCGGC	688
COS723	No	GPI:C:745074	159,6	2	2AL|2BL|2DL	ATCAACAAGATCTTCGACGG	CTTTGTCTGAACATTGCTGC	849
COS724	No	GPI:C:770952	161	2	2AL|2BL	ACGTCCTAGCTGCCACTCC	CCTGTACCACTTGAGGAACG	483
COS725	Yes	GPI:C:795544	162,4	2	2AL|2DL	TCATTTGGTTGCATAGTTGC	AATTTAGCAGTATTCTTAGCTTCCC	358
COS726	No	GPI:C:725554	162,5	2	2AL|2BL	GGACGAGGCTTGAACTTGG	CCAACTTCAGTGTCTGAAAGC	616
COS727	No	GPI:C:763094	162,7	2	2AL|2BL|2DL	CAACCAGGTAAATGGTGTGC	TGGTTTTCGTGGATTATTGC	271
COS728	No	GPI:C:753377	164,3	2	2AL|2DL	CTTGTTCAGTGTTTCCCTGG	AAGACCTTGGACATCGACC	329
COS729	No	GPI:C:759269	164,3	2	2AL	AGGTGACCCCGAAGAAGG	ATCAGGAGCTTCCAGTTGC	275
COS730	No	GPI:C:760814	167,4	2	2DL	TTTCTCATCGAACTTCAGGC	TCATTTTCCACCTTCTTTCG	540
COS731	No	GPI:C:753773	168	2	2AL|2BL|2DL	GGACAATGAGAAAGCAAAGG	CTTTGCAAGAGCATCAGAGG	309
COS732	Yes	GPI:C:753251	168,3	2	2AL|2DL	GGAAATCTACGGTGACCTCG	AGCATAAAAGATGCAGCAGG	633
COS733	No	GPI:C:765591	170,5	2	2DL	TGGTGAGGTATACTCCGTCG	CTGTTTCATACAAGCCTGGG	321
COS734	No	GPI:C:756792	172,5	2	2AL|2BL|2DL	ATAAACCAGCTCCCCGCAC	GTGGTGAGGAGAGAGGACG	274
COS735	Yes	GPI:C:747571	173,2	2	2AL|2BL|2DL	AACCTTCTGTTTTGGAGGTTC	TGGTAAAAAGCCCAGCTTC	463
COS736	Yes	GPI:C:743473	173,5	2	2DL	ACGTCTACTTCGGACAGGC	GCACCTCAGGAAATGATCC	254
COS737	No	GPI:C:779794	176	2	2AL|2BL	GTGAGGATTCCTGATTGTGG	ACGGTTAACACGAAGAATCG	519
COS738	No	BE495372	176,9	2	2AL|2BL|2DL	TTGTAGAAACTACGGACGGC	GGTACCAGCTCTGGAACAGC	571
COS739	No	GPI:C:795564	178,5	2	2AL	GAGTGCTCCACTGTAAAGCC	CACCTTTGTAGACAGTCCCG	546
COS740	No	GPI:C:730585	178,9	2	2AL|2DL	AGGGAAAAGGTTAAGGATGC	TGAAACTCATCTCTTAGGCCC	279
COS741	No	GPI:C:730704	179,4	2	2DL	CTGCAGCTCATCGAGTCC	ACTTGATGTCGAACACGTCC	539
COS742	No	GPI:C:760321	179,6	2	2AL|2DL	CTACGAGGACCAGCTCTACG	TCCATACATCAGCTTCTGGC	893
COS743	No	GPI:C:745441	181,5	2	2AL|2BL|2DL	TTGTACTGGTATCAAGCGGC	CGAGAACAGGGAAACATAGG	894
COS744	No	GPI:C:756174	183,2	2	2AL|2BL|2DL	GTTCAGCTACTAACTGCGGC	AGAGCATATGAAGTGGACGC	862
COS745	No	GPI:C:766721	184	2	2BL|2DL	AAGATACACTGCTCCGATGG	CACCTGATCTCTTTGCTTGG	863
COS746	Yes	GPI:C:735499	186,9	2	2AL|2BL|2DL	ACCCTTTCATTTACACACCG	ACAAGTACCTACCGCTCTCG	442
COS747	No	GPI:C:741642	187,3	2	2BL|2DL	CACACATGGCAAGTTACAGG	ATCAGACTTGCTTGCTCACC	426
COS748	Yes	GPI:C:746642	189,5	2	2AL|2BL|2DL	GTGCTGCTGCCATTACTTTAG	AGCAGCAGCCAATTGAAG	808

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS749	Yes	GPI:C:747195	190,2	2	2AL|2BL|2DL	GAAGGAGGAGGAGACATGGT	CTTCTGCTCGTGGCTGCG	759
COS750	No	GPI:C:731690	191,7	2	2BL|2DL	AGGAGCCCAGGAAGATGG	CAAAAGAGTGCCAGAGAAGC	789
COS751	Yes	GPI:C:754545	192,6	2	2BL|2DL	GTTGGTGGTTGAAAAGATGG	AGTATGCACCTTCGATTTGC	601
COS752	Yes	GPI:C:756123	192,9	2	2AL|2BL|2DL	CACGGTGGAAGTCACTAACC	CAGTTTCCAAGGCATAGGG	498
COS753	No	GPI:C:758373	194,1	2	2AL|2BL|2DL	CTGTCGGACTACCACATTCC	AGTTGGCATACAACCTGTGC	336
COS754	Yes	GPI:C:760130	194,1	2	2AL|2DL	GATTGCTTTGTCGTTTGTCC	TCATTGACTCAAGCATCTGG	860
COS755	Yes	GPI:C:758992	194,9	2	2AL|2BL|2DL	AAGGGGTTCATGGATAAAGG	ACAGACAGAGCTTGTGAGCG	807
COS756	No	GPI:C:771366	195,7	2	2DL	CGAACAAACTTATTCCACCG	GAGAAAATCGTGATGGATGG	563
COS757	No	GPI:C:772258	196,2	2	2DL	AAACCTACGAGGCGCTGC	GGAGTCCCAGGATCCTTCC	280
COS758	Yes	GPI:C:770859	196,8	2	2AL|2BL|2DL	TGACGAAGAAGATCGAAAGG	AAGAATGTTCAGCAACAGCC	741
COS759	No	GPI:C:785000	198,2	2	2AL	TGTGGTGGGGAAGACTAACC	ATGAAGCATAAGCCATGAGG	590
COS760	No	GPI:C:736528	199	2	2DL	CCCCTTAACGGTTGTATTGG	GGTGTGGCTATCAGTATACGC	250
COS761	No	GPI:C:803367	199,4	2	2AL|2BL	TTTTGGTCCTGGTGTTGC	GCCACAGAACAGAGTTGAGG	267
COS762	No	GPI:C:736528	199,5	2	2DL	TCCTCGATAGCTATTGTCCG	TGAGTAGCCCAGTAGCATCC	495
COS001	Yes	BE404977	79,7	4	4AL|4BS|4DS	TCAAGTGGTGAGAGGAGGG	CCCAGCATTCTCTTGTTCC	262
COS002	No	BE496216	85,2	4	4AS|4BL|4DL	CCGCTAGGCTATATGTTTGC	AGCTTGTCCACCATGTCG	339
COS003	Yes	GPI:C:718131	156,6	4	4DL	GCTTTGTTAATGTTGCCAGC	CGTGAACTTTGAGTTCTGGG	738
COS004	No	GPI:C:721932	101,8	4	4DL	GTTTCTTTGTTTCACGAGCG	AATAGATTCAAAGAGCCGCC	714
COS005	No	GPI:C:721946	94,4	4	4AS|4BL|4DL	GCGACCGCTACGTCTTCC	GGCCACTCCGTATATCTTGG	316
COS006	No	GPI:C:724813	160,3	4	4BL|4DL	GCACGTTTAAGTGATGTCCC	CACAAGACAGTTCACATGGC	450
COS007	No	GPI:C:725135	120,8	4	4AS|4BL|4DL	AGTCGGAGCTCCTCACCC	AAGGCCACATTTCACATACG	334
COS008	No	GPI:C:725506	157,2	4	4DL	AGAGCAAACAGGACAAGAGC	ACAACTATTGCACCCGACC	315
COS009	No	GPI:C:725531	123,5	4	4AS|4BL|4DL	CGCATACAGTGAAATAAAAGGTC	GGATGCTCAGCTTCTCGTC	290
COS010	No	GPI:C:726171	165,7	4	4BL|4DL	GTCTGCATTGTCCGGCTG	GAATGCTGTGTCCACCAAG	268
COS011	No	GPI:C:727651	134	4	4DL	CCGCCTTTAATGAGGAGC	CTTCGATCAGCTTCTTCAGC	287
COS012	No	GPI:C:728248	121,9	4	4AS|4BL|4DL	ATCCAAAACATGACTGGAGC	AGAATCTAGTGCAGCATCGG	374
COS013	Yes	GPI:C:731424	109,9	4	4AS|4BL|4DL	GATCCTCTTTGAAGGAAATCG	GCTGCTGTCAAAGGTAGAGC	257
COS014	No	GPI:C:733003	56,9	4	4AL|4BS|4DS	GGCTTTCTTAAACTCGAGGG	ATATGGAGACTTCACCTGCG	629
COS015	Yes	GPI:C:733709	85,3	4	4AS|4BL |4DL	CCATACAGGGAGGTCATGC	GTCGGTGAAGAATATCGAGC	569
COS016	No	GPI:C:734360	123,1	4	4AS|4BL|4DL	CGAGGTCTTCTTCTTCCTCC	CCGTTCCAGAAGACGTAGG	742
COS017	Yes	GPI:C:737898	86,9	4	4AS|4BL |4DL	CAGAAGTGAAGCAGTCAATGG	CTGGATCATCATCTTCCACC	439
COS018	Yes	GPI:C:738073	25,8	4	4BS|4DS	AAGAGAGCACACTGGAGAGC	ATAGCGCCTTTCTTGATTCG	275
COS019	No	GPI:C:738583	86,8	4	4AL|4BS	ATTAGGTTCCGAGAAGTCCG	AAGCTGCACCATTTATCAGC	528
COS020	Yes	GPI:C:738833	94,6	4	4AS|4DL	AGATCCTTCTCTAGGCTCGC	TGAGGTAGAGCACGGTGG	550
COS021	Yes	GPI:C:739175	84,7	4	4AL|4DS	TGAAGATGAAATAATCCGGC	CTCCTTCAGTACCCTTTTTGC	251
COS022	No	GPI:C:739747	166,7	4	4BL|4DL	ATCCACGGCAAAGACTACC	GAAACTCATTATCCGGAACG	333
COS023	Yes	GPI:C:740051	105,9	4	4AL	GCCAACTCTCATTCTTTTGG	GGCACTTTAGACACCTGTGC	871
COS024	No	GPI:C:740215	137,5	4	4DL	TGGCTATGACACTCATGTGG	GACCAGGTCACCTTTCTGC	588
COS025	Yes	GPI:C:740946	63,2	4	4BS|4DS	AATCGTAAACCCTAGCCCC	TGATCTCCTTCTTGAGGTCG	385
COS026	No	GPI:C:742089	101,4	4	4AS|4BL|4DL	TAAACCCTCAACAAAAACGC	CTTCCCTTCTAGGTCAACCC	832
COS027	No	GPI:C:743217	106,9	4	4BS|4DL	ACCTTATCCAAGAGGCGG	AAGTGGAGATCGACTAGCCC	657
COS028	No	GPI:C:743745	83,2	4	4AL|4DS	AATGTCATTACATCCGCCAC	TGGAGCTTTGATTCTAAAGGAAG	365

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS029	No	GPI:C:744613	107,4	4	4AS|4BL|4DL	CAAAGAGTCAGACAGCTCGG	AATTTCCAGCTTAAGCACCC	548
COS030	No	GPI:C:744968	101,3	4	4AL|4BL|4DS	TAATTTTGAACGTTACCGGG	TTTGCTGCCAACTCTTGC	301
COS031	No	GPI:C:746429	78,2	4	4AL|4BS|4DS	AGATCGATCGAATCAACAGC	GTAATGACTTGAAGTGCTCGC	861
COS032	No	GPI:C:746929	86,1	4	4AL|4BS	CTGGTATAAACGAGGATCGC	CAGAAGGTATTAGGCCTCCC	499
COS033	No	GPI:C:747145	99	4	4AS|4BL|4DL	CACGAGGGGGTGGAGGAC	ATCTCTCGCAGATGCTTGAT	279
COS034	No	GPI:C:747657	158	4	4BL|4DL	TTCTGGAGGAGTCTCATTCG	CAGAACCCACCATCAGTAGC	321
COS035	Yes	GPI:C:748004	140,5	4	4DL	AGGGGTACATCTCTGCGG	ACGTCGCAAGCAACAAGC	261
COS036	Yes	GPI:C:748122	160	4	4DL	AAAATTGGAGCCCTACTTGG	AAAGCAGCATAGAGTGACGC	260
COS037	No	GPI:C:748813	141,5	4	4DL	TCAGCACAGTCCTAGAAGGC	TCAAGGTACATCAACTGGGC	578
COS038	No	GPI:C:748932	77,8	4	4AL|4DS	TCCTGTTCCTTCTCATCTGC	TGAAGTCGTACACAAGCAGC	499
COS039	Yes	GPI:C:749454	120,4	4	4AS|4BL|4DL	CTGAAGAGGGGTACACATGC	ATCGCAAGTCCAATATCAGC	469
COS040	No	GPI:C:749717	110,3	4	4AS|4BL	GGTGACAACCTCAGACATGG	GAATCTGGAAGTAGAGCTCGG	252
COS041	Yes	GPI:C:749921	90,5	4	4AS|4BL|4DL	ACACCTTCTGCAAGGAGTGC	GCTTCTCCTCCTCCTCTTCC	878
COS042	No	GPI:C:749992	60,6	4	4AL|4BS|4DS	TCCCCGATTTTTATTAAGGG	ATTTGGAAGCTGAGCATAGG	663
COS043	Yes	GPI:C:750766	114	4	4AS|4BL |4DL	CCTCTCCTCTCCTCTCTTGC	CAGGGGAGAAGAAGTCAACG	744
COS044	No	GPI:C:751351	115,3	4	4AS|4DL	TACACCATGTTTGTTGCTGG	TCTGTCGGTCTATTGAAATGC	820
COS045	No	GPI:C:751739	116,1	4	4AS|4BL|4DL	TGGAGATCAGGAATCTCACC	GTCTCCGGAGGAGGTAAGC	352
COS046	No	GPI:C:751871	107,5	4	4BL|4DS	CATCGCTACAACTTCAACGC	GCCACCGCATCACAGTCC	451
COS047	No	GPI:C:751871	17,9	4	4BL|4DS	CATCGCTACAACTTCAACGC	ATCACAGTCCGACGAAACG	822
COS048	No	GPI:C:751999	110,8	4	4AS|4BL|4DL	TCCTCCCTTCTCATAAACCC	TTGACAGTGGATGACTTCCC	460
COS049	No	GPI:C:752143	113,1	4	4AS|4DL	CAAGCCTCACTTTGTTCTCG	TCTCCTTATCCATGCAAACC	649
COS050	No	GPI:C:752225	57,7	4	4AL|4BS|4DS	CTGCTCACCTCGTCCTCG	ACGGTGATGAGCCAGTAGG	326
COS051	Yes	GPI:C:752511	177,9	4	4BL|4DL	CTGGTACCAATACGAGTGGC	GTAAATTTGCGCTTCTTTGG	810
COS052	No	GPI:C:752858	64,2	4	4AL|4BS|4DS	CAGAGTCCATCAAAACCGC	AGGATGAGGAGGTCCTTGG	441
COS053	No	GPI:C:752990	133,5	4	4AL|4BL|4DL	GACTCTCCGATGGCTTCC	GTGGTGGGTTTGTAGTCGG	283
COS054	No	GPI:C:753244	145	4	4DL	CACTGCCTCTTACTTGTCCC	CATCGAAGAGGGGAGGGG	279
COS055	No	GPI:C:753407	125,2	4	4AS|4BL|4DL	CACCCCATAACCCCTAACC	TGGATCTCCTCGTACGGC	260
COS056	No	GPI:C:753433	169,8	4	4DL	GAAGCAAAAGCAACACGC	GGATGTGAAGATGAACCACC	646
COS057	No	GPI:C:753511	178,6	4	4BL|4DL	TCTTGAAGCTGGTGCCGA	AGGTAGGCTCCATAGCTTCC	331
COS058	No	GPI:C:754556	128,2	4	4BL|4DL	TACCGAAGATAAGGTTTGCG	AAAAGGTTGGGTTAAGCTGG	726
COS059	No	GPI:C:754584	145,5	4	4DL	GACCTTCCGTTTAACGTTCC	GTAATTTCATTGCAGGTCCG	265
COS060	No	GPI:C:754586	116,4	4	4DL	CTCCATGTTCAACCTCATCC	AAGGAAAAGATATGCAGGGG	801
COS061	No	GPI:C:754728	123,7	4	4AS|4BL|4DL	TCCATCACCCTCCTGTCC	TCCAAGGAAATCTCATCGC	702
COS062	No	GPI:C:755521	113,3	4	4AS|4BL|4DL	ATACATCTGCTGAAGGTCGG	ACTGGAGGAGGTTGAGAAGC	816
COS063	No	GPI:C:756221	146,3	4	4BL|4DL	CGGTCACCCTCCTACACC	CCATGTCAAAGAACCTGTCC	395
COS064	Yes	GPI:C:756425	134,9	4	4AL|4DL	CAACAACTTGAGAGCAGTAAGC	ATGAGTTTGATCCCCAGAGC	251
COS065	Yes	GPI:C:756869	108,5	4	4AS|4BL |4DL	TCTTCGGACTTGTATTTGCC	GTAACCGTGACAATATCCGC	498
COS066	No	GPI:C:756989	85,1	4	4AL|4BS|4DS	TGTCTCTCTCTCCTTCGTGG	GATCTTCTCTAGGATCGCGG	418
COS067	Yes	GPI:C:757265	138,2	4	4AS|4BL|4DL	CTTTTCCCCTCATCTTCTCC	CGACCTCCTTCTGAGAGTCC	460
COS068	Yes	GPI:C:757404	109,5	4	4AS|4BL|4DL	CGATGGATGAGGAGTACGAC	GGATGTAGATTCTCCACCGTAG	592
COS069	No	GPI:C:757631	151	4	4BL	ACAGACAATCCTGATCTGCG	TTAACACGGCTAACAAAGGC	305
COS070	Yes	GPI:C:757750	152,8	4	4BL	ACAGCAAGGTCTCTCTCTCG	ATTTCACTGAATGCCACCC	765

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS071	No	GPI:C:757859	88,2	4	4AL|4DS	ATTACAACCTCTCTCGCCG	GGAACTGGTAGAGGTGGAGG	476
COS072	No	GPI:C:758192	151,8	4	4BL|4DL	AGGATGAACAAGGAGAAGGG	CCCTAAGAACTTGAGGAGGC	796
COS073	No	GPI:C:758472	27,9	4	4BS|4DS	ACCAAGGAGTACGGCTACG	TGTTCTCCGACTCGATGG	267
COS074	No	GPI:C:758556	126,1	4	4AS|4BL|4DL	CTCTCCACCCTTTTTGTTCC	ACGGAGAGCTTCTGGTCG	293
COS075	No	GPI:C:758628	110,5	4	4AL|4BS|4DL	CAACTGTAGCAACATAGGCG	AGCTTGGCGTTAGGAATGG	726
COS076	No	GPI:C:759324	150	4	4DL	TTGTGTTACGGTCCTTCTCC	ACAATGACTTGTTCTTGGGG	737
COS077	No	GPI:C:759345	133,5	4	4AS|4BL|4DL	CTCTCACTCCCCACTCTCCT	CTTTCCTCCATCAATATCTCCT	894
COS078	No	GPI:C:759427	113,4	4	4AS|4BL|4DL	AGTCAATCATCAGCTCAGCC	TTTGCGATCTTCTTGATTGC	628
COS079	Yes	GPI:C:759492	134,4	4	4AS|4BL|4DL	GAGGAGAGCAGAGCAGTACG	TTTGCAGAGAGAATGGAAGG	814
COS080	Yes	GPI:C:760004	60,8	4	4AL|4BS|4DS	AAGAAGTACGTGGACATCGG	GGTAATTGAACTTGATGGCG	781
COS081	No	GPI:C:760103	139,3	4	4BL|4DS	GGCCAGCTGCAAAGAATC	AAGCACCAAATGAACTGCTC	258
COS082	No	GPI:C:760373	85,9	4	4DS	ACATCGGCGTCAACTACG	GGGAGAAGAGGTAGGTCTCG	900
COS083	No	GPI:C:760930	26,4	4	4DS	GTTACTGCTCCGACTTGTGG	GATCTCTCCTCCGTCGCC	417
COS084	No	GPI:C:761282	89,4	4	4DS	CTTCTCGATGGTCATCTTGG	GTACAACAAGGAGGAGAACCC	507
COS085	No	GPI:C:761443	174	4	4BL|4DL	CTGAATTTCTGATCCGATGG	ACCGGTCAATCTTTAGCACC	893
COS086	No	GPI:C:761804	174,3	4	4BL|4DL	AAGCCAGAGCCATAAGACG	GCAGACGGAGTCCTTGCC	431
COS087	Yes	GPI:C:763059	155	4	4DL	ATGTTCACAAGAAACCAGGC	GAGAATGGCCTCTATGATGG	877
COS088	No	GPI:C:763332	92	4	4AS|4DL	TTTCTGACAGGACCGATACC	TGATTGAAGTGCATCTTTGC	299
COS089	No	GPI:C:765452	115,6	4	4AS|4BL|4DL	CCTCTTTCAGAAGGGAAAGC	GTACACCGCGTAGATGATCC	354
COS090	No	GPI:C:765787	122,8	4	4AS|4BL|4DL	CTAAGCTTGAAGACAACCGC	TGACGTTATCAGCAGAACCC	671
COS091	No	GPI:C:767341	76,3	4	4BS|4DS	CGACATGCTACAGAGCATCC	TCCTTTACATTCTCCAACGC	276
COS092	No	GPI:C:769451	79,5	4	4AL|4DS	TCTTCGACCTCGACTACACC	CTTGTCAATGAAGACCCTGG	336
COS093	No	GPI:C:769451	78,4	4	4AL|4DS	GAAGGAGAGGAGGAGGGG	CTTGTCAATGAAGACCCTGG	404
COS094	Yes	GPI:C:769630	108,8	4	4AS|4BL|4DL	CTTTCAATCCCATCCTTGG	TCAACTTCGATGTGATGTCG	258
COS095	No	GPI:C:769633	77,1	4	4AL|4BS|4DS	AGAGCGACGTCCTCAACC	GACCTTGAACACCGACTCC	852
COS096	No	GPI:C:769650	84,8	4	4AL|4DS	CCTCAAGTCTCCAACCACC	TTCTGAAGAATCTTGGACGG	862
COS097	No	GPI:C:769725	93,6	4	4AS|4DL	ACATTAGAAACGTGAAGCCG	GTCTGATGATATGGGCATCC	471
COS098	No	GPI:C:770014	123,1	4	4AS|4BS|4DL	ACGTGGCGATCTACTACGAG	GATGGCGCAAGCCTGAAC	475
COS099	Yes	GPI:C:770094	138	4	4AS|4BL|4DL	AAAGAAACGATGTAGCAGGC	CTCCCCATATAGAGGATGCC	537
COS100	No	GPI:C:771467	89,7	4	4AL|4BS|4DS	CTTATGCATTTGCTCCTGC	ATATCACGCTCTTGTGGAGC	274
COS101	No	GPI:C:771529	113,6	4	4AS|4BL |4DL	CGGACAATCTGGAGACTAAGC	AGGTCAGATAGTGTGCGAGG	362
COS102	Yes	GPI:C:771614	97,3	4	4AL|4BS|4DS	AAGATTCTTTCCCCCTTCC	AGAGGAAGAAGACGCTCTCG	327
COS103	No	GPI:C:777247	95	4	4AS	ATGATTGCTTCAGTCGATCC	TCTCTCTCTTAAGCCAATCGC	586
COS104	Yes	GPI:C:785017	113,2	4	4AS|4BL|4DL	CAACTTAACCAGCATGGACC	CAGAAGTAGAGGATGGAGCG	361
COS105	Yes	GPI:C:797119	169,3	4	4AS|4BL|4DL	GCTTGCCATCAATTTGTACC	CTTGAGGAGGCCATAGAAGG	474
COS107	No	GPI:C:726959	23,2	5	5AS|5BS|5DS	AGGTCAAAAATTTTCCCATTG	ATCAAGGATAGGACCAAGGC	250
COS108	Yes	GPI:C:728036	22	5	5AS|5BS|5DS	CATTGCCAGAAGTCGTTACC	ACAGCTCCATTTACCTTCCC	624
COS109	No	GPI:C:728956	49,7	5	5AS|5BS|5DS	ATTAAAGATCCTGTTCGCCC	AGTGTATGCTTATCCAGCGG	363
COS110	No	GPI:C:729592	41,1	5	5AS|5BS|5DS	GAGGACCACCCTTAAGAAGC	CTTTGTTCCCATCAGTTTCG	276
COS111	No	GPI:C:730781	23	5	5AS|5BS	GTTGCACCTCCTGTTAATGG	GTGGTGTCTGGAGAGTCAGG	342
COS112	No	GPI:C:739811	22,1	5	5AS|5BS|5DS	TTATGATGTTTTGGGCATCC	TTCCCCATGTTTTTATCAGG	253
COS113	Yes	GPI:C:739859	33,4	5	5AS|5BS	CAAGAAGTCGAAGAAGCACC	CTTCTTGGCCTGTATCATCG	528

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS114	No	GPI:C:741009	49,7	5	5AS|5BS|5DS	TTGAAGCAGAACTTGAAGCC	GAGCACTATCGTGGTCATCC	865
COS115	No	GPI:C:743567	33,7	5	5AS|5BS|5DS	CATGTAACCAACTCCTTGGC	GACTATCGTTGTTCCTTGGC	774
COS116	No	GPI:C:744654	31,8	5	5AS|5BS|5DS	TGACCTGTGGAAGAATGAGG	AAACTTCTATGGTGCATGGC	322
COS117	Yes	GPI:C:746088	48,5	5	5AS|5BS	ACCACCTTCTGTTTTGATGG	ACATGCTTCAAGCTCAATGG	269
COS118	No	GPI:C:746150	41,6	5	5AS|5BS	CACAGGCTCAACAGTATCCC	CCAGTTGCATATCTTGGTCC	581
COS119	Yes	GPI:C:746156	24,6	5	5AS_|5BS	TTACAAAGGCTTCAAGTGGC	TTTACGTATCTGCTTTCGCC	338
COS120	No	GPI:C:746971	48,5	5	5AS|5BS|5DS	ATCTTGGAGGCCTACTCTGG	AATGTGTCTGCCAGATCTCC	284
COS121	No	GPI:C:748146	45,3	5	5AS|5BS	TGAGAAGCTTGAGGAGTTGG	TCTCATGCAAACTATCTGCG	466
COS122	Yes	GPI:C:751972	37,6	5	5BS	AGTTCCCCACGTTAGTCTCC	GAGCTTCAAAAGGAAGGTCC	777
COS123	No	GPI:C:756188	48,9	5	5AS	TGATTTTGAATTATCCGAGGG	CGTAAGAAATGAAGATCGCC	294
COS124	No	GPI:C:758029	30	5	5AS|5BS|5DS	CACGCAAGGGAATAAGAAGC	GCAAGAGGTCACAATGGG	840
COS125	No	GPI:C:758334	25,1	5	5AS|5BS|5DS	CCCTTGTCTTGATCTCCTCC	CTTGGAGAGGTCCTGGTAGG	362
COS126	Yes	GPI:C:762599	18,6	5	5AS|5BS|5DS	CAGACCGTTACTCATGGACC	CCTCCTTGTACTCATTGATAGCC	295
COS127	Yes	GPI:C:762599	16,9	5	5AS|5BS|5DS	TGCTTTCTACTTGGACTGGG	GCTGCTATACGGCTTACTGC	533
COS128	Yes	GPI:C:796275	28,8	5	5AS|5BS	ATGAGCTGATGAAGTTGAAGC	TGGTGATTAATTCGTAGCTGG	461
COS129	Yes	GPI:C:806204	29,7	5	5AS|5BS	TTAGGACCACGTCTTCTTGG	GCCTCAAAGCAAATGACTTC	294
COS130	No	GPI:C:717465	176,5	5	5BL|5DL	TATATTGCTTGGACATTCTTTGG	GTTCACTAGCATGCCTTTCC	250
COS131	Yes	GPI:C:728443	180,4	5	5BL|5DL	CATATGGCCCGTATCGCC	GCAGTCGAGGAGGAAACG	264
COS132	No	GPI:C:739528	160,9	5	5DL	TGAGAGCAAAGATTGAAGCG	TCTTGCTCTTCCTTGTCACC	362
COS133	No	GPI:C:743337	166,1	5	5AL|5BL|5DL	CTACACGGACGTGGACTACC	ATGAGGATCCAGAGCATGG	866
COS134	Yes	GPI:C:745899	181,1	5	5AL|5BL|5DL	GCATAGTTGGACTGGAAACC	TCCATCATTCTGTTCAACCC	644
COS135	Yes	GPI:C:748166	172,8	5	5AL|5BL|5DL	GGAGAGGGAAGGAAAGCG	ACATGTGCGCTCCGTAGG	266
COS136	No	GPI:C:748436	169,2	5	5AL|5BL	TCGACACCAACGAGAAGC	GCTGCGTTCAAGTACTCTCC	493
COS137	Yes	GPI:C:749645	182,8	5	5AL|5BL|5DL	GACTTCATCGACTGGACCG	TGAAGGGGATGTAGATGAGC	383
COS138	No	GPI:C:750154	168,5	5	5AL|5DL	GCTGGTAGGAAGTATGCAGC	TCGATGAGCTTTGATTTTCC	637
COS139	Yes	GPI:C:750235	174,5	5	5AL|5BL|5DL	GAGAAATCTTGCCTTGATGG	GAATTCCAACCCAACAACC	277
COS140	No	GPI:C:750957	174	5	5AL|5DL	TTCATTCATTCGTTCGTTCC	GGTATGCACCACGATCTGC	775
COS141	No	GPI:C:754632	181,2	5	5BL|5DL	TCAATCTGCAGAAGACGGAG	GTTCAGCAGCAGCGACATC	881
COS142	No	GPI:C:754904	165,6	5	5AL	CTTCTCAGGCTTACTCACGC	GGTGTCAACCAGCTAAAAGC	427
COS143	No	GPI:C:755811	165,8	5	5BL|5DL	CCTCTTCTCCGAACTCCG	ACTCCTCCGTCGTGATGC	317
COS144	Yes	GPI:C:756721	174,7	5	5AL|5BL|5DL	TAACAAGAACAGCAATGGGC	GGCTTGTAGATCTCTATGTATTGTTG	301
COS145	No	GPI:C:756738	177,5	5	5BL	TACAAGGACCTCTCCATTGC	AAGCATCCAGTCTCCATCC	280
COS146	No	GPI:C:757699	172	5	5BL|5DL	GGCAAACCCCAGCTGCAT	GACGTCGCCGGACTTCTT	250
COS147	Yes	GPI:C:759134	183,6	5	5BL|5DL	TCCGATCCCCTCCTATAAAC	GTGCCGATCCATGATTTG	735
COS148	No	GPI:C:760115	184,3	5	5AL|5BL|5DL	CAACGATCACGAATCAAACC	GATGATGATCTCCCTCATGG	831
COS149	No	GPI:C:761162	170,2	5	5AL	GAACTTAGTGGGAACAACGC	CCTTTTCCATAGGATCCCC	286
COS150	Yes	GPI:C:762245	178,7	5	5BL	CGACTTCAACGACTTCGG	TGGTCTTGAGGAAGAACTCG	389
COS151	No	GPI:C:762245	178,5	5	5BL	CAAGGACCTCCCCTACCC	GTAGAGCTTGAGGATGTCGG	573
COS152	Yes	GPI:C:764126	173,1	5	5AL|5BL	TCATCCAACCCTAAGTCTGC	CACTGCAATCTCTTGGAAGG	316
COS153	No	GPI:C:765220	176,9	5	5AL|5BL|5DL	TTTGAGTGACCCTGAGAAGC	GATGGACAACATCCTCTCCC	305
COS154	No	GPI:C:765220	177,3	5	5AL|5DL	TTTGAGTGACCCTGAGAAGC	GATGGACAACATCCTCTCCC	377
COS155	No	GPI:C:765518	181,3	5	5AL|5BL|5DL	AGCTCAGGCTTATGAGGTCC	ATGAACCACATCCTCACCC	368

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS156	No	GPI:C:765930	175,8	5	5BL	ACGCGTCGATTTACTCTCC	GCTAAGTGCGGAGTAGAGGG	285
COS157	No	GPI:C:765930	175,9	5	5BL	TCATCCAACCCTAAGTCTGC	CCATTAGTTTGGAAGTCCCC	361
COS158	No	GPI:C:771643	172	5	5AL|5BL|5DL	CCAAGTACTTCCTCAATGCG	AATATTACCAGAAAGAAACTTGAAGG	392
COS438	No	GPI:C:749398	0,7	6	6BS|6DS	CTGTCTCAGAGGGATGATCG	GCTTCAGCTATCATGTTGCC	639
COS439	No	GPI:C:717631	2,6	6	6AS|6BS|6DS	AAGTGGGACATGATGGAGC	CTCCTTCAAAATAACATCCAGC	895
COS440	Yes	GPI:C:741456	4	6	6AS|6BS	TGAGAAATCAACCACATCTGC	TCCTGTCTTCAGACCTTTGG	541
COS441	No	GPI:C:739714	5,2	6	6AS|6DS	TCAGCTCCTCTTCTACCTCG	GGAGTTACATTGACTCCCCC	529
COS442	No	GPI:C:738609	8,3	6	6AS	AGCCAAGCGCTCTCTCTC	GCTGAGGTAGCGGGAGAC	258
COS443	No	GPI:C:747871	8,6	6	6AS|6BS|6DS	GAACAATGAAGGTTGGTTGC	TTTCCAATCTCTTGTGTCACC	705
COS444	Yes	GPI:C:756680	12,5	6	6AS|6BS|6DS	GGTCTTGTCCCCTCCTCC	CCTTGAGCTTGAGGTAGGC	439
COS445	No	GPI:C:745208	15	6	6AS	CATTCCCAGCCACCTACC	ATGATGAGCGAGTCGATGC	313
COS446	No	GPI:C:748899	41,5	6	6AS|6BS|6DS	ACTGCTTCTGTTTGGTGAGC	ATATTCCTCCAAGGTACGGC	824
COS447	Yes	GPI:C:745606	46,1	6	6AS	AAAACAAGCTTTTCCCAAGG	GCTGCTGTAAGCCATCTAGG	802
COS448	No	GPI:C:747157	47,6	6	6AS	AGATGAACAATGCTGAGAAGC	ATAAGTTGTGAATCGCCACG	489
COS449	No	GPI:C:716746	50,8	6	6AS|6BS	TGCTCCTCCTGTCTAAATGG	GGAAATAAGCCAGAGAAGCC	286
COS450	Yes	GPI:C:750133	50,8	6	6AS	CTTGGAAAACCAAATGAAGC	TCATATGCAGCAATTTCACG	558
COS451	No	GPI:C:770912	55,7	6	6AS|6BS|6DS	CAGGGACGAACATACTGAGC	CTCGTCGATCACCTCATAGC	458
COS452	No	GPI:C:753637	57	6	6AS|6BS|6DS	TCCAATCTCTCTGCTCTTGC	GGTCCCTGCTCTCTTGTGG	505
COS453	Yes	GPI:C:769135	57,1	6	6AS	GCATTGTTTTCGATGATGC	CCTGTTTGCACATACACACG	252
COS454	No	GPI:C:753640	58,9	6	6AS|6BS |6DS	CACAAATCCTCGACACAACC	GGTCCCTGCTCTCTTGTGG	499
COS455	Yes	GPI:C:725537	61,7	6	6AS|6BL|6BS|6DS	GAACTCTTGTCTGCGAGAGG	GTCCTTCAAAAGAGGTCCG	559
COS456	No	GPI:C:750237	65,6	6	6AS|6BS|6DS	ACCCAATACTTAACCCCAGC	ATCCGGTACATGTGGTCG	440
COS457	Yes	BE496986	119	6	6AL|6BL|6DL	AGTACATTGGCATGGTCAGG	CGTTGGAGCTGTCGTAGTGG	823
COS458	No	GPI:C:749465	121,4	6	6AL|6DL	ATCTTCGATGGCAACAAGC	GGCTGTACATGTCGATGAGG	354
COS459	No	GPI:C:750539	124,3	6	6AL|6BL	GCTGGTGGGTAGACTGTACG	ACGACAAAGGACTGCTCG	428
COS460	No	GPI:C:737067	127,1	6	6AL|6BL|6DL	TACGTGTTCATCTGGTGGC	TCCACAGCTACGATACATTCC	571
COS461	No	GPI:C:742110	128,2	6	6AS|6BL|6DL	CCATCCCGAGTCAGACATAC	CTTCTCCTGCTGGGCGCT	814
COS462	No	GPI:C:754674	130	6	6BL|6DL	AAAAGACCATCAAATTCCCC	TCTTGGTGTTCTTGTCCAGG	402
COS463	Yes	GPI:C:744097	130,7	6	6AL|6BL|6DL	AGACAGCAAACGAGCAGG	ATGGAGGTGGAGACGACC	284
COS464	No	GPI:C:747730	131,8	6	6AL|6BL|6DL	TTGAAGAACGAGGAGTCTGG	TCTTCCAGTGCATCTTTTCC	894
COS465	Yes	GPI:C:726714	132,2	6	6DL	GTAGTACCACCGGAGAAGGG	TGCTCTTGTCATTGAGGGG	491
COS466	Yes	GPI:C:724406	134,1	6	6BL|6DL	GCAGCAATCAAAAGAAATGG	CTCCTCTTCCATTCTCCTCG	634
COS467	Yes	GPI:C:741435	135	6	6AL|6BL|6DL	GGCATCTTCGTCATCACC	GTGTACAAGTATGCTCCGCC	789
COS468	Yes	GPI:C:749639	135,2	6	6AL|6BL|6DL	GTGCATGAAATTGCATATCG	CATTGCTTGATTCCTCATCC	433
COS469	No	GPI:C:744967	135,4	6	6DL	CGGGATGACCACTGTTGC	CATACGCCGTTGTTTCTAGG	365
COS470	Yes	GPI:C:751613	137	6	6AL|6BL|6DL	GTCGATTTCCTCTTCGTGG	ACTTCTCTGCATTTACTTTCGC	608
COS471	Yes	GPI:C:750412	137,5	6	6BL|6DL	CTTCCACCTCCAGACTTCG	AATAGCAGCCTTCACAGTGG	783
COS472	No	GPI:C:750638	138	6	6DL	ATAACAAGCAGCTGGAATCG	AAATAAGCAGACTGCCAACG	867
COS473	No	GPI:C:721456	138,7	6	6BL|6DL	GTCCTCCTCACCCTCTTCC	GAAGACGTCCTTCCAGGG	494
COS474	No	GPI:C:752614	139,1	6	6DL	CGTGTATCCCTGACAGAACC	CAACTGGCCACTTCTACGC	307
COS475	No	GPI:C:759557	139,2	6	6BL	CTTCCTCCCCTGCTAGATTC	TGATATTACCTCCAATATCACTCTTC	827
COS476	Yes	GPI:C:755292	141,7	6	6DL	CGCTCGTCTACTACTCGTCC	TTGCTTTAGGAGACTGACGG	417

COS Lab code	Polimorphic *A. cristatum* / wheat	Wheat accession	Wheat position	Wheat chromosome	Chromosome arm	Primer Forward	Primer Reverse	Amplicon size
COS477	No	GPI:C:759035	141,8	6	6DL	AGGGTGGTAAAAAGTCCAGC	CTCAGCAAGCTCAAGAATCC	888
COS478	No	GPI:C:760549	142,4	6	6AL|6BL|6DL	CAGAATAACATCAGACCCGC	AAACTTCCTTGACCTCCTGC	433
COS479	No	GPI:C:744182	144,5	6	6AL|6BL|6DL	GCTGAGTCATCACCTATCGC	ACTGCTATGCCAACTGAACC	745
COS480	Yes	GPI:C:717360	144,9	6	6BL	ACCAGATGGTGCTAATGCC	GGTGAGACAGGTGTTGAAGG	426
COS481	Yes	GPI:C:770793	147	6	6AL|6DL	GCCGAACCCATTGAAATA	TCTCCTTTAAGTCGAAAATACG	900
COS482	No	BE495607	148,2	6	6AL|6BL|6DL	GGACTCCATATCAGACACCG	CATGTTGATCCCTATCCTCG	437
COS483	No	GPI:C:752861	151,6	6	6BL|6DL	GCATGAAGCAGAGAGACAGG	TCACCTCATGGCAGATAACC	343
COS484	Yes	BM136727	162,8	6	6AL	TCACCTGAAGAAGTTACGGC	TGTGTCCAGACAAAGAATGC	267
COS485	No	GPI:C:753405	163,7	6	6AL|6BL|6DL	CTTGTAGAATTTCCCAAGCG	GAAGGTGCTTATGGTCATGC	392
COS486	No	GPI:C:757249	164,6	6	6AL|6BL|6DL	CAACGTCATCGTCGAGTACC	TTGAGCTTATCCAGCAAACC	869
COS487	No	GPI:C:756265	166,6	6	6AL|6BS|6DL	TTCGAGTGACGAAGAAGAGC	TGATATGTAGGACAAGAAACTTGC	650
COS488	No	GPI:C:718605	168,8	6	6AL|6BL	ACAACTATGGCAGTGGAAGG	GCTTTGCTTTGATAAGCTCG	467
COS489	No	GPI:C:733286	170,5	6	6BL|6DL	GAAATGAACGTTGGTCTTGG	TGCTAATTTCAGATCCTGCC	437
COS490	No	GPI:C:751109	170,9	6	6AL|6DL	TCCATATCCTTCCCAAATCC	GTAGTTGGCCTTGAGCACC	275
COS491	No	GPI:C:759098	171,1	6	6AL|6BL|6DL	ATGCTGCTAAACAAAGTGCC	ATTAAAACAAGGGCAGGAGG	470
COS493	No	GPI:C:756945	173,6	6	6AL|6BL|6DL	AACCCTAGCCAGCCAACC	CTCCTTCCTGTACTTCTGCG	610
COS494	No	GPI:C:739776	173,8	6	6AL|6BL|6DL	CAAGAAGCAGAGCAGAACG	GTGGTGGAGCTTGATCCC	423
COS495	No	GPI:C:766328	175,3	6	6AL|6BL|6DL	TCGGCGAGTACTACCAGTTC	CCAAATGCCCTCTGCTATC	423
COS496	No	GPI:C:745905	175,7	6	6AL|6BL|6DL	AACTACGCCAGTGGAAAGC	TTGCTTTGATAAGCTCAGCC	454
COS497	No	GPI:C:736210	176,2	6	6AL|6BL|6DL	CCTCTTGAATGATGACCACC	AAAAGGAAGATTTTGGGTGC	888
COS498	No	GPI:C:770671	178,1	6	6AL|6BL|6DL	AAGCTGAAGCACAAGGAGC	GAATTTCGTCCAAATCCTCC	526
COS499	No	GPI:C:754028	179,9	6	6AL|6BL|6DL	GAACCTCTACTACATGGGCG	GGACTGGAGGATGAAGAAGC	464
COS500	Yes	GPI:C:746902	180,1	6	6AL|6BL|6DL	GAACATGATCGCGGTCCC	GTAGTAGGCGTAGGCGGG	500
COS501	Yes	GPI:C:755465	181	6	6AL|6BL|6DL	CACTCTCCCACTCTTCTCCC	CAGTGGTTGCTCTGGATACC	856
COS502	No	GPI:C:750815	181,1	6	6AL|6BL|6DL	TGTTGAGCTAGGTCTTAACCG	CTTTGCTGTAGTTCAAGCACC	435
COS503	Yes	GPI:C:771209	181,2	6	6AL|6BL|6DL	GGAATCATTGTCTACCGTGG	TTGAAGAGAGACTTGGCACC	342
COS504	No	GPI:C:771134	182,4	6	6AL|6BL|6DL	AACTGTTGCCTTTGTTCTGC	GGAAACAAACTGCTTGAACC	467
COS505	No	GPI:C:768875	183,8	6	6AL|6BL	CTCTGCTCGCTCTGCCTC	GCTCGTTGAGGAACACCAC	262
COS506	No	GPI:C:753982	184	6	6AL|6BL|6DL	GATCCACCGACACCCCAC	GGTTGACCTGGTACATCTCG	517
COS507	Yes	GPI:C:756643	186,4	6	6AL|6BL|6DL	TCCAACTCCAGATGACCG	AAGTTTTTAAACAAAATAGGCCC	268
COS508	Yes	GPI:C:754910	187	6	6DL	ATCCTCATCCACGTCCTACC	AGGATCTCCAGCACCTCC	296
COS509	No	GPI:C:766555	189,4	6	6BL|6DL	CGTCTTCTACAGCGACCAG	GAGGTCCTCGATGGCGAC	280
COS510	No	GPI:C:760225	189,9	6	6AL|6BL|6DL	GCATCTTCGACAAGTTCACC	TCCTTCTTCTTCTGCTGGG	337
COS511	No	GPI:C:745934	190,2	6	6AL|6DL	TGCAGTTCGGCTACGACG	CCAACCATATGTTTTCCTGC	864
COS512	No	GPI:C:817780	190,7	6	6AL|6BL|6DL	TCACTGATTTCAGAGTGTGAGG	CTCGGGTGATGAAACAGC	270
COS513	No	GPI:C:803496	191,9	6	6BL	CTTTTTGAAGGATTGGATGG	GAAACATTCTTGATAGAATCTGAGC	256
COS514	No	GPI:C:794088	194,2	6	6AL|6BL	CATTGAGGGAGAATTTGAGC	ACAGAAGGAAGAAGGTTCGG	307
COS515	No	GPI:C:758815	197	6	6AL	AAATTCTCCCACCGAATCC	CGAAGAACATGAGGAAGACG	617
COS516	No	GPI:C:762451	198,4	6	6BL	CACTTTCAGCTCGTGTCAGG	GATTGTCCTGGTTTACTATCGG	324