**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Tropical forage breeding from classic to new genomic tools: an
example with interspecific tetraploid *Urochloa* spp. hybrids**

Filipe Inácio Matias

Thesis presented to obtain the degree of Doctor in
Science. Area: Genetics and Plant Breeding

**Piracicaba
2018**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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“Não é o mais forte que sobrevive, nem o mais inteligente,
mas o que melhor se adapta às mudanças.”

Leon C. Megginson

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pelo exemplo de pessoas nas quais sempre me espelho e
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RESUMO

Melhoramento de forrageiras tropicais do clássico as modernas ferramentas genômicas: um exemplo em híbridos interespecíficos tetraploides de *Urochloa* spp.

Um programa de melhoramento de forragem tropical contém várias peculiaridades, especialmente quando se trata de espécies poliplóides e de apomixia facultativa. Apesar de sua importância, atualmente, faltam informações sobre estudos genéticos de características forrageiras e sobre o emprego de ferramentas genômicas quando comparadas a outras culturas e forragens de clima temperado. O gênero *Brachiaria* é o mais importante para formação de pastagens nas regiões tropicais, principalmente para produção de carne bovina. As espécies comerciais deste gênero são excelentes forrageiras perenes, e a identificação de genótipos superiores depende da seleção de muitas características sob controle genético complexo, com alto custo e avaliação demorada. Portanto, o conhecimento sobre usos e aplicações de ferramentas clássicas e genômicas em características forrageiras pode ser útil para apoiar programas de melhoramento e o desenvolvimento de novas cultivares. Nesse contexto, objetivou-se avaliar diversas ferramentas clássicas e genômicas a serem empregadas como estratégias de seleção em um programa tradicional de melhoramento de forrageiras tropicais. Um painel de híbridos tetraplóides obtidos do cruzamento *Urochloa brizantha* x *Urochloa ruziziensis* foi fenotipado e genotipado para avaliar parâmetros genéticos e realizar estudos genômicos. Para a análise fenotípica clássica, concluímos que não havia uma tendência clara da importância dos efeitos genéticos aditivos e não-aditivos para características agrônômicas e nutricionais. O índice de Mulamba e Mock deve ser usado no nível univariado, devido à promoção de uma resposta mais equilibrada à seleção para todas as características na seleção multivariada. Na extração e nas avaliações genômicas, as leituras que foram alinhadas ao genoma de referência 'simulado', criado a partir dos dados de GBS da cultivar 'Marandu', tiveram a maior porcentagem de descoberta de marcadores SNP comparado aos genomas de referência mais próximos, *Setaria viridis* e *S. italica*. Recomendamos diferentes limiares de profundidade de leitura e qualidade de genótipo (GQ) para eliminar leituras de baixa qualidade sem introduzir viés de chamada de genótipo. A validação cruzada revelou que os genótipos ausentes foram imputados com uma precisão mediana de 0,85 pelo algoritmo *Random Forest* para produzir uma matriz genotípica completa, independentemente da frequência de heterozigotos. A análise de associação genômica ampla (GWAS) revelou genes candidatos associados a muitas características forrageiras tropicais, o que poderia ser o primeiro passo em direção à seleção assistida por marcadores (MAS). Além disso, nossos resultados sugerem que a contabilização da dosagem alélica é essencial, uma vez que o nível tetraploide fornece mais informações sobre o verdadeiro estado biológico. Portanto, nossos achados revelam a complexidade da arquitetura genética de características de *Urochloa* spp. e fornecem informações importantes para a aplicação de GWAS em espécies poliplóides. A análise de seleção genômica revela que o GBLUP-A (aditivo) e o GBLUP-AD (aditivo + dominância) mostraram capacidades de predição semelhantes, considerando tanto os modelos simples quanto os multi-característica. Por outro lado, combinando-se

GBLUP-AD e informação tetraploide foi possível melhorar a coincidência de seleção. Além disso, o esquema de validação multi-característica 2 (VS2), onde uma característica não é avaliada para alguns indivíduos, pode fornecer um incremento de até 30% da capacidade de previsão. Portanto, é uma estratégia útil para características com baixa herdabilidade. No geral, todos os modelos de seleção genômica considerados proporcionaram maiores ganhos genéticos do que a seleção fenotípica tradicional. Da mesma forma, a dosagem do alelo associado a fatores aditivos, de dominância e multicares aumentou a acurácia dos modelos genômicos de predição para híbridos poliploides interespecíficos. Finalmente, ferramentas genômicas devem ser utilizadas em programas de melhoramento de forragens para reduzir custos e tempo.

Palavras-chave: Seleção genômica; Associação genômica; Modelos mistos; Genotipagem por sequenciamento; Dosagem alélica; *Brachiaria* spp.

ABSTRACT

Tropical forage breeding from classic to new genomic tools: an example with interspecific tetraploid *Urochloa* spp. hybrids

A tropical forage breeding program contains several peculiarities, especially when it involves polyploid species and facultative apomixis. Despite their importance, there is still a lack of information on genetic studies of critical forage traits and on the employment of genomic tools when compared to other crops and temperate forages. The genus *Brachiaria* is the most important for forage in tropical regions mainly beef production. The commercial species in this genus are excellent perennial forage, and the identification of superior genotypes depends on the selection of many characteristics under complex genetic control, with high cost and time-consuming evaluation. Therefore, the knowledge about uses and applications of classic and genomic tools in forage traits may be useful to support breeding programs and the development of new cultivars. In this context, the aim was to evaluate several different classic and genomic tools to be employed as selection strategies in a traditional tropical forage breeding program. A panel of tetraploid hybrids obtained from crossing *Urochloa brizantha* x *Urochloa ruziziensis* was phenotyped and genotyped to evaluate genetic parameters and perform genomic studies. The classic phenotypic analysis showed no clear trend of the importance of additive and non-additive genetics effects for agronomical and nutritional traits. The Mulamba and Mock index should be used in the univariate level, due to the promotion of a more balanced response to selection for all traits in the multivariate selection. In the genomic extraction and evaluations, the reads that were aligned to a ‘mock’ reference genome, created from GBS data of the cultivar ‘Marandu’, had more SNP discovered compared to the closest true reference genomes, *Setaria viridis* and *S. italica*. We recommended different thresholds of sample depth and genotype quality (GQ) to eliminate poor quality reads without introducing genotype bias. Cross-validation revealed that missing genotypes were imputed with a median accuracy of 0.85 using *Random Forest* algorithm to produce a complete genotype matrix, regardless of heterozygote frequency. The genome-wide association analysis (GWAS) revealed candidate genes associated with many tropical forage traits across all cutting seasons, which could be the first step toward marker-assisted selection (MAS). Moreover, our results suggest that accounting for allele dosage is essential, since the tetraploid level provided more information about the true biological state. Therefore, our findings revealed the complexity of the genetic architecture of *Urochloa* spp. traits and provided important insights towards the application of GWAS in polyploids species. The genomic selection analysis revealed that GBLUP-A (additive) and GBLUP-AD (additive + dominance) showed similar prediction abilities considering both single and multi-trait models. Conversely, combining GBLUP-AD and tetraploid information could improve the selection coincidence. Furthermore, the multi-trait validation scheme 2 (VS2), where one trait is not evaluated for some individuals, provided an increment of up to 30% to the prediction ability. Therefore, it is an useful strategy for traits with low heritability.

Overall, all genomic selection models considered provided greater genetic gains than the phenotypic selection. Similarly, the allele dosage associated with additive, dominance and multi-trait factors increased the accuracy of genomic prediction models for interspecific polyploid hybrids. Finally, genomic tools should be used in forages breeding programs in order to reduce cost and time.

Keywords: Genomic selection; Genomic association; Mixed models; Genotyping-by-sequencing; Allele dosage; *Brachiaria* spp.

1. INTRODUCTION

Animal protein is an important nutritional source for humans' health due to the high biological value (World Health Organization and United Nations University, 2007). The consumption of animal protein increased following the population growth, and meat and milk from cattle are undoubtedly the favored and most important protein sources (Tilman and Clark, 2014; Henchion et al., 2017; FAO, 2018). Despite improved animal production technologies and sustainable animal production systems there is still an association between a negative impact on the environment and cattle production (Henchion et al. 2017). Thus, the use of native and cultivated pastures rather than animal confinement has contributed to animal welfare, product quality, environmental protection and costs reduction, resulting in a more natural protein source (Lupo et al., 2013; Picasso et al., 2014; Grandin, 2015; O'Callaghan et al., 2016a; b; Henchion et al., 2017).

Brachiaria is the most cultivated genus as pasture on tropical livestock farms and *U. decumbens* (syn. *Brachiaria decumbens*) and *U. brizantha* (syn. *B. brizantha*) the most used species (Jank et al., 2014; Pessoa-Filho et al., 2017). Cultivars from this genus have many advantages such as tolerance to poor and acid soils, good carrying capacity, tolerance to insects and good nutritional value. The economically important *Brachiaria* cultivars are tetraploid ($2n = 4x = 36$), but this genus also has diploid ($2n = 2x = 18$), pentaploid ($2n = 5x = 45$) and hexaploid commercial genotypes ($2n = 6x = 54$) (Mendes-Bonato et al., 2002; Jank et al., 2014). Due to the cytogenetic behavior of the *Brachiaria* polyploid species, Mendes-Bonato et al. (2002) and Worthington et al. (2016) suggested that they may be complex segmental allopolyploids. The segmental allopolyploids have partial homology among chromosomes and sets of chromosomes with differentiated behavior, varying between allopolyploid and autopolyploid (Sybenga, 1996). Segmental allopolyploids also are common for other plants as already described in the literature for buffelgrass (Jessup et al., 2003) and *Lencaena benth* (Boff and Schifino-Wittmann, 2003).

Initially, the commercial cultivars used in tropical regions came from introductions from Africa (Jank et al., 2011, 2014; Maass et al., 2015). The hybridization between *U. brizantha* tetraploid cultivars was impossible due the apomictic reproduction and lack of compatible sexual source (Miles, 2007). Then in 1981 the *Urochloa ruziziensis*, that is originally a diploid species, had the genome duplicated by colchicine (Swenne et al., 1981) opening a new possibility for *Brachiaria* breeding programs. Now it is possible to hybridize in a recurrent selection schemes (Miles, 2007; Worthington and Miles, 2015)

between sexual “polyploidized” *U. ruziziensis* with apomictic commercial pollen donor *U. decumbens* or *U. brizantha* (Lutts et al., 1991; Souza-Kaneshima et al., 2010; Monteiro et al., 2016; Worthington et al., 2016). Thus, it is possible to select superior males (apomictic) and females (sexual) genotypes to provide a unique and distinctive diversity interspecific hybrids of *Urochloa* spp to be explored.

An ideal forage plant should produce large quantities of dry matter, especially of leaves, good regrowth ability, which allows for more grazing cycles throughout the year, and high nutritional value regarding the content of protein, less fiber and lignin content, which allows for good digestibility (Resende et al. 2008). Although selection on a single trait provides for higher genetic progress, its use may be detrimental due to the occurrence of unwanted correlations between the traits of interest under improvement (Bauer and Léon 2008). Thus, multivariate methods, such as principal component analysis, allow multivariate patterns of interest to be shown graphically, assisting in the simultaneous selection of agronomic and nutritional characteristics.

In a breeding program, it is necessary to estimate genetic parameters in order to establish the best strategy of hybridization, selection and germplasm organization considering traits of interest. Estimation of genetic parameters and correlations have been described in the literature for *U. decumbens* (Mateus et al. 2015; Matias et al. 2016) and *U. humidicola* (Figueiredo et al. 2012). However, studies about genetic parameters such as additive and dominance variance, as well as general and specific combining ability in interspecific hybrid populations are not yet available in the literature.

The molecular studies in *Brachiaria* populations were restricted before to microsatellites (Jungmann et al., 2009, 2010; Vigna et al., 2011; Silva et al., 2013) and more recently in the use of genotyping-by-sequencing methods (GBS) (Worthington et al., 2016; Ferreira et al., 2018). However, little is known about the influence of filtering parameters criteria on GBS data in polyploids forages. New breeding panels and polyploid species can be evaluated genomically using GBS (Poland et al., 2012; Worthington et al., 2016) which is an interesting approach to reduce the complexity of polyploid genomes by restriction enzyme digestion (Huang et al., 2014). Different methods and softwares were developed to perform genotype calling of polyploid species using GBS. All these methodologies have in common the use of the ratio between the number of reads and alleles (Grandke et al., 2014). However, estimating polyploid genotypes require higher read depth compared to diploids. For instance, different genotyping call requirements can be found in the literature for tetraploid data. Genotype call could require a depth of at least 48x as described for *Poa*

grasses (Griffin et al., 2011) or among 60-80x as described for potato (Uitdewilligen et al., 2013). In addition, the genotype call for the single nucleotide polymorphisms (SNP) discovery is an inference by the allele depth, where alleles with shallow depth could be biased. Then, it is necessary to choose carefully the genotype quality score to prevent bias in genomics studies (Anderson et al., 2010; Laurie et al., 2010; McKenna et al., 2010).

Among many quantitative genetics tools, genome-wide association studies (GWAS) have been used to discover genetic regions in significant association with essential traits (Zargar et al., 2015). However, in polyploid species, GWAS is complicated, mainly due to the number of genotype classes and possible modes of gene action which, until recently, were under supported with appropriate analysis methods (Rosyara et al., 2016). Consequently, GWAS use in polyploids is relatively new (Bourke et al., 2018; Ferrão et al., 2018) and is predominantly applied by disregarding the allele dosage and then applying diploid models and software (Sun et al., 2016; Mourad et al., 2018). However, little is known about the consequences of using diploid models on the GWAS results compared to the use of the adequate allele dosage. Furthermore, despite the noteworthy importance of *Urochloa* spp. for livestock in tropical regions, as far as we know, there are no studies of GWAS using SNP markers performed on this genus.

Usually, the whole selection process, from the generation of segregating populations to the release of new cultivars in tropical perennial forages, takes around 10-15 years. Furthermore, it is a hard-working and expensive process due to the evaluation of animal performance apart from plant performance (Jank et al., 2014). For instance, one selection cycle in these species demands on average of two years, where phenotypic records of seven to ten cuttings are employed to evaluate the genetic value, stability, and adaptability of genotypes. Hence, genomic prediction methods can be a useful tool to reduce the costs due to the phenotyping expenses and the length of *Urochloa* spp. breeding cycle. In this sense, a simulation study of the feasibility of GP in a traditional forages breeding program (Resende et al. 2014), concluded that the individual genomic prediction method (INDG) could be useful when marker effects have been previously estimated. However, the genomic prediction may be ineffective depending on the heritability of the target trait (de los Campos et al., 2013). Thus, an alternative is the use of the correlation between traits to improve the predictive ability of the models by using the multi-trait approach (MTM). Through this approach, it is possible to use traits with higher heritability to improve the power to predict the others trait (Bauer and Léon, 2008; Dos Santos et al., 2016; Fernandes et al., 2018). It has been successfully implemented using single by single

trait (Jia and Jannink, 2012; Guo et al., 2014) or indices (Schulthess et al., 2016; Lyra et al., 2017).

In this context, our aims were (i) to estimate the contribution of additive and non-additive effects on agronomical and nutritional traits; (ii) to estimate the accuracy of multivariate index selection efficiency; (iii) the influence of different quality filters to select markers from GBS analysis; (iv) to infer the phylogeny of *Brachiaria* species by GBS reads alignment and SNP discovery using five different grass genomes; (v) to compare the missing data imputation accuracy using the Random Forest and Mode methods; (vi) to perform GWAS analysis for forage traits of different cutting seasons using SNP markers from genotyping by sequencing (GBS); (vii) to verify the influence of allele dosage through diploid and tetraploid configuration markers in the GWAS approach; (viii) to empirically evaluate the influence of multi-trait and the allele dosage information in genomic prediction accuracy in a diversity panel of *Urochloa* spp. hybrids.

2. GENERAL CONCLUSION

The classic phenotypic analysis showed no clear trend of the importance of additive and non-additive genetics effects for agronomical and nutritional traits. The Mulamba and Mock index should be used in the univariate level, due to the promotion of a more balanced response to selection for all traits in the multivariate selection. In the genomic extraction and evaluations, the reads that were aligned to a ‘mock’ reference genome, created from GBS data of the cultivar ‘Marandu’, had more SNP discovered compared to the closest true reference genomes, *Setaria viridis* and *S. italica*. We recommended different thresholds of sample depth and genotype quality (GQ) to eliminate poor quality reads without introducing genotype bias. Cross-validation revealed that missing genotypes were imputed with a median accuracy of 0.85 using *Random Forest* algorithm to produce a complete genotype matrix, regardless of heterozygote frequency. The genome-wide association analysis (GWAS) revealed candidate genes associated with many tropical forage traits across all cutting seasons, which could be the first step toward marker-assisted selection (MAS). Moreover, our results suggest that accounting for allele dosage is essential, since the tetraploid level provided more information about the true biological state. Therefore, our findings revealed the complexity of the genetic architecture of *Urochloa* spp. traits and provided important insights towards the application of GWAS in polyploids species. The genomic selection analysis revealed that GBLUP-A (additive) and GBLUP-AD (additive + dominance) showed similar prediction abilities considering both single and multi-trait models. Conversely, combining GBLUP-AD and tetraploid information could improve the selection coincidence. Furthermore, the multi-trait validation scheme 2 (VS2), where one trait is not evaluated for some individuals, provided an increment of up to 30% to the prediction ability. Therefore, it is an useful strategy for traits with low heritability. Overall, all genomic selection models considered provided greater genetic gains than the phenotypic selection. Similarly, the allele dosage associated with additive, dominance and multi-trait factors increased the accuracy of genomic prediction models for interspecific polyploid hybrids. Finally, genomic tools should be used in forages breeding programs in order to reduce cost and time.

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