Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8621
Doi: 10.4025/actasciagron.v41i1.42603

Genetic linkage map and mapping of the locus of biological nitrogen fixation inefficiency in cowpea

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ABSTRACT. The objectives of the present study were to construct a cowpea genetic map using the F_2 population resulting from the cross IC-1 x BRS Marataoã, based on single nucleotide polymorphism (SNP) markers, and to map the *cpi* gene, with additional reference to introgression with the consensus map of species, aiming to identify markers for assisted selection to develop more efficient cultivars for BNF. The parents and 89 F_2 plants were genotyped with 51,128 SNP markers, of which 910 polymorphic markers were used to construct the map. The results revealed 11 linkage groups, with an average of 82 markers per chromosome and average distance of 1.26 cM between markers. Recombination analysis of the SNPs indicated that markers 2_12850 and 2_00188, located in linkage group 11, flanked the *cpi* gene at a distance of 6.7 cM and 5.64 cM, respectively. The introgression of linkage group 11 with the cowpea reference map revealed short distances (from zero to 0.6 cM) for these markers, indicating a strong association with the *cpi* gene. The constructed map and *cpi* mapping provide basic information that can assist the genetic breeding of more efficient cowpea plants for BNF by marker-assisted selection.

Keywords: *Vigna unguiculata*; nitrogen fixation; marker-assisted selection.

Received on November 28, 2017.
Accepted on March 16, 2018.

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an autogamous diploid species (2n = 2x = 22) with a genome size estimated at 620 Mb (Arumuganathan & Earle, 1991). This legume species can fix N_2 because of the species symbiotic relation with rhizobia (Leite et al., 2009). This economically important characteristic for cowpea provides nitrogen and mineral accumulation by the symbiotic association with N_2-fixing bacteria, depending on the macro- and microsymbiont interactions and environmental conditions (Mohammadi, Sohrabi, Heidari, Khalesro, & Majidi, 2012; Belane, Pule-Meulenberg, Makhubedu, & Dakora, 2014). Although both parties of the association are susceptible to genetic variation, little information is available on the host plant (Bladergroen & Spaink, 1998; Shamseldin, 2013).

Initial interactions for nodule formation have increasingly improved due to the identification of non-nodulating plants (Nod^-) in the presence of nitrogen-fixing bacteria. Non-nodulation is controlled by the host plant through the release of phenolic compounds incompatible with the nodulation promoter regions (nod-box), which are responsible for the induction of transcription of the bacterial genes essential to nodulation (Geurts, Fedorova, & Bisseling, 2005; Madsen et al., 2010; Okazaki et al., 2016). Non-nodulating plants have been observed in several legume species and are usually determined by recessive alleles (Nigan, Nambiar, Dwivedi, Gibbons, & Dart, 1982; Vest & Caldwell, 1972; Ceccatto, Gomes, Sarries, Moon, & Tsai, 1988; Novák, 2003).

Nitrogen fixation efficiency (Fix^-) after nodulation has been reported in several legume species. Studies have demonstrated that this trait can be controlled by dominant (Vest, 1970; Markwei & LaRue, 1992) and recessive (Pedalino, Kipe-Nolt, Frusciante, & Monti, 1993; Park & Buttery, 1994; Sagan, Huguet, & Duc, 1994) alleles. Pemberton, Smith, and Miller Jr. (1990) and Purdom and Trese (1995) developed preliminary studies with the inefficient mutant IC-1, which presents small white nodules and small shoots compared with plants with effective symbiosis. The gene that confers BNF inefficiency was denominated *cpi*, and no studies have developed marker-assisted selection (MAS) for this gene.

Limitations for cowpea genetic linkage maps, such as low density and long distances between markers (Ouédraogo et al., 2002; Muchero et al., 2009; Agbicodo et al., 2010; Lucas et al., 2011; Adetumbi, Akinyosoye, Olowolafe, Oloyede-Kamiyo, & Agbeleye, 2016), were overcome by the consensus map provided by Muñoz-Amatriaín et al. (2017), which presents 37,372 SNP markers and spans 873.11 cM, with an average distance of 0.26 cM between markers. This consensus map allows the introgression of low-density genetic linkage groups, mapping, and the development of markers for MAS.

The objectives of this study were to construct a cowpea genetic map for the 11 linkage groups in the F_2 population of the cross IC-1 x BRS Marataoã, based on SNP markers, and to map the *cpi* gene, with additional reference to introgression with the consensus map of the species, aiming to identify markers that allow assisted selection for the development of more efficient cultivars for BNF.

Material and methods

Plant material

A mapping population $F_{2n} = 89$, resulting from the selfing of a single F_1 progeny of the cross between a mutant line inefficient for nitrogen fixation (fix^-) (IC-1) and a commercial variety efficient for nitrogen fixation (fix^+) ("BRS Marataoã"), was used to map the *cpi* gene. All crosses, as well as the obtained populations (P_1, P_2, F_1, and F_2), were cultivated in a protected environment with 70% shade cloth at Embrapa Semiárido, Petrolina, Pernambuco State, Brazil.

All plants were grown in plastic pots containing 3 L of nonsterile soil. Rhizobial colonization was provided by the inoculation of a mixture of BR 3267, BR 3262, and BR 3299 bacteria, which are recommended for cowpea. Bacteria were cultured in YM liquid medium (Vincent, 1970). At planting and 5 days after emergence, the seeds and seedlings, respectively, were inoculated via soil with 1 mL of inoculum, adjusted to 10^9 cells mL^{-1}. Rhizobia strains were provided by Embrapa Agrobiologia, Seropédica, Rio de Janeiro State, Brazil. Efficient (green leaves) and inefficient (yellow leaves) plants for BNF in the F_2 population were visually identified at 40 days after emergence. Inefficient plants for BNF showed typical symptoms of nitrogen deficiency (yellow leaves) and reduced shoot size.

Genotyping

Genomic DNA was extracted from young leaves using the modified CTAB protocol (Doyle & Doyle, 1990). The extracted DNA was analyzed in a spectrophotometer (NanoDrop® ND-1000 UV-Vis) to estimate the DNA quality and concentration. The material was diluted in sterile water to a concentration of 50 ng μL^{-1} and stored at -20°C for subsequent analyses. Eighty-nine F_2 plants and three replications for each parent were genotyped using The Cowpea iSelect Consortium Array which consists of 51,128 SNPs (WG-401-1002; Illumina, Inc.). Genotyping was performed on an iSCAN System bead chip reader at the Center for Public Health Genomics (University of Virginia School of Medicine). The SNPs were called and filtered in GenomeStudio 2.0 (Illumina, Inc.) according to the guidelines outlined in the Illumina genotyping technical notes (https://www.illumina.com/documents/products/technotes/technote_infinium_genotyping_data_analysis.pdf) and a previously developed cowpea cluster file (Muñoz-Amatriaín et al., 2017). Genotype data were exported from GenomeStudio, and additional filtering was applied based on parental calls and expected F_2 segregation. SNP markers with different genotypic classifications between parent replications, as well as monomorphic SNP markers between parents, were discarded. An F_2 plant was discarded from the analysis due to failures in SNP reactions greater than 10%. The chi-square test was performed to identify and discard markers with Mendelian segregation distortion in the F_2 population greater than $\chi = 0.01$.

Linkage map development

The linkage map was constructed using the IciMapping QTL version 4.1 (Meng, Li, Zhang, & Wang, 2015). Phenotypic data for efficient (chlorotic) plants and phenotypic data for inefficient (nonchlorotic) plants were converted to AH and B, respectively, for inclusion in the mapping analyses, based on software instructions.

Redundant SNP markers were discarded from the analysis using the "Binning" command in the software. The map order for each F2 individual was verified for the formation of the linkage groups using the "Grouping" command. The ordering algorithm nnTwoOpt (the nearest neighbor) was used to calculate the distances in the linkage groups. The sum of adjacent distances (SAD) criterion was applied using the function "Rippling." The values obtained for the recombination frequencies were converted to genetic map distance (centimorgans) using the Kosambi function (Kosambi, 1943). The number and orientation of the linkage group were assigned based on the cowpea consensus map (http://harvest.ucr.edu/).

Introgression of the linkage group 11 with the cowpea consensus map (Muñoz-Amatriaín et al., 2017) was used to saturate the region containing the *cpi* gene. Introgression was performed in the IciMapping QTL software, using the function "consensus map construction."

Results and discussion

Efficient and inefficient plants for BNF were easily visualized. Small white nodules co-segregated with yellowish leaves, typical of the IC-1 line, which is inefficient for BNF, as reported by Pemberton et al. (1990). The segregation of the 169 F_2 plants resulting from the cross IC-1 x "BRS Marataoã" revealed 44 inefficient and 125 efficient plants for BNF (Table 1). The 3:1 ratio observed in this cross indicates that BNF inefficiency is controlled by a recessive gene, as described by Pemberton et al. (1990).

Table 1. Chi-square (χ^2) test for number of efficient (green leaf) and inefficient (yellow leaf) plants, means and variances for the accumulated nitrogen-NA in cowpea in the parents and F_1 and F_2 generations for the cross IC-1 x BRS Marataoã.

Cross	Generation	No. of plants			Hypothesis	$\chi^{2(1)}$
		Total	Green	Yellow		
IC-1 x BRS Marataoã	P_1	40	0	40	0:1	0 (100%)
	P_2	43	43	0	1:0	0 (100%)
	F_1	54	54	0	1:0	0 (100%)
	F_2	169	125	44	3:1	0.09^{ns} (75.%)

^{ns} Not significant at 5% probability level by the square test.

Linkage map and identification of SNP markers linked to the *cpi* gene

After the elimination of monomorphic markers with different genotypic classifications in the parent replications or in cases where the loss of information was greater than 10%, 7,112 polymorphic markers were selected in the population. Afterward, markers in repetitive positions were discarded. The linkage map was constructed with 910 SNP markers, distributed in 11 linkage groups, with LOD scores ranging from 4 to 9 (Figures 1 and 2).

The 910 polymorphic SNPs detected in this study spanned 1,140.12 cM, with an average of 82 markers for each genetic linkage group and an average distance of 1.26 cM between markers, presenting high saturation compared with some maps available for cowpea. The longest and shortest lengths were observed for groups 3 and 10, respectively (Table 2). The largest gap devoid of markers (29.75 cM) was observed in linkage group 4, and the smallest gap devoid of markers (5.19 cM) was observed in linkage groups 2 and 6 (Table 2).

Studies on genetic linkage maps with molecular markers in cowpea are recent. Menéndez, Hall, and Gepts (1997) constructed the first genetic map of this species, associating the markers with pests, diseases, and morphological characteristics using an intraspecific cross. They used the markers 133 RAPD, 19 RFLP, and 25 AFLP to identify 12 linkage groups spanning 972 cM, with an average distance of 6.4 cM between markers, and linkage groups ranging from 3 to 257 cM. Ouédraogo et al. (2002), using the same mapping population with recombinant inbred lines (RIL) that was employed by Menéndez et al. (1997), constructed a linkage map with 441 AFLP, RFLP, and RAPD markers, spanning 2,670 cM, with an average distance of 6.43 cM between markers. This map associates several traits with resistance to viruses, diseases, and races 1 and 3 of *Striga gesnerioides*.

The linkage group in cowpea has progressed with Illumina GoldenGate SNP marker technology. This platform was developed and implemented to map 928 SNPs derived from expressed sequence tags (EST) of cowpea (Muchero et al., 2009). The map spanned 680 cM with 11 linkage groups and an average distance of 0.73 cM between markers. This map showed the evolutionary closeness between cowpea and soybeans and identified regions for synteny-based functional genomics studies in legume species.

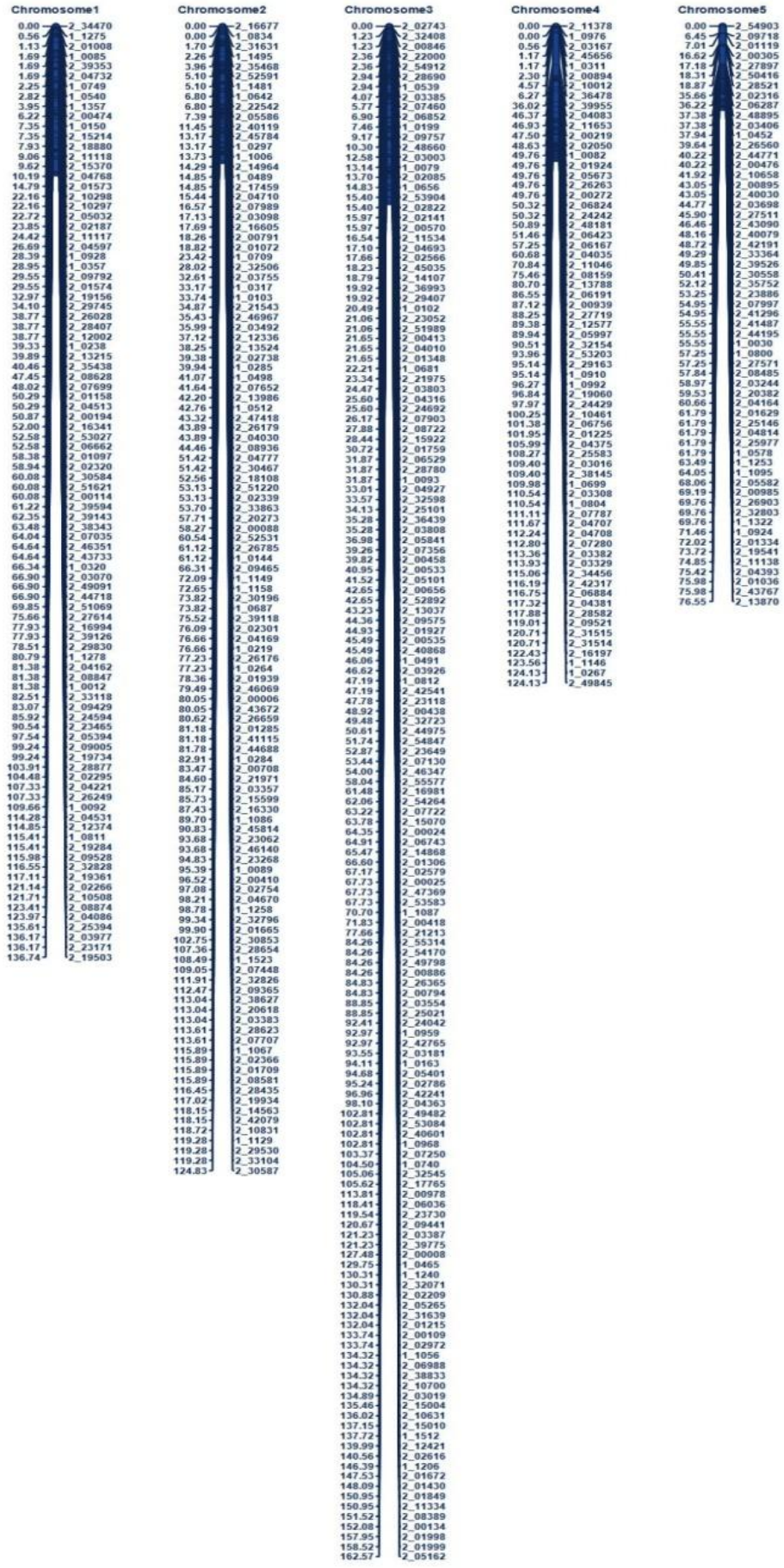

Figure 1. Linkage groups (Chr) 1, 2, 3, 4, 5, and 6 constructed with SNPs and *cpi* gene markers in the F_2 population of the IC-1 x BRS Marataoã cross.

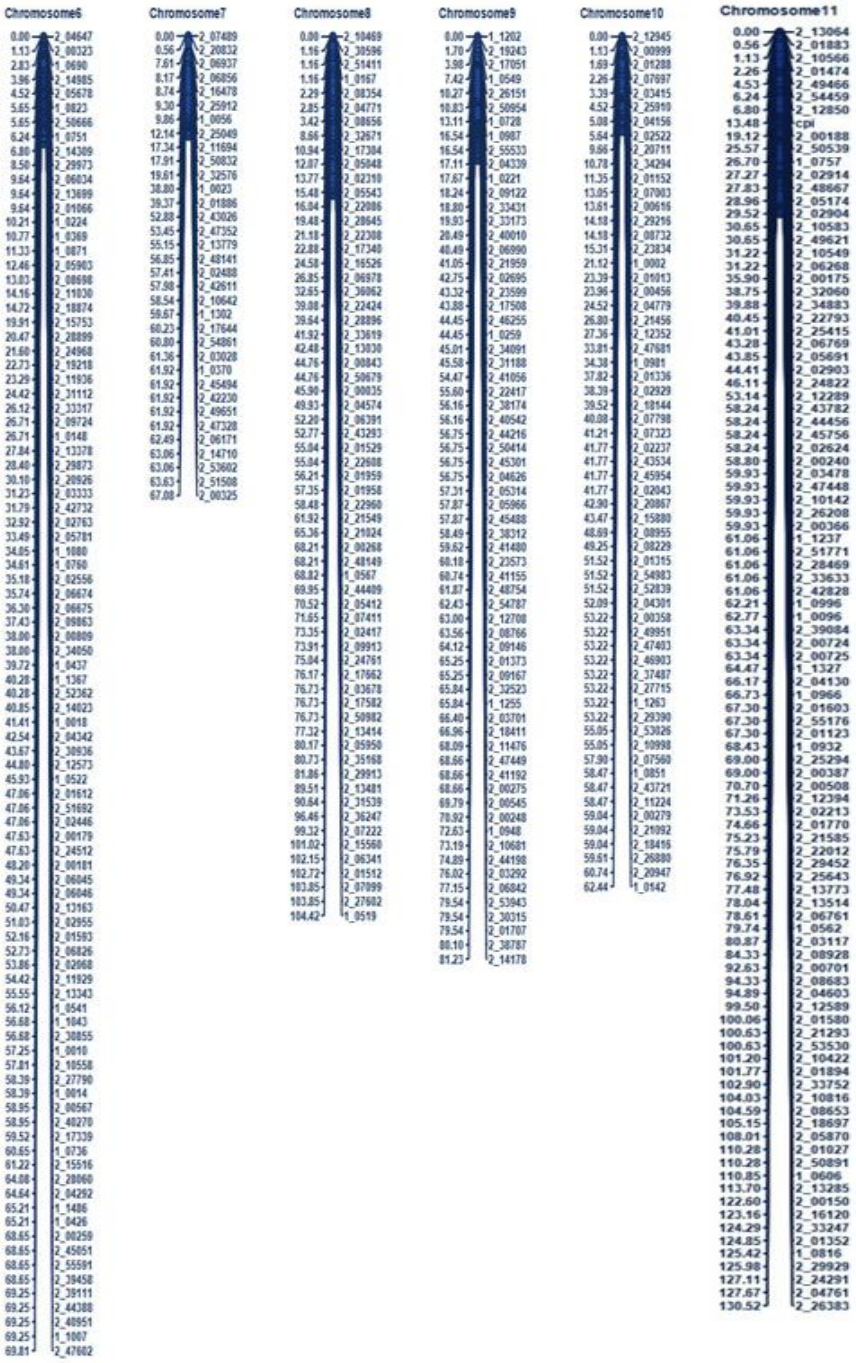

Figure 2. Linkage groups (Chr) 6, 7, 8, 9, 10, and 11 constructed with SNPs and *cpi* gene markers in the F_2 population of the IC-1 x BRS Marataoã cross.

Table 2. Genetic linkage groups with 910 SNP markers in the F_2 population of the cross IC-1 x BRS Marataoã.

Linkage group	Number of SNP marker	Size (cM)	Average distance (cM)	Longest distance (cM)
1	94	136.74	1.45	11.00
2	115	124.83	1.08	5.19
3	153	162.57	1.06	5.80
4	67	124.13	1.85	29.75
5	59	76.55	1.29	16.79
6	93	69.81	0.75	5.19
7	34	67.08	1.97	19.20
8	63	104.42	1.65	7.64
9	66	81.23	1.23	20.00
10	61	62.44	1.02	6.45
11	105	130.32	1.24	8.89
Total	910	1140.12	1.26	12.35

Agbicodo et al. (2010) constructed a genetic linkage map of cowpea with 113 recombinant lines using 282 SNP markers selected from the cowpea consensus map of Muchero et al. (2009). This map consisted of 11 linkage groups, totaling 633 cM, with an average distance of 2.24 cM between markers and was associated with resistance to the bacterium *Xanthomonas axonopodis* pv. Vignicola (Xav). Lucas et al. (2011) constructed the consensus map of cowpea, allocating 1,107 SNP markers in 11 linkage groups, spanning 680 cM of the genome, with a distance of 0.62 cM. Muñoz-Amatriaín et al. (2017) presented a consensus map with 37,372 SNPs and a span of 873.11 cM, with an average distance of 0.26 cM.

The linkage map of the F_2 population IC x BRS Marataoã (Figures 1 and 2) corresponded well to the consensus map of cowpea previously reported by Lucas et al. (2011) and Muñoz-Amatriaín et al. (2016). Although the genome size in this study is larger than the consensus map of 680 cM and 837.1 cM, the present result is consistent and shows good accuracy, even using a smaller population and with fewer recombination events observed in the F_2 population compared with the RIL populations used in the development of the consensus map.

Map introgression

For introgression markers in the *cpi* gene region, 3027 SNP markers of the linkage group 11 were used, which are available on the consensus map of cowpea (Muñoz-Amatriaín et al., 2017). The introgression of the maps for chromosome 11 resulted in a shorter distance because introgression was nonexistent for the locus with SNP 2_12850 and was 0.56 cM for the locus with SNP 2_54459, which was not included in the present analysis of the F_2 population (Figure 3; Table 1).

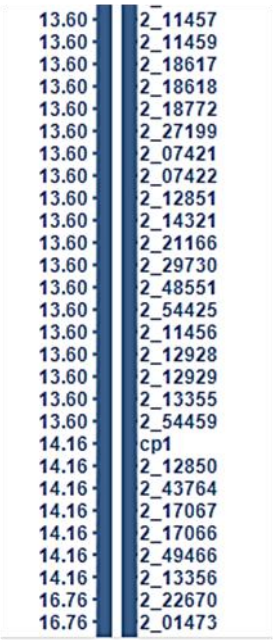

Figure 3. Position of the *cpi* gene in group 11 of the consensus linkage map of cowpea.

The mapping of simple inheritance traits in cowpea was reported by Rodrigues, Santos, and Santana (2012), who identified three AFLP markers linked to the resistance gene for cowpea golden mosaic virus, with two markers flanking this gene. Pottorff et al. (2012) mapped the resistance locus of Fusarium oxysporum (Fot3-1) race 3 to a 1.2 cM region and identified the SNP marker 1_1107 as co-segregating with Fot3-1 in cowpea. These studies show that candidate genes can be identified for simply inherited agronomic traits.

The present study identified, in a pioneering way, the genomic region related to inefficient plants for nitrogen fixation, and the markers 2_12850 and 2_00188 were located at a distance of 6.7 cM and 5.64 cM, respectively, flanking the *cpi* gene region. The introgression with the cowpea consensus map reduced the distance between markers and the *cpi* gene, possibly due to the wide spanning, favoring the joint analysis of the dominant marker (*cpi*) with the codominant marker (SNP). In the scenario provided by the introgression of linkage group 11, the distances of the SNP flanking the *cpi* gene were reduced to zero or 0.6 cM, indicating that this is a chromosomal region strongly associated with the gene of the nitrogen fixation inefficiency in cowpea.

Conclusion

Despite the genetic maps available for cowpea in the literature, no marker linked to the BNF inefficiency gene has been identified. Therefore, this is the first map using a *V. unguiculata* population segregating for N_2 fixation. The markers 2_12850 / 2_54459, identified in the present study, emerge as strong candidates for use in molecular marker-assisted selection, given their greater proximity to the *cpi* gene, which confers inefficiency to nitrogen fixation in cowpea.

Acknowledgements

Sirando L. Seido has a CAPES scholarship. Carlos A. F. Santos is a CNPq researcher. Arthur T. Trese provided the IC-1 seeds.

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