Scientific Paper

Application of molecular tools for pasture breeding

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Abstract

Objective: To ratify the importance of the application of molecular tools in the pasture breeding programs.

Materials and Methods: Fifty-nine papers were consulted and reviewed, which approach the methods based on molecular (genetic markers, CRISPR technology and molecular cytogenetics), available in databases (Google Scholar, Dialnet, Redalyc, SciELO, REDIB, DOAJ and Latindex), in order to obtain information about their application in pasture breeding.

Results: Information was compiled about the main molecular markers (microsatellites, randomly amplified polymorphic DNA, single-nucleotide polymorphism, amplified fragment length polymorphism), utilized to analyze the genetic diversity in pastures. Which ones are more effective and versatile was researched. It was noted that the phenomenon of apomixis can be utilized to maintain the hybrid vigor in pasture lines and that molecular marks contributed to identify apomictic plants at early ages. It was validated that the CRISPR (clustered regularly interspaced short palindromic repeats) technology can be applied in pasture species to improve agronomic attributes.

Conclusions: Molecular markers, especially SNPs, are ideal for genetic diversity studies in pastures and for the identification of apomictic plants at early ages. The CRISPR technology of genomic edition constitutes a versatile tool applicable in pastures.

Keywords: molecular markers, apomixes, CRISPR, genetic diversity, genomic edition

Introduction

Pastures show modifiable traits hereditable in time, besides a high variation in growth habits and reproduction systems. They are important as forages species, besides being fast colonizers of degraded environments, and having high ornamental potential as turfgrasses (Capstaff and Miller, 2018). In these species, belonging to the Poaceae family, a wide range of reproductive behaviors (self-pollination, cross pollination, apomixis and vegetative propagation) is observed (Acuña *et al*., 2019). The complexity and variation in reproductive systems is usually accompanied by high plasticity degree, which indicates significant levels of genotype-environment interaction for the agronomic traits (Peltier *et al*., 2018).

Traditionally, pasture breeding is based on conventional techniques, dependent on the variability that occurs naturally in adapted ecotypes, naturalized populations and old cultivars. Conventional breeding, based on morpho-physiological traits, is laborious and requires experiments repeated in multiple environments (Collard and Mackill, 2008). That is why molecular tools can substantially accelerate and improve breeding programs.

Since the discovery of DNA as bearer of the genetic information of the cell, scientists have been constantly dedicated to develop tools that allow to manipulate and modify the genome. The development of diverse molecular techniques allowed an advance in the breeding of crops of agricultural interest and the introduction of new approaches, to shorten the reproductive cycles of plants. It has been proven that the novel techniques developed in the last 20 years, such as genomic selection and high throughput phenotyping (HTP) accelerate plant breeding (Ahmar *et al*., 2020).

Genetic engineering and molecular methods also play an important role to develop crops with desirable characteristics through genetic transformation, mutagenesis, protoplast fusion, *in vitro* cultivation, embryo rescue, among others (Watson *et al*., 2018). Recent studies propose techniques, such as large-scale genomic sequencing and

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high-yield molecular markers, to improve the reproduction of commercially important crops (Mujjassim *et al*., 2019).

From the above-described antecedents, the objective of this review is to ratify the importance of the application of molecular tools in pasture breeding programs.

Use of molecular methods to evaluate genetic diversity in pastures. The characterization of the genetic diversity of a population is necessary for a better use of the genetic resources in biodiversity breeding and conservation programs. For such reasons, the knowledge of the genetic diversity of the available germplasm is essential in the selection of materials for cultivation or parents for the development of cultivars (Kuwi *et al*., 2018). For the evaluation of genetic diversity different tools can be used, among which molecular markers stand out. They are developed through fragment analysis, hybridization matrices or methods based on DNA sequencing (Loera-Sánchez *et al*., 2019).

Molecular markers are DNA segments, which are associated to a part of the genome and which can be identified through a simple essay (Nadeem *et al*., 2018). They are based on differences in the DNA sequence, for which they are not subject to environmental influence (Hamouda, 2019). They are abundant in all the genome and the corresponding essays can be conducted at any moment during plant growth (Nadeem *et al*., 2018).

Methods based on fragment analysis. The DNA-based methods are classified into fragment (markers) analysis techniques, hybridization matrices (they detect DNA polymorphisms through hybridization of a DNA sample and a matrix marked with probes) and sequencing-based methods that detect DNA polymorphisms by sequencing (Loera-Sánchez *et al*., 2019).

Among the methodologies that are based on fragment analysis, which detect DNA polymorphisms through the comparison of sequence size, are amplified fragment polymorphism length (AFLP), randomly amplified polymorphic DNA (RAPD), inter-primer binding sites (iPBS), sequence-related amplified polymorphisms (SRAP), inter simple sequence repeats (ISSR), restriction fragment length polymorphisms (RFLP) and simple sequence repeats or microsatellites (SSR).

Molecular markers can be dominant or codominant, depending on the alleles they can identify. Dominant markers (AFLP, RAPD, iPBS, SRAP and ISSR) are not capable of identifying heterozygous individuals, which constitutes a limitation, but have as advantage that they can be produced at low cost, without needing information about the sequence of the target species. This turns them into the choice systems for many pasture species that have not been sequenced (Loera-Sánchez *et al*., 2019).

Codominant marker systems, such as SSR, allow the estimation of genetic diversity and are based on allelic frequencies. The development of these markers requires *a priori* sequencing information, which constitutes a limitation in the case of genomes that have not been sequenced. However, once obtained, the sets of SSR primers can be easily standardized and used by multiple laboratories (Loera-Sánchez *et al*., 2019; Romero *et al*., 2019). For such reasons, they are considered ideal markers in genetic diversity studies, due to the feasibility of their application, high reproducibility, fast analysis, low cost and higher allelic diversity (Tibihika *et al*., 2019).

Molecular markers are widely used in the identification of cultivars and species, in the establishment of evolutionary relations among different plant groups, in the evaluation of genetic variability among populations, genetic mapping and assisted selection. That is why they are considered basic and useful tools in breeding programs (Carrodeguas-Gonzalez and Zuñiga-Orozco, 2020).

In order to evaluate genetic diversity in grasses, several authors have used different molecular markers that are shown in figure 1.

In recent years, the interest in the evaluation of genetic diversity in pasture populations through the use of molecular markers has grown, because this type of study constitutes the previous requisite for success in any selection program (Carrodeguas-Gonzalez and Zúñiga-Orozco, 2020). Recently, Luo *et al*. (2020) through the use of morphological markers (SNPs, SRAP and ISSR) analyzed genetic diversity in 49 accessions of *Stenotaphrum secundatum* (Walt.) Kuntze, a popular turfgrass in gardens of tropical and subtropical regions, in order to compare the efficiency of such techniques. These authors proved that in the analyzed species the three molecular markers used are very effective for this type of study.

Hybridization matrices. DNA hybridization matrices are high yield techniques, based on the hybridization of DNA sequences with a matrix of marked probes, which are bound to a solid surface. After eliminating non-hybridized DNA, successful hybridizations are visualized through fluorescence

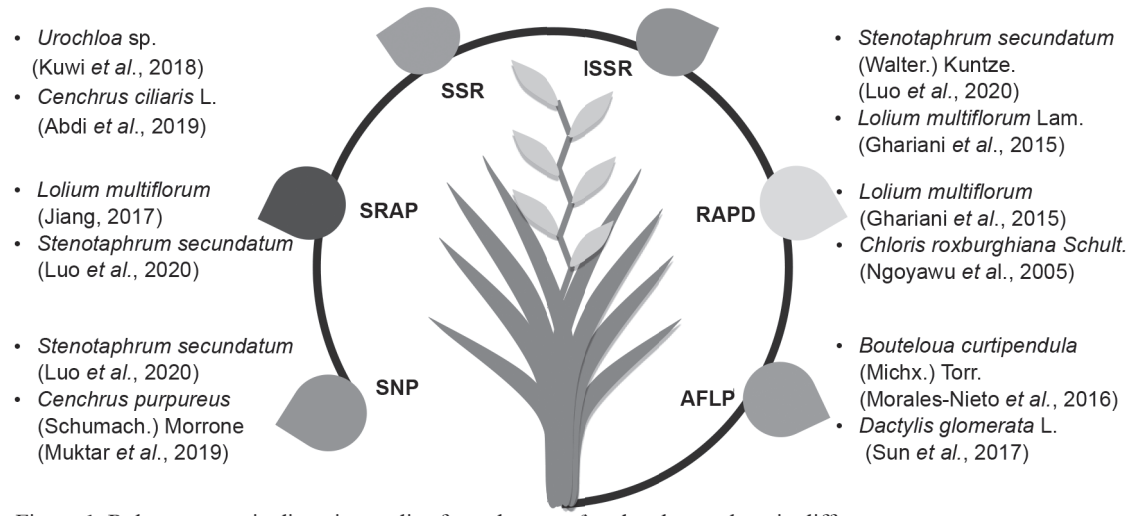

Figure 1. Relevant genetic diversity studies from the use of molecular markers in different grasses.
Source: Elaborated by the author

or chemo-luminescence (Loera-Sánchez *et al*., 2019). The methods based on hybridization matrices, such as SNP matrices and diversity arrays technology (DArT), have been used to measure the genetic variation of pasturelands (Blackmore *et al*., 2015).

The technology based on diversity arrays (DArT) utilizes genomic representations of the populations object of analysis. Such representations are generated through DNA cuts from multiple plants with restriction enzymes. Then, certain fragments are enriched with the use of selective primers and, finally, the fragments are cloned in a plasmid library. Unlike the SNP matrices, this technology of matrices does not require *a priori* sequencing information, which reduces to a large extent its development costs (Loera-Sánchez *et al*., 2019).

Single-nucleotide polymorphisms (SNP) are inherited codominant mutations, which occur at the unique basis level in codifying or non-codifying regions of the genome. SNPs are identified comparing the sequences of multiple plants from one population and can be utilized to study genetic diversity (Nybom *et al*., 2014). In the next years, with the reduction of the sequencing cost, it is expected that these markers are the most used and ideal to evaluate variability in pastures.

Sequencing-based methods. Next generation sequencing (NGS) groups a set of technologies, designed to sequence a large quantity of DNA fragments in a massive way and in parallel, in lower amount of time and at lower cost per base. Initially it was used to detect variants of single nucleotide, and it has been developed for another type of variants, such as insertions, deletions and large rearrangements (Raza and Shahi, 2020).

NGS provides the opportunity to explore genetic diversity in pastures and their wild relatives, at a much higher scale than what was possible with previous technologies. The analysis of the primary genetic stock and of the most distant wild relatives has the potential to identify genes and alleles that can be used to improve pasture yield.

Molecular markers for the identification of apomictic plants. During the sexual reproduction of angiosperms, pollen is essential for fertilization and to ensure the formation of sexual seeds. Nevertheless, in many plants known as apomictic, seed formation occurs without needing the double fertilization characteristics of angiosperms.

Apomixis is a form of asexual reproduction through seeds, which originates genetically identical plants to the mother plant, that is, it constitutes a natural cloning method. These seeds are formed from maternal tissues of the ovule, and avoid meiosis and fertilization processes. This phenomenon is closely related to the ploidy level, so that diploid genotypes show, generally, sexual reproduction; while polyploid genotypes are apomictic (Soliman *et al*., 2019). Apomixis can be gametophytic or sporophytic. In the sporophytic one (adventitious embryony), the embryo is directly developed in the ovule from a somatic cell (generally in the nucele or tegument), outside the sexual embryonal sack. This

type of apomixis has been described in the genera *Poa, Oryza* and *Paspalum* (Fiaz *et al*., 2021). In gametophytic apomixis, the embryonal sack is obtained by apospory or diplospory. The embryo is later formed by embryogenesis, independently from fertilization (parthenogenesis), and the endosperm is developed autonomously or after the fertilization of the polar nuclei (pseudogamy) (Henderson *et al*., 2017).

Apomixis is present in more than 400 plant species, which represent approximately, 40 families. The appearance of adventitious embryos in 148 genera, apospory in 110 and diplospory in 68 genera, has been reported (Hojsgaard *et al*., 2014).

Diplospory consists in the mitosis experienced by the mother cells of megaspores to form a non-reduced embryonal sack (meiosis does not occur, for which the chromosome number is not reduced. The cells of apomictic initiation are originated from the mother cells of megaspores and they finally become embryos. This type of apomixis has been observed in the family Poaceae, in the genera *Paspalum, Tripcacum, Eragrostis* and *Elymus* (Quero-Carrillo *et al*., 2010).

During apospory, the somatic cells that are found near the mother cells of the megaspore are the ones that form a non-reduced embryonal sack. These cells are subject to three rounds of mitosis and finally become embryos (Schmidt, 2020). They have been recorded in the family Poaceae, in the genera *Megathyrsus, Paspalum, Brachiaria, Bouteloua, Cenchrus* and *Pennisetum* (Quero-Carrillo *et al*., 2010). Figure 2 shows the types of apomixis.

In agriculture, apomixis constitutes an advantage to maintain higher genotypes of different crops. The perspective of cloning hybrid genotypes with characteristics of agronomic interest can represent an important help for agricultural farmers of developing countries. This would allow them to support high yields, by using part of the harvested seeds without losses in production, due to the segregation of traits and depression by endogamy.

Among other advantages, the expression of apomixis reduces to the minimum the required physical isolation to preserve homozygous genetic lines. The new interspecific and intergeneric hybrids can be easily obtained and propagated, which allows the development of genotypes better adapted to the different environments.

At the beginning, in pasture breeding programs, the generation of hybrids between apomictic and non-apomictic species was hindered by the ploidy differences. This phenomenon constituted a problem for breeders until the advance allowed the

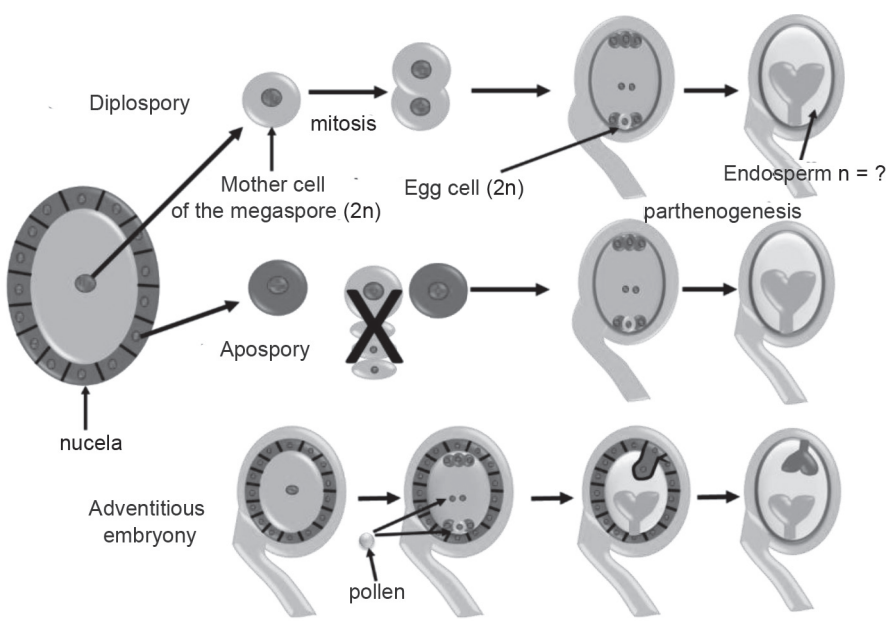

Figure 2. Types of apomixis in angiosperms. First diplospory is observed, present in Poaceae, where the mother cells of megaspores experience mitosis to form a non-reduced embryonal sack. In apospory, also present in Poaceae, the sack is formed from nucele cells. Lastly, adventitious embryony is observed.
Source: Elaborated by the authors

creation of polyploidized biotypes, of interest for their crossing with the apomictic ones.

The generation of hybrids in apomictic species includes the following procedures, as described by Quero-Carrillo *et al*. (2010):

a. The diploid sexual individuals of the species of agronomic interest are polyploidized in laboratory through inhibiting agent of the formation of the achromatic use during mitosis, which induces polyploidy sexuality.
b. Apomictic polyploids are used as pollinators and fertilization does not represent a problem, when individuals with the same level of ploidy with regards to the female plant are used.
c. The segregation of apomictic and sexual individuals maintains the Mendelian proportion 1:1 in the offspring. Sexual, genetically recombined, individuals can be integrated to the group of future parents.
d. Apomictic hybrids are evaluated for the attributes of interest, given the fact that hybrid vigor is integrated in the genome.

From this scheme, the precocious identification of the reproductive way in hybrid populations would help to identify apomictic genotypes with attributes of interest for them to become new cultivars, capable of maintaining the desired traits.

Traditionally, in pastures morphological descriptors are used, as well as the analysis of embryonal sack under the microscope to differentiate sexual from apomictic genotypes (Savidan, 2000). Since 1990, with the development of biotechnological techniques molecular markers begin to be used, with the advantage that they provide results in little time, besides the fact that any plant tissue can be used, without heeding the growth status (Poblete-Vargas *et al*., 2018). Diverse studies have been conducted in the search for molecular markers associated to apomixis in pastures. Table 1 shows the most relevant ones.

At present, single nucleotide polymorphisms (SNP) are the most used molecular markers in genetic studies, and as support to them genotyping by sequencing (GBS) emerges in recent years (Zappacosta *et al*., 2019). The first binding maps were developed by Worthington *et al*. (2016) for a polyploid apomictic pasture species (*Brachiaria decumbens* Stapf.) through the utilization of SNP markers, generated by GBS. This binding map has been used to identify markers linked to the apospory-specific genomic region (ASGR).

In a study conducted by Zappacosta *et al*. (2019) the first saturated binding map of *Eragrostis curvula* ws constructed, where traditional molecular markers (AFLP y SSR) of high yield (GBS-SNP) were used. These authors identified the locus that controls diplospory and putative regulatory regions that affect the expressiveness of such trait. The analyses of quantitative trait locus (QTL), which are related to the expressiveness of diplospory in F1 hybrids, between a sexual variety and an apomictic one, revealed the presence of two main QTL, located at 3,27 and 15 cM of the diplospory locus.

The molecular analysis of F1 hybrid populations, from homozygous individuals, for the apomixis allows

Table 1. Molecular markers used for the identification of apomictic or sexual plants in Poaceae.

Species	Type of apomixis	Molecular marker	References
Brachiaria sp.	apospory	CAPS	Poblete-Vargas *et al*. (2018)
		RAPD	Zorzatto *et al*. (2010)
Megathyrsus maximus (Jacq.) B. K. Simon & S. W. L. Jacobs	apospory	RAPD	Bluma-Marques *et al*. (2014)
Eragrostis curvula (Schrader) Nees.	diplospory	SSR, AFLP y GBS-SNP	Zappacosta *et al*. (2019)
Hieriacium	apospory	AFLP	Catanach *et al*. (2006)
Poa pratensis L.	apospory	AFLP, SAMPL and SCARs	Porceddu *et al*. (2002)
Taraxacum officinale Wigg.	diplospory	SSR, AFLP	Mayesky *et al*. (2012)
Hypericum perforatum L.	apospory	RAPD, AFLP and SSR	Barcaccia *et al*. (2006)

the construction of genetic maps and the location of molecular markers associated with apomixis. These populations are composed by genetically close individuals, but which have different reproduction ways, which allows to conduct expression studies for the identification of candidate genes that could regulate apomixis.

Much effort has been made to transfer apomixis to other crops via genetic transformation, but no good results have been reached. To apply transgenesis it is important to know the molecular pathways and the genes implied in apomixis. With this objective, several studies were conducted based on interspecific hybridizations between sexual and apomictic plants: the analysis of the process in natural apomictic species and the identification of mutants of sexual species that imitate apomixis (Garbus *et al*., 2017; Selva *et al*., 2017).

The development process of apomictic seeds is complex and comprises three components: apomeiosis (which leads to the formation of non-reduced ovules), parthenogenesis (development of embryos without fertilization) and functional development of the endosperm (Kaushal *et al*., 2019). The apomeiosis and parthenogenesis components of apomixis in other grasses are generally inherited as only one dominant locus, known as apospory specific genomic region (ASGR), identified in *Pennisetum* (Ozias-Akins and Van Dijk, 2007). ASGR contains gene-rich and poor segments, where several genes can play a certain role in apomictic development, as well as many classes of transposable elements (Fiaz *et al*., 2021).

Conne *et al*. (2015) found in *Cenchrus* and *Pennisetum* a candidate gene for parthenogenesis: ASGR-BABY BOOM (ASGR-BBML). In later studies, this gene was combined with others, and a methodology was produced for obtaining seeds in rice asexually (Khanday *et al*., 2019; Wang *et al*., 2019). *Applications of CRISPR in pasture breeding.* During 1987, researchers from Osaka University, in Japan, discovered in the genome of *Echerichia coli* five repetitions of 29 nucleotides, spaced by 32 nucleotides. Afterwards, they were identified in other bacteria, such as *Haloferax mediterranei, Streptococcus pyogenes, Anabaena* sp. PCC 7120 and *Mycobacterium tuberculosis*. The term CRISPR (clustered regularly interspaced short palindromic repeats) was designated to make reference to these repeated sequences. Genes associate to CRISPR, called *Cas*, were also identified, which codify for restriction endonucleases, known as caspases (Concepción-Hernández, 2018).

Three main CRISPR systems have been identified, which have in common the presence of the CRISPR component, a guide RNA and the Cas component, which differs for the type of system. In CRISPR I Cas3 is activated, in the second system, Cas9, and in CRISPR III, Cas6 (Liu *et al*., 2020).

The second system, CRISPR-Cas9, has two components: the first one is an enzyme (Cas9), which works as scissors that cut DNA (bacteria use it to disarm the genome of invading viruses). The other component (CRISPR) is an RNA, which leads the scissors towards a specific sequence of nucleotides, known as aim or target. Once the target is found, Cas9 opens the DNA chain, and allows the modification of the nucleotide sequence (Zúñiga-Orozco, 2018). Figure 3 shows the way of action of the CRISPR-Cas9 complex.

The system CRISPR / Cas9 of type II performs double-strand breaks (DSB), just before a protospacer adjacent motif (PAM) of three length nucleotides (NGG). DSB can be repaired via non-homologous end joining (NHEJ) or by homology-directed repair (HDR), which can generate mutants (Svitashev *et al*., 2016). Because CRISPR functions in trans, a mutation can be created in a distant locus from the insertion site of the transgene can be eliminated without affecting the mutation (Xu *et al*., 2016). These mutants are very different from traditional transgenic plants, and can require less regulatory supervision or none (Liu *et al*., 2018).

In many pasture species, genetic studies are hindered due to the high self-incompatibility and the polyploidy condition, such as for example *Megathyrsus virgatum* L. (Martínez-Reyna and Vogel, 2002). To overcome these limitations, Liu *et al*. (2018) explored the viability of utilizing CRISPR/Cas9 for directed mutagenesis in a tetraploid *M. virgatum* cultivar. For such purpose, they established a temporary essay through the use of mesophyll protoplasts, in order to validate the activity CRISPR/Cas9. These authors also proved that this methodology is efficacious to create directed mutations simultaneously.

CRISPR/Cas9 has been widely used for specific mutagenesis in a large number of species of agronomically important herbaceous plants, which include rice, sorghum, corn and wheat (Svitashev *et al*., 2016). For pastures, Capstaff and Miller (2018) refer to candidate genes related with important physiological processes for the plant. This is the case of the genes associated to the family of superoxide ferric dismutase, responsible for the accumulation of biomass, as well as of the genes

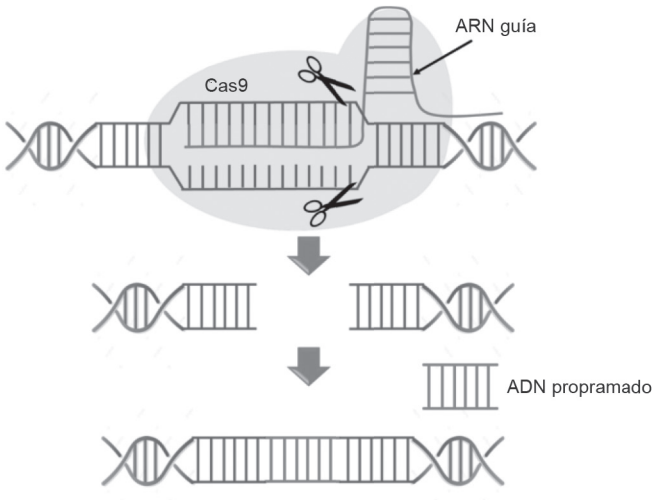

Figure 3. Genomic edition technology CRISPR/Cas9. The polymerase next to guide RNA opens the DNA molecule in a specific point or target, which can be closed again by homologous/ non-homologous recombination.
Source: Elaborated by the author

that are identified in dwarfism, which can be used to reduce size in tall species.

In forage species that are not grasses, such as *Medicago sativa* L., candidate genes to improve traits of economic interest were identified. From them the program Biomercator could be used to analyze orthologous genes in well-studied grasses, such as rice, and thus increase the possibility of finding these genes in grasses that are used as pastures. In *M. sativa* genes such as CONSTANS-LIKE, associated to flowering and height of the floral stem, were found, which can be important for tall species. Li *et al.* (2017) make reference to genes like *Arabidopsis Enhanced Drought Tolerance1, HSP23* and to genes of the MsHSP family, which improve biomass accumulation, sucrose and chlorophyll content under hydric stress. Similarly, it has been recorded that the genes MsERF9 and MsERF11 confer tolerance to salinity (Chen *et al*., 2012).

Conclusions

Molecular markers allow studies of genetic diversity in pastures. With the advance of technology, the most used markers would be SNP.

Apomixis is a way of reproduction in many pasture species and constitutes a necessary tool to maintain and reproduce hybrids with characteristics of agronomic interest. Through molecular markers it is possible to recognize apomictic plants at early ages.

With technologies of genomic edition, such as CRISPR, the possibility opens up to improve traits in pastures. The candidate traits are the ones related to: higher tolerance to biotic and abiotic stress (especially drought), acceleration of the regrowth rate, higher quantity and speed of biomass accumulation and protein content for forages.

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Conflict of interests

The authors declare that there is no conflict of interests among them.

Authors' contribution

- Ayerin Carrodeguas-González. Carried out the search for literature, and the document writing and creation.
- Andres Zúñiga-Orozco. Carried out the search for literature, and the document writing and creation.

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