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In silico characterization of putrescine N-methyltransferase in Solanaceae species

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Genet. Mol. Res. 21 (4): gmr19076
Received July 07, 2022
Accepted September 27, 2022
Published October 30, 2022
DOI http://dx.doi.org/10.4238/gmr19076

ABSTRACT. The Solanaceae family comprises about 100 genera and 2,500 species, with a cosmopolitan distribution and greatest diversity in the Neotropical region. In Brazil, 36 genera and 506 species have been identified, including 236 endemic species. The family has a high diversity of species of economic importance as a source of food, medicinal extracts, and for ornamental use. The species are sources of bioactive secondary metabolites, with diverse applications. We made ae structural and functional characterization and developed three-dimensional models of putrescine N-methyltransferase (PMT) proteins, a key enzyme of secondary metabolism, involved in biosynthesis of nicotine, tropane alkaloids, and calistegines. We examined 48 PMT sequences from Solanaceae species available in public databases. A hydrophilic characteristic of PMT was found for all species, and the isoelectric point demonstrated a somewhat acidic character (5.4 - 6.6). We identified four functional domains in the PMT sequences. Cluster analysis by Neighbor-Joining was consistent with recent taxonomic classifications of the species.

Key words: Bioinformatics; Computational analysis; Homology modeling; Secondary metabolites; Solanaceae

INTRODUCTION

The Solanaceae family has a cosmopolitan distribution, comprising about 100 genera and 2,500 species, with the neotropical region being its most important center of diversity (Olmstead, 2013). According to D'Arcy (1991), South America is considered one of the family's leading centers of taxonomic diversity and endemism, where the widest species variety is found. In Brazil, there are 36 genera and 506 species of the family, 236 of which are endemic to the country (Stehmann et al., 2015). Approximately half of the species in the family belong to only five genera, *Capsicum*, *Cestrum*, *Lycianthes*, *Physalis*, and *Solanum*, the last being the largest and most morphologically diverse (Knapp, 2008; Sampaio et al., 2019).

In view of the great diversity of species belonging to Solanaceae that are present in the Brazilian flora, it is known that many of these have economic importance used as food, pharmacological substances production, and ornamentation. Additionally, plants belonging to this family are also sources of bioactive secondary metabolites of different chemical classes, such as steroidal alkaloids, tropane alkaloids, pyridine alkaloids, vitanolides, glycoalkaloids, flavonoids, among others, which have the most diverse applications (Corrêa, 2015).

Putrescine N-methyltransferase (PMT) is a crucial enzyme of secondary plant metabolism in initiating the specific biosynthesis of nicotine, tropane alkaloids, and calistegines, which are glucosidase inhibitors with nortropane structure. Studies claim that PMT proteins likely evolved from spermidine synthases (SPDSs), ubiquitous polyamine metabolism enzymes (Teuber et al., 2007; Junker et al., 2013; Moallem, 2017).

According to Leite et al. (2012), polyamines are fundamental molecules for the growth and functioning of cells. They interact with many macromolecules in the biological environment, both electrostatically and covalently. Such molecules play an essential role in cell growth and proliferation and the synthesis of proteins and nucleic acids, being also involved in extracellular matrix repair, cell adhesion, and signaling processes.

Methyltransferases have essential biological functions in nucleic acid methylation and protein methylation, which alter the activity of DNA, RNA, or protein, and the methylation of small molecules, such as mammalian and plant hormones or plant secondary metabolites, is of fundamental importance for the organism (Teuber et al., 2007). According to Stenzel et al. (2006), the transfer of N-methyl from the first specific steps catalyzes putrescine N-methyltransferase for the biosynthesis of tropane and nicotine alkaloids. The enzyme uses S-adenosyl-1-methionine as the methyl group donor and putrescine as the substrate, with only one or a few other diamines methylated less efficiently.

Geng et al. (2018) state that although the role of PMT in nicotine biosynthesis is clear, knowledge of PMT in the biosynthesis of tropane alkaloids and the regulation of polyamines remains limited. Biastoff et al. (2009) state that the biological history of PMT is still unclear, given the emergence of a concept for the evolution of this enzyme at the branch point between primary and secondary metabolism. According to Junker et al. (2013), it remains unclear whether putrescine N-methyltransferases (PMTs) occur in more unrelated families of higher plants and whether the hitherto undetected PMTs are similar to current PMT sequences, or whether new groups form.

According to the above, the present study aimed to: (1) structurally and functionally characterize the putrescine N-methyltransferase sequences of species of the Solanaceae

family by bioinformatics methodologies; (2) develop three-dimensional models of the putrescine N-methyltransferase of representatives of the Solanaceae family, based on the homology modeling methodology, in order to enable a better understanding of their molecular structures and functions.

MATERIAL AND METHODS

Sequences retrieval

The putrescine N-methyltransferase sequences were obtained from the NCBI (National Center for Biotechnology Information) platform, using the BLAST (Basic Local Alignment Search Tool) search algorithm, downloading them in FASTA format. BLASTp tool recovered protein sequences in Solanaceae species to search for homologs, where a total of 48 sequences were found.

Sequence Analysis

The ProtParam analyzed physicochemical parameters of PMT present in species of the Solanaceae family (http://web.expasy.org/protparam) (Gasteiger et al., 2005). CELLO2GO server predicted subcellular locations (Yu et al., 2014). The identification of the functional domains of the protein using the Prodom server to its classification and ontology, a family database of protein domains of homologous segments (http://prodom.prabi.fr/prodom/) (Servant et al., 2002). The estimation of the functional effects caused by mutations of amino acid sequences through the SNAP2 server (https://rostlab.org/services/snap2web/) (Hecht et al., 2015).

Alignment and Phenetic Analysis

The PMT enzyme sequences were aligned using the ClustalW algorithm. Then, MEGA 7.0.21 software performed the cluster analyses (Kumar et al., 2016) using the neighbor-joining method with a bootstrap test with 1000 replicas.

Tertiary structure prediction, evaluation, and validation of the model

The tertiary structure prediction to find 3-D models of PMTs used the Phyre2 server in multi-template mode (http://www.sbg.bio.Ic.ac.uk/phyre2) (Kelley et al., 2015). This server uses advanced homology detection methods to build a 3D model. The 3D structure visualization of the protein used the UCSF Chimera package (Pettersen et al., 2004). Model quality assessment using the Molprobity server (http://molprobity.biochem.duke.edu/) by Ramachandran analysis (Chen et al., 2010). The ProSA-web interactive server calculated the Z-score (https://prosa.services.came.sbg.ac.at/prosa.php) to recognize errors in three-dimensional structures (Wiederstein et al., 2007). The energy minimization and correction of minor errors in the three-dimensional model were performed by the Yasara force Field server (Krieger et al., 2009) and the KiNG software (Chen et al., 2009).

RESULTS AND DISCUSSION

The forty-eight Putrescine N-methyltransferase sequences of Solanaceae species analyzed from protein sequences available in the NCBI database, retrieved in FASTA format, are listed in Table 1.

Table 1. Putrescine N-methyltransferase sequence analysis in species of the Solanaceae family.

Species	GB-ID	GenBank definition	Family
Anisodus acutangulus	ACF21005.1	putrescine *N*-methyltransferase 1	Solanaceae
Anisodus acutangulus	ACF21006.1	putrescine *N*-methyltransferase 2	Solanaceae
Anisodus luridus	AGL76988.1	putrescine *N*-methyltransferase	Solanaceae
Anisodus tanguticus	AAT99576.1	putrescine *N*-methyltransferase	Solanaceae
Atropa belladonna	BAA82261.1	putrescine *N*-methyltransferase 1	Solanaceae
Atropa belladonna	BAA82262.1	putrescine *N*-methyltransferase 2	Solanaceae
Capsicum annuum	XP_016557670.1	putrescine *N*-methyltransferase 1	Solanaceae
Capsicum annuum	XP_016553600.1	putrescine *N*-methyltransferase 2- like	Solanaceae
Capsicum annuum	KAF3624529.1	putrescine *N*-methyltransferase 3	Solanaceae
Capsicum annuum	PHT78552.1	putrescine *N*-methyltransferase 3	Solanaceae
Capsicum baccatum	PHT30221.1	putrescine *N*-methyltransferase 3	Solanaceae
Capsicum baccatum	PHT45224.1	putrescine *N*-methyltransferase 3	Solanaceae
Capsicum chinense	PHU08967.1	putrescine *N*-methyltransferase 3	Solanaceae
Capsicum chinense	PHU14344.1	putrescine *N*-methyltransferase 3	Solanaceae
Datura inoxia	CAJ46253.1	putrescine *N*-methyltransferase 1	Solanaceae
Datura inoxia	CAJ46254.1	putrescine *N*-methyltransferase 2	Solanaceae
Datura metel	AAQ94738.1	putrescine *N*-methyltransferase	Solanaceae
Datura stramonium	CAE47481.1	putrescine *N*-methyltransferase	Solanaceae
Hyoscyamus niger	BAA82263.1	putrescine *N*-methyltransferase	Solanaceae
Nicotiana attenuata	AAK49870.1	putrescine *N*-methyltransferase 1	Solanaceae
Nicotiana attenuata	OIT40667.1	putrescine *N*-methyltransferase 1	Solanaceae
Nicotiana attenuata	XP_019258257.1	putrescine *N*-methyltransferase 2	Solanaceae
Nicotiana attenuata	XP_019258255.1	putrescine *N*-methyltransferase 3	Solanaceae
Nicotiana benthamiana	ABY25273.1	putrescine *N*-methyltransferase	Solanaceae
Nicotiana sylvestris	BAA74544.1	putrescine *N*-methyltransferase	Solanaceae
Nicotiana sylvestris	XP_009771961.1	putrescine *N*-methyltransferase 2	Solanaceae
Nicotiana sylvestris	XP_009771962.1	putrescine *N*-methyltransferase 3	Solanaceae
Nicotiana sylvestris	XP_009786665.1	putrescine *N*-methyltransferase 4 isoform X1	Solanaceae
Nicotiana sylvestris	XP_009786666.1	putrescine *N*-methyltransferase 4 isoform X2	Solanaceae
Nicotiana tabacum	XP_016446557.1	putrescine *N*-methyltransferase 1	Solanaceae
Nicotiana tabacum	NP_001312037.1	putrescine *N*-methyltransferase 1	Solanaceae
Nicotiana tabacum	XP_016478024.1	putrescine *N*-methyltransferase 2	Solanaceae
Nicotiana tabacum	XP_016478023.1	putrescine *N*-methyltransferase 3	Solanaceae
Nicotiana tabacum	XP_016445029.1	putrescine *N*-methyltransferase 4	Solanaceae
Nicotiana tomentosiformis	XP_018627768.1	putrescine *N*-methyltransferase 1 isoform X2	Solanaceae
Nicotiana tomentosiformis	XP_009606202.1	putrescine *N*-methyltransferase isoform X1	Solanaceae
Physalis divaricata	CAJ46255.1	putrescine *N*-methyltransferase	Solanaceae
Solanum dulcamara	CAQ19733.1	putrescine *N*-methyltransferase	Solanaceae
Solanum lycopersicum	NP_001233790.2	putrescine *N*-methyltransferase	Solanaceae
Solanum lycopersicum	CAJ46251.1	putrescine *N*-methyltransferase	Solanaceae
Solanum lycopersicum	XP_004244762.1	putrescine *N*-methyltransferase 2	Solanaceae
Solanum pennellii	XP_015084559.1	putrescine *N*-methyltransferase 2	Solanaceae
Solanum pennellii	XP_015078654.1	putrescine *N*-methyltransferase 3-like	Solanaceae
Solanum tuberosum	CAE53633.1	putrescine *N*-methyltransferase	Solanaceae
Solanum tuberosum	XP_006350699.1	putrescine *N*-methyltransferase 3-like	Solanaceae
Solanum chilense *	TMX00678.1	putrescine *N*-methyltransferase	Solanaceae
Solanum commersonii *	KAG5589663.1	putrescine *N*-methyltransferase	Solanaceae
Solanum commersonii *	KAG5600829.1	putrescine *N*-methyltransferase	Solanaceae

GB-ID: Genbank Identification. *: Hypothetical sequence.

Observing parameters such as molecular weight, theoretical isoelectric point (pI), and the average hydrophobicity (GRAVY) of the evaluated protein sequences was possible through the ProtParam program (Table 2). The isoelectric point of the sequences varied between 5.36

(*Datura metel*) and 6.6 (*Solanum pennellii*), indicating their acidic character. These results indicate that the pIs evaluated were hydrophilic. Since the isoelectric point (pI) is the pH at which a given molecule carries no net electrical charge, pI has wide use in currently used proteomic and biochemical techniques, such as during electrophoresis, where the direction of migration of proteins in the gel depends on the charge, so it is possible to separate proteins in a gel-based on their Ip (Kozlowski, 2017).

Table 2. Analysis of the primary structure of putrescine N-methyltransferase sequences from species of the Solanaceae family.

Species	Number of amino acids	MW (kDa)	pI	GRAVY
Anisodus acutangulus	347	38.10	6.51	-0.001
Anisodus acutangulus	338	37.19	5.86	-0.034
Anisodus luridus	338	37.17	5.73	-0.02
Anisodus tanguticus	338	37.17	5.73	-0.009
Atropa belladonna	340	37.46	6.18	-0.032
Atropa belladonna	336	36.97	5.86	-0.032
Capsicum annuum	341	37.33	6.06	-0.023
Capsicum annuum	355	39.04	5.81	-0.134
Capsicum annuum	355	39.07	5.81	-0.141
Capsicum annuum	354	38.97	5.81	-0.139
Capsicum baccatum	341	37.37	6.14	-0.036
Capsicum baccatum	355	39.03	6.12	-0.148
Capsicum chinense	341	37.30	5.68	-0.018
Capsicum chinense	353	38.88	5.93	-0.111
Datura inoxia	340	37.37	5.88	-0.075
Datura inoxia	341	37.47	5.76	-0.071
Datura metel	343	37.46	5.36	-0.043
Datura stramonium	344	37.72	6.00	-0.066
Hyoscyamus niger	388	37.20	5.78	-0.051
Nicotiana attenuata	388	42.56	5.95	-0.288
Nicotiana attenuata	371	40.69	5.84	-0.223
Nicotiana attenuata	388	42.62	5.95	-0.279
Nicotiana attenuata	405	44.57	5.73	-0.119
Nicotiana benthamiana	388	42.82	6.34	-0.322
Nicotiana sylvestris	353	38.76	5.62	-0.104
Nicotiana sylvestris	430	47.17	6.20	-0.466
Nicotiana sylvestris	381	41.79	5.95	-0.244
Nicotiana sylvestris	419	45.98	6.22	-0.416
Nicotiana sylvestris	386	42.34	6.1	-0.256
Nicotiana tabacum	353	38.74	5.74	-0.083
Nicotiana tabacum	375	41.14	5.74	-0.219
Nicotiana tabacum	375	41.11	5.74	-0.226
Nicotiana tabacum	403	44.20	6.21	-0.364
Nicotiana tabacum	386	42.28	6.10	-0.266
Nicotiana tomentosiformis	364	39.87	5.69	-0.16
Nicotiana tomentosiformis	375	41.15	5.84	-0.218
Physalis divaricata	344	37.58	5.81	-0.066
Solanum dulcamara	340	37.33	6.05	-0.032
Solanum lycopersicum	340	37.56	6.06	-0.079
Solanum lycopersicum	340	37.47	6.15	-0.069
Solanum lycopersicum	340	37.51	6.15	-0.074
Solanum pennellii	340	37.48	6.60	-0.066
Solanum pennellii	342	37.71	6.15	-0.082
Solanum tuberosum	340	37.35	5.99	-0.019
Solanum tuberosum	339	37.43	6.15	-0.097
Solanum chilense *	340	37.48	6.37	-0.063
Solanum commersonii *	340	37.43	5.88	-0.094
Solanum commersonii *	333	36.76	5.93	-0.062

MW: Molecular Weight. pI: Isoelectric point. GRAVY: Grand Average of Hydrophobicity. *: Hypothetical sequence.

The GRAVY value calculation for a peptide or protein used the sum of the hydrophobicity values of all amino acids divided by the total number of residues in the sequence (Bezerra et al., 2018) which presents a negative average for the hydrophobicity index (GRAVY). The values ranged from -0.001 (*Anisodus acutangulus*) to -0.466 (*Nicotiana sylvestris*), which suggests a hydrophilic character of this protein for all species because, as defined by Kyte and Doolittle (1982), the hydrophobicity index reveals the potential of residues to interact with constituents of membrane regions or for protein stabilization through interactions between its domains.

The PMT sequences ranged in length from 336 (*Atropa belladonna*) to 430 (*Nicotiana sylvestris*) amino acids. The molecular weight (MW) ranged from 36.97 kDa (*Atropa belladonna*) to 47.17 kDa (*Nicotiana sylvestris*) (Table 2). The analysis of the subcellular localization using the CELLO2GO server predicted that the evaluated PMTs are intracellular proteins classified as cytoplasmic.

Four conserved domains were identified based on the results obtained (Figure 1). Only the PD002785 domain occurs in all analyzed sequences and associates with thermospermine synthases and spermidine synthase activity. PMTs show similarities between amino acid sequence and spermidine synthases (SPDSs), which are different from other plant methyltransferases (Junker et al., 2013). Furthermore, spermidine synthase (SPDS) is known to accept the same substrate as PMT, putrescine, despite a slightly different co-substrate, decarboxylated S-adenosyl methionine (dcSAM), which forms spermidine, which is an essential polyamine in all eukaryotic organisms (Teuber et al., 2007).

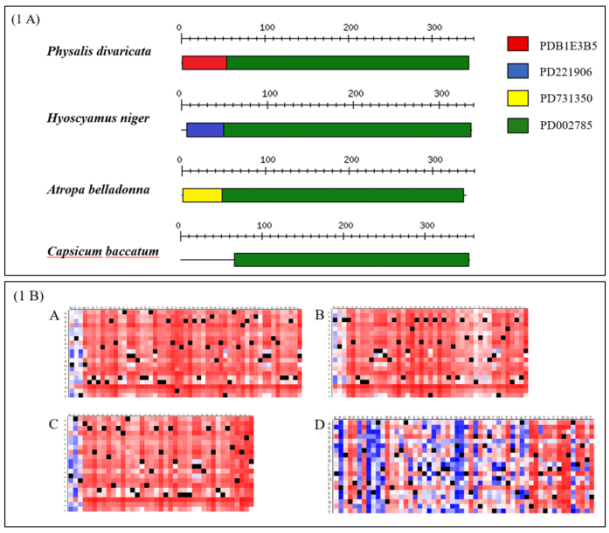

Figure 1. (1A) Functional domains observation through the ProDom server. (1B) Prediction of functional effects of amino acid mutations by SNAP2 server for domains PDB1E385 (A) *Physalis divaricata*, PD221906 (B) *Hyoscyamus niger*, PD731350 (C) *Atropa belladonna*, and PD002785 (D) *Capsicum baccatum*.

The PDB1E3B5 domain is present in 28 of the 49 sequences analyzed, transferring a methyl group to diamine putrescine from S-adenosyl-1-methionine (SAM) as a coenzyme. The PD221906 domain was found in six sequences, while the PD731350 domain is present

only in *Atropa belladonna*, in the sequence BAA82262.1, and both do not yet have a characterized function.

The SNAP2 server analyzed that the functional domains PDB1E385, PD221906, and PD731350 are the protein regions most sensitive to mutations. This server provided a map with the presence of different shades with possible substitution at each position of these proteins. The red color indicates strong mutation signals, the white color indicates weak signals, and the blue indicates neutrality. A smaller number of mutation-sensitive sites in the PD002785 domain explains its conservation and occurrence in all analyzed sequences (Figure 1).

The creation of the phenetic tree aimed to characterize the PMT sequences more efficiently and better understand the phylogenetic relationship between the different species belonging to the Solanaceae family under study through the MEGA 7 server. The species have the PD002785 domain, allowing the first group formation observation by sequences with the PDB1E385 domain present in the genera *Capsium, Datura, Nicotiana, Physalis*, and *Solanum*. This domain is involved in transferring a methyl group to the diamine putrescine from S-adenosyl-1-methionine (SAM) as a coenzyme. However, the second group formed included proteins with domains that remain uncharacterized by function.

The formation of a group with 100% support was observed, which groups the species of *Nicotiana*, belonging to the subfamily Nicotianoideae. The other genera under study comprise the subfamily Solanoideae. Such results are similar to the results obtained by Olmstead et al. (2008), who proposed a phylogeny of Solanaceae based on the ndhF and trnLF chloroplast DNA regions, comprising 89 genera and 190 species. Having the formation of the clade "x = 12" with all species joined by a base chromosome number 12, with the referred clade being one of the most strongly supported branches in the tree (99% BS), composed of two strongly supported sister groups, which would be Nicotianoideae (*Nicotiana* plus Anthocercideae 99%) and Solanoideae (95%). Subsequently, Särkinen et al. (2013), through molecular and fossil data, analyzed 40% of the total species of Solanaceae, with 34% of the sampling referring to species within the genus *Solanum*, "x = 12" is established with solid support, with Nicotianoideae resolved as sister to the clade formed by Solanoideae.

Regarding the Solanoideae subfamily, Olmstead et al. (2008) defined four clades, all with bootstrap support ≥ 78%, namely (1) Atropina (*Hyoscyameae, Lycieae, Jaborosa, Latua, Nolana*, and *Sclerophylax*), (2) Juanulloeae, (3) Solaneae, Capsiceae, Physaleae and Datureae, and (4) Salpichroina (*Salpichroa* and *Nectouxia*), these data later corroborated by Särkinen et al. (2013). Such placements are similar to the results obtained in the present study. One can observe the formation of a cluster between *Hyoscyamus, Atropa*, and *Anisodus*, which comprise the Atropina clade. Other groups formed by the genus *Solanum, Capsicum, Datura*, and *Physalis* comprise the clade, as mentioned earlier, composed of Solaneae, Capsiceae, Datureae, and Physaleae.

Figure 2 illustrates the cluster analysis of Solanaceae with the inclusion of species of the subfamily Nicotianoideae, with representatives of the tribe Nicotianeae, and of the subfamily Solanoideae, with representatives of the tribes Hyoscyameae, Solaneae, Capsiceae, Datureae, and Physaleae.

The modeling of the tertiary structure of proteins uses different methodologies, one of them being homology modeling, which uses software as tools that allow the obtaining of models through information on similarity between the sequence of the protein of interest

and deposited structures. In databases experimentally determined to extract later information on common ancestry between them (Rego, 2012). Model validation occurs through tools such as the Ramachandran chart and Z–score (Santos, 2021).

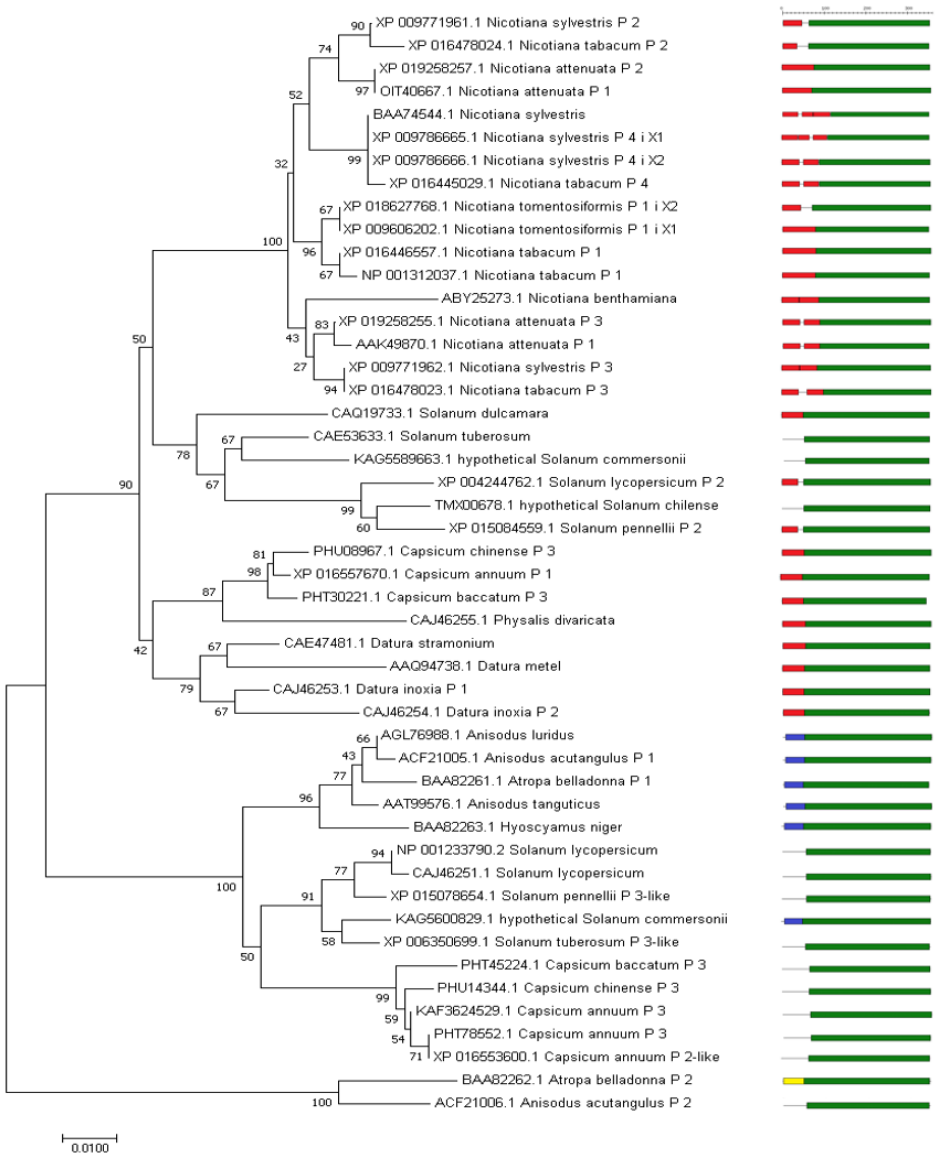

Figure 2. Cluster analysis obtained by the Neighbor-Joining method of Solanaceae species, based on putrescine N-methyltransferase sequences. The four functional domains (PD002785; PDB1E385; PD221906 and PD731350) are represented for each species.

Putrescine N-methyltransferase sequences from *Atropa belladonna*, *Capsicum baccatum*, *Hyoscyamus niger*, and *Physalis divaricata* used the Phyre2 server to predict the tertiary protein structures (Figure 3). After refinement of the 3D structures by KiNG and

Yasara Force Field software, the evaluation of models by the MOLPROBITY server for error recognition in tertiary structures. The PD221906 and PD731350 domains have one alpha-helix formation, while the PDB1E3B5 domain formed two alpha-helices. The PD002785 domain has a conserved conformation explained by the fact that it occurs in all species analyzed.

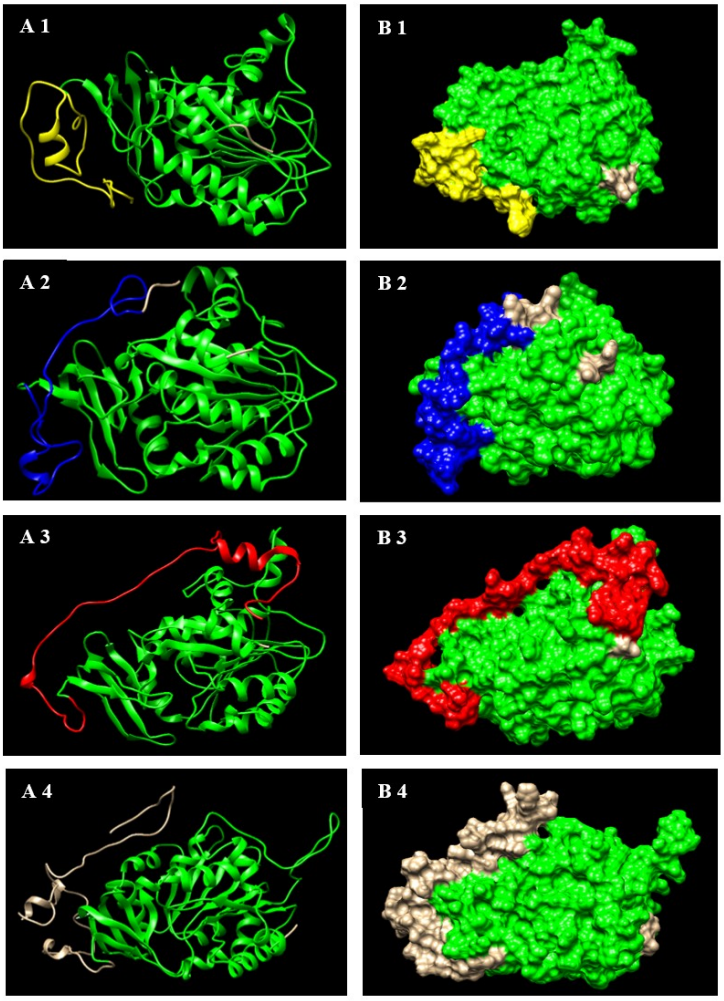

Figure 3. Three-dimensional structure of putrescine N-methyltransferase generated by Phyre2 server for *Atropa belladonna* (A1 and B1); *Hyoscyamus niger* (A2 and B2); *Physalis divaricata* (A3 and B3) and *Capsicum baccatum* (A4 and B4).

Based on the data obtained from the Prosa server, the quality of the models proved to be satisfactory, as they presented Z-score results of -8.12 for *Atropa belladonna*; -7.34 for *Capsicum baccatum*; -8.41 for *Hyoscyamus niger*; -8.05 for *Physalis divaricata*, respectively (Figure 4). According to Abreu (2015), Z-score values can be considered important from a thermodynamic and structural quality point of view. Furthermore, they can

present values very close or even higher concerning experimentally elucidated proteins, thus showing the reliability of the models.

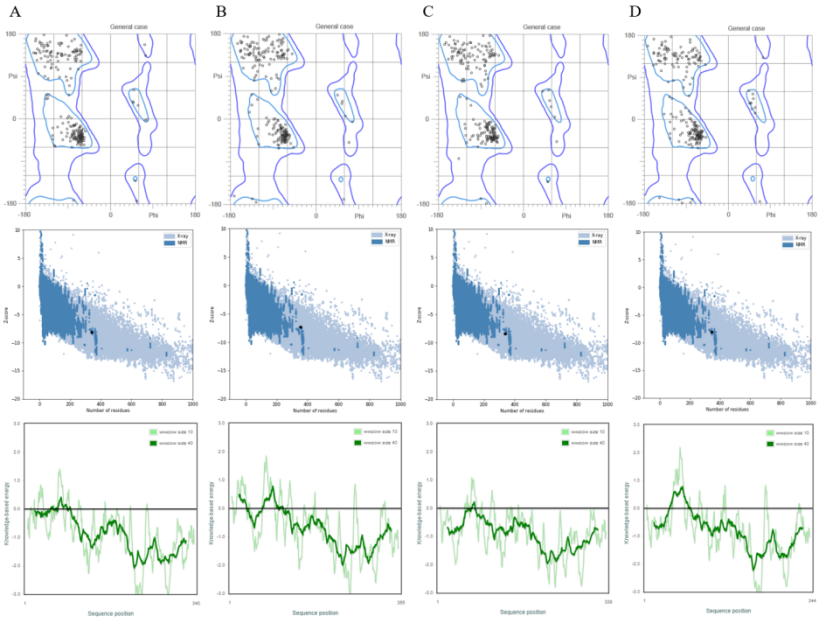

Figure 4. Analysis of the Ramachandran graph generated by the MolProbity server from the three-dimensional model built by the Phyre2 server for *Atropa belladonna* (A1); *Capsicum baccatum* (B1); *Hyoscyamus niger* (C1), and *Physalis divaricata* (D1). Z-score value by ProSA-web for *Atropa belladonna* (A2); *Capsicum baccatum* (B2); *Hyoscyamus niger* (C2) and *Physalis divaricata* (D2). Use of ProSA-web for putrescine N-methyltransferase showing residue energy scatter plot of native protein structure for *Atropa belladonna* (A3); *Capsicum baccatum* (B3); *Hyoscyamus niger* (C3) and *Physalis divaricata* (D3).

The data generated from the refined models showed 98.5, 98.9, 99.1, and 98.5% of residues in regions of propitious shape by the analysis of the Ramachandran graph for the species *Atropa belladona, Capsicum baccatum, Hyoscyamus niger*, and *Physalis divaricata*, respectively (Figure 4).

Coding sequences generate three-dimensional structures; therefore, biological effects can be studied and evaluated more precisely. Predicting three-dimensional structures of proteins usually results in practical applications of significant therapeutic and biotechnological impact (Capriles et al., 2014; Abreu, 2015). Computational methods have become increasingly used for protein prediction, bringing a more significant basis for the experimental part, aiming at a previously determined result. Homology or comparative modeling is the most used because it effectively generates good results and the quality of the predicted models, which have a reasonable evolutionary relationship and present a greater precision than those produced with different techniques (Abreu, 2015).

CONCLUSIONS

The developed study allowed a better understanding of the functions of putrescine N-methyltransferase in the regulation of alkaloid synthesis. Allied to this, the grouping

obtained through the PMT sequences corroborates the phylogeny of species currently accepted for the Solanaceae family.

The *in silico* methodology proved to be valid and reliable in the generation of 3D models, building models within the standards, which can be considered a very close representation of the protein's actual structure.

ACKNOWLEDGMENTS

This research has financial support from the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) including master's scholarship and institutional support from UFRPE.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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