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MICROBIOLOGY

Genotyping of South American clinical isolates of *Pythium insidiosum* based on single nucleotide polymorphism-based multiplex PCR

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ABSTRACT: *We aimed to genotype the South American clinical isolates of Pythium insidiosum using the single nucleotide polymorphisms (SNP) of the ribosomal DNA sequences (rDNA). Previously, an SNP-based multiplex-PCR was able to distinguish three different clades of P. insidiosum isolates. Thus, we used this assay to evaluate South American clinical isolates of P. insidiosum (n=32), standard strains from Costa Rica (n=4), Thailand (n=3), Japan (n=1), and India (n=1), a standard strain of Pythium aphanidermatum, and Brazilian environmental isolates of Pythium torulosum, Pythium rhizo-oryzae and Pythium pachycaule voucher (n=3). It was possible to allocate each American P. insidiosum isolate to clade I, the isolates of India, Japan, and Thailand to clade II, and the Thai isolate to clade III. P. aphanidermatum, P.torulosum, P.rhizo-oryzae and P.pachycaule voucher isolates were not amplified. For the first time, a P. insidiosum isolate from Uruguay, South America, was included in molecular analyzes. By SNP-based multiplex-PCR, it was possible to perform the identification and genotyping of the South American isolates of P. insidiosum, demonstrating similar genetic characteristics of these isolates.*
Key words: *Pythium insidiosum, Pythiosis, molecular detection, genotype, single nucleotide polimorphisms.*

Genotipagem de isolados clínicos de *Pythium insidiosum* da América do Sul utilizando polimorfismos de nucleotídeo único baseado em PCR multiplex

RESUMO: *O objetivo deste estudo foi genotipar isolados clínicos de Pythium insidiosum da América do Sul utilizando polimorfismos de nucleotídeo único (SNP) de sequências de rDNA. Anteriormente, um multiplex-PCR baseado em SNP foi capaz de distinguir P. insidiosum em três diferentes clados. Dessa forma, utilizamos este método para avaliar isolados clínicos de P. insidiosum da América do Sul (n=32), cepas padrão da Costa Rica (n=4), Tailândia (n=3), Japão (n=1) e Índia (n=1), uma cepa padrão de Pythium aphanidermatum e isolados ambientais brasileiros de Pythium torulosum; Pythium rhizo-oryzae e Pythium pachycaule voucher (n=3). Os isolados analisados foram alocados aos clados: I (americanos), II (isolados da Índia, Japão e Tailândia), e III (um isolado tailandês). P. aphanidermatum, P.torulosum, P.rhizo-oryzae e P.pachycaule voucher não foram amplificados. Pela primeira vez, um isolado de P. insidiosum do Uruguai foi incluído em análises moleculares. Através da multiplex-PCR baseada em SNP, foi possível realizar a identificação e genotipagem dos isolados sul-americanos de P. insidiosum, demonstrando características genéticas semelhantes entre esses isolados.*
Palavras-chave: *Pythium insidiosum, Pitiose, detecção molecular, genótipo, polimorfismos de nucleotídeo único.*

INTRODUCTION

Pythium genus is an ecofriendly oomycete found in a varied ecosystem. Most species are saprobic or pathogens of plants, algae, fishes, insects, and mammals (ADHIKARI et al., 2013). *Pythium insidiosum* causes pythiosis, a relevant infectious disease in human and animals that is widely distributed throughout the world (GAASTRA et al., 2010). In Brazil, this oomycete is present predominantly in the swampy areas of Pantanal Mato-Grossense and Rio Grande do Sul

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State (RS) (SANTOS et al., 2014; WEIBLEN et al., 2016). Little is known about the presence of pythiosis in some countries of South America, such as Uruguay, where the first case in an equine was recently reported in Costas del Tacuarí, Departamento de Treinta y Tres (LABORATORIO REGIONAL ESTE DE DILAVE, 2012).

Due to the difficulty of diagnosing pythiosis and the high costs for laboratory identification of *P. insidiosum*, as well as the similarity to other agents, especially other oomycetes and filamentous fungi, there is a need for diagnostic tools that can identify rapidly this relevant microorganism. VILELA et al. (2015) proposed a biochemical assay for identification of oomycetes; however, this technique should still be used carefully for the evaluation of *P. insidiosum* isolates (KRAJAEJUN et al., 2018).

Molecular biology tools have been successfully employed for diagnosing pythiosis, mainly using polymerase chain reaction (PCR) targeting the *P. insidiosum* internal transcribed spacer (ITS) of the rRNA locus, i.e., the ribosomal DNA (rDNA region) that consists of 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA, internal transcribed spacer 2 (ITS2), and 28S rRNA (GROOTERS & GEE, 2002). Phylogenetic studies of *P. insidiosum* have already been used with different genetic markers to elucidate aspects related to epidemiology, pathogenesis, and hosts (SCHURKO et al., 2003a,b; KAMMARNJESADAKUL et al., 2011; AZEVEDO et al., 2012; RIBEIRO et al., 2017). The first phylogenetic analyses grouped *P. insidiosum* in three clusters: cluster I North, Central, and South America; cluster II Australia, North America, Southeast Asia, and Thailand; and cluster III North America and isolates from Thailand (SCHURKO et al., 2003a,b). However, these analyses have limitations owing to the high costs, time required for DNA sequencing, and the delay in obtaining results.

Simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers have been used for studies of diversity and relationship in different microorganisms, including *P. insidiosum* (SUPABANDHU et al., 2007; RUJIRAWAT et al., 2017). The purpose of our study was genotyping American clinical isolates of *P. insidiosum* using a fast, simple, and low-cost tool based on SNP multiplex PCR.

MATERIALS AND METHODS

Thirty-one clinical isolates of *P. insidiosum* from Brazil, one from Uruguay, standard strains from Costa Rica (n=4), Thailand (n=3), Japan (n=1), and India (n=1) were analyzed (Table1). Additionally, one standard strain of *Pythium aphanidermatum* and three environmental species of *Pythium* (*P.torulosum*, *P.rhizo-oryzae* and *P.pachycaule voucher*) were included in this research. All isolates were cultivated and submitted to total DNA extraction and amplification of rDNA region were according to AZEVEDO et al. (2012) using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-CTTCCGTCAATTCCTTTAAG-3′) (WHITE et al., 1990). The primers used for SNP multiplex PCR amplification were ITS1, R1 (5′-CCTCACATTCTGCCATCTCG-3’), R2 (5′-ATACCGCCAATAGAGGTCAT-3′), and R3 (5′-TTACCCGAAGGCGTCAAAGA-3′) (RUJIRAWAT et al., 2017). Amplifications were performed according to RUJIRAWAT et al. (2017) with modifications. Briefly, in a final volume of 25µL, the PCR reaction contained 1µM of the forward primer ITS1, 0.5µM each of the reverse primers (R1, R2, and R3), 1.5 units of Taq DNA polymerase (Invitrogen), 200µM of each deoxynucleotide, 1.5mM $MgCl_2$, 1x enzyme buffer, and 100ng of DNA sample. The amplifications were carried out in a programmable thermal cycler (PTC-100, MJ Research), with initial denaturation at 95°C for 5min, 20 cycles of denaturation at 95°C for 30s, annealing at 53°C for 30s, and extension at 72°C for 45s, and then a final extension at 72°C for 10min. A 5µL aliquot of the PCR product was submitted to electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

The PCR products of rDNA region presenting a single band with the desired length (approximately 500-800pb) were purified with PureLink PCR Purification Kit (Invitrogen), and the DNA was sequenced in an automatic sequencer (ABI-Prism 3500 Genetic Analyzer) using the primers ITS1 and ITS4 (WHITE, 1990). Phylogenetic analysis for rDNA region was conducted by Neighbor-joining (NJ) method with 10,000 bootstrap replicates. All rDNA sequences from *P. insidiosum* clinical isolates from South America and standard strains of Costa Rica, India, Japan and Thailand, as well as *P. aphanidermatum*, *P.torulosum*, *P.rhizo-oryzae* and *P.pachycaule voucher* and *Phytopythium vexans* (outgroup) were used to construct the phylogenetic tree.

RESULTS AND DISCUSSION

Based on the multiplex PCR targeting the three SNPs identified in the rDNA region, all the thirty-six South and Central American isolates of *P. insidiosum* and the five standard strains from

Thailand, India and Japan were grouped in their respective clades, as suggested by RUJIRAWAT et al. (2017) (Table 1, Figure 1). We observed that the American clinical isolates, grouped in clade I, generated amplicons of approximately 490 and 660bp when using the primers ITS1/R1 and ITS1/R2, respectively. *P. aphanidermatum*, *P.torulosum*, *P.rhizo-oryzae* and *P.pachycaule voucher* were not amplified since these isolates do not belong to any *P. insidiosum* clade.

The multiplex PCR targeting the three SNPs identified in the rDNA region was developed by RUJIRAWAT et al. (2017) and has many advantages, such as 100% of sensitivity, and specificity, rapid and cost-effective identification, and genotyping of *P. insidiosum*. As these authors evaluated only one Brazilian isolate of *P. insidiosum* in their study, we proposed to evaluate an expressive number of *P. insidiosum* clinical isolates from South America using this technique.

The molecular phylogeny obtained for the rDNA region showed *P. insidiosum* as paraphyletic in relation to other *Pythium* species. However, it was observed that South and Central American *P. insidiosum* isolates were grouped together, forming a monophyletic group. In addition, isolates from other countries formed a basal-positioning group in relation to the American isolates (Figure 2). These results were consistent with AZEVEDO et al. (2012) and RIBEIRO et al (2017) that used rDNA (ITS) and cytochrome c oxidase subunit II as molecular markers and exo-1,3-β glucanase gene in phylogenetic analyses of Brazilian *P. insidiosum* isolates, respectively. Moreover, all isolates of *P. insidiosum* from India, Japan and Thailand were grouped in different clades as proposed by SCHURKO et al. (2003 a,b) and lately supported by SUPABANDHU et al. (2008).

According to RUJIRAWAT et al. (2017) multiplex PCR targeting the three SNPs identified in the rDNA (ITS) region were able to allocate *P. insidiosum* to clade-I provided two amplicons (approximately 490 and 660bp), whereas the clades-II and –III showed only one amplicon (approximately 660 and 800bp, respectively). The same results were obtained in this study, allowing to assign each American *P. insidiosum* isolates to clade I, isolates from India, Japan, and Thailand to clade II, and one Thai isolate to clade III. In addition, *P. aphanidermatum, P.torulosum*, *P.rhizo-oryzae* and *P.pachycaule voucher* were not amplified. Thus, these results evidenced that this molecular biology methodology is specific and sensitive for identification and genotyping of *P. insidiosum*, in agreement with RUJIRAWAT et al. (2017).

The genome sequences of *P. insidiosum* recently available can be a useful genetic resource for exploring aspects related the biology and evolution

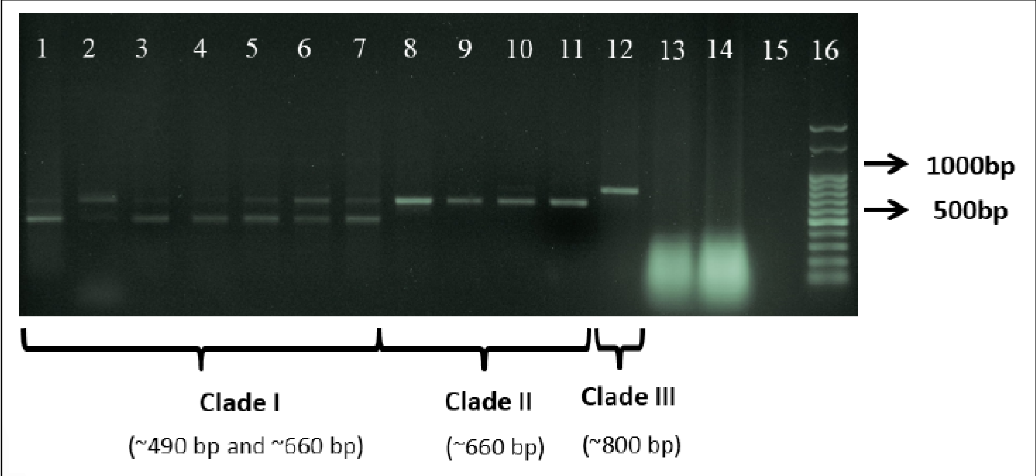

Figure 1 - Agarose gel electrophoresis of PCR amplification of the rDNA sequences of *Pythium insidiosum* from strains of each clade and controls. The amplicon size generated by SNP were: primers ITS1/R1 (~490bp) + primers ITS1/R2 (~660bp)=Clade I - 1: *P. insidiosum* 0-44, 2: *P. insidiosum* 138, 3: *P. insidiosum* 219, 4: *P. insidiosum* 247, 5: *P. insidiosum* 260, 6: *P. insidiosum* 152, 7: *P. insidiosum* 152, primers ITS1/R2 (~660bp)=Clade II - 8: *P. insidiosum* 1H, 9: *P. insidiosum* 2H, 10: *P. insidiosum* 6H, 11: *P. insidiosum* 7H, primers ITS1/R3 (~800bp) = Clade III - 12: *P. insidiosum* 8H, 13: *P. aphanydermatum*, 14: *P. tolurosum,* 15: Negative control: ddH_2o (PCR grade), 16: 100-bp DNA ladder marker.

Table 1 - Isolates of *Pythium. insidiosum* (n=36) and other species of *Pythium* (n=4) used for evaluation of the multiplex PCR assay and their information of GenBank acession number of rDNA sequence, isolate source, geographic origin and phylogenetic clade.

Isolate	GenBank[#]	Isolate source	Geographic origin	Amplicon (bp)[##]	Clade
0-44	MF767408	Equine	Uruguay	~490 and 660pb	I
118	JN126280	Equine	Jaguari[*]	~490 and 660pb	I
121	JN126282	Equine	Santa Maria[*]	~490 and 660pb	I
123	JN126283	Equine	Cachoeira do Sul[*]	~490 and 660pb	I
126	JN126286	Equine	Corumbá[*]	~490 and 660pb	I
135	MH813295	Equine	Corumbá[*]	~490 and 660pb	I
137	MH813296	Equine	Corumbá[*]	~490 and 660pb	I
138	JN126289	Equine	Corumbá[*]	~490 and 660pb	I
142	MH813297	Equine	Corumbá[*]	~490 and 660pb	I
143	JN126290	Equine	Corumbá[*]	~490 and 660pb	I
152	MH813298	Equine	Santa Maria[*]	~490 and 660pb	I
156	JN126293	Equine	Santa Maria[*]	~490 and 660pb	I
178	JN126295	Equine	Corumbá[*]	~490 and 660pb	I
187	JN126296	Equine	Jari[*]	~490 and 660pb	I
210	JN126298	Equine	Uruguaiana[*]	~490 and 660pb	I
219	JN126299	Equine	São Lourenço do Sul[*]	~490 and 660pb	I
223	JN126300	Equine	Cachoeira do Sul[*]	~490 and 660pb	I
232	JN126302	Equine	Uruguaiana[*]	~490 and 660pb	I
247	JN126304	Equine	Restinga Seca[*]	~490 and 660pb	I
252	MH813299	Equine	Uruguaiana[*]	~490 and 660pb	I
254	MH813300	Equine	Pelotas[*]	~490 and 660pb	I
259	JN126306	Equine	Santa Vitória do Palmar[*]	~490 and 660pb	I
260	JN126307	Equine	Santa Vitória do Palmar[*]	~490 and 660pb	I
268	JX675977	Canine	Canguçu[*]	~490 and 660pb	I
271	MH813301	Equine	Silveira Martins[*]	~490 and 660pb	I
290	KJ176713	Equine	Santa Maria[*]	~490 and 660pb	I
291	MH813302	Equine	Pelotas[*]	~490 and 660pb	I
293	MH813303	Equine	Rio Grande[*]	~490 and 660pb	I
295	MH813304	Canine	Pelotas[*]	~490 and 660pb	I
296	MH813305	Equine	Pelotas[*]	~490 and 660pb	I
337	MH813306	Equine	Jaguari[*]	~490 and 660pb	I
339	MH813307	Equine	Uruguaiana	~490 and 660pb	I
ATCC 58637	JN126310	Equine	Costa Rica	~490 and 660pb	I
CBS 57485	AY598637	Equine	Costa Rica	~490 and 660pb	I
3H - CBS 57685	AB898106	Equine	Costa Rica	~490 and 660pb	I
4H- CBS 57585	AB971178	Equine	Costa Rica	~490 and 660pb	I
1H-CBS 119453	EF016853	Human	Thailand	~660	II
2H-CBS 119455	EF016855	Human	Thailand	~660	II
6H- CBS 70283	AY151170	Equine	Japan	~660	II
7H- CBS 77784	AY151169	Mosquito larva	India	~660	II
8H- CBS 119454	AB971185	Human	Thailand	~800	III
P.aphanidermatum CBS 128995	JF412451	Human	Afghanistan	NA	-
P.torulosum	MH813308	Environmental	Capão do Leão[*]	NA	-
P.rhizo-oryzae	MH813309	Environmental	Santa Vitória do Palmar[*]	NA	-
P.pachycaule voucher	MH813310	Environmental	Santa Vitória do Palmar[*]	NA	-

[#]GenBank acession number corresponding to rDNA (ITS) sequences. [##]Amplicon size generated by SNP (base pair) using: Primers ITS1/R1 (~490 bp) + Primers ITS1/R2 (~660bp)=Clade I; Primers ITS1/R2 (~660bp)=Clade II; Primers ITS1/R3 (~800bp)=Clade III; and no amplification)=No *P. insidiosum* genotype. =This isolate does not belong to *P. insidiosum* clade. [*]Municipality of Brazil.

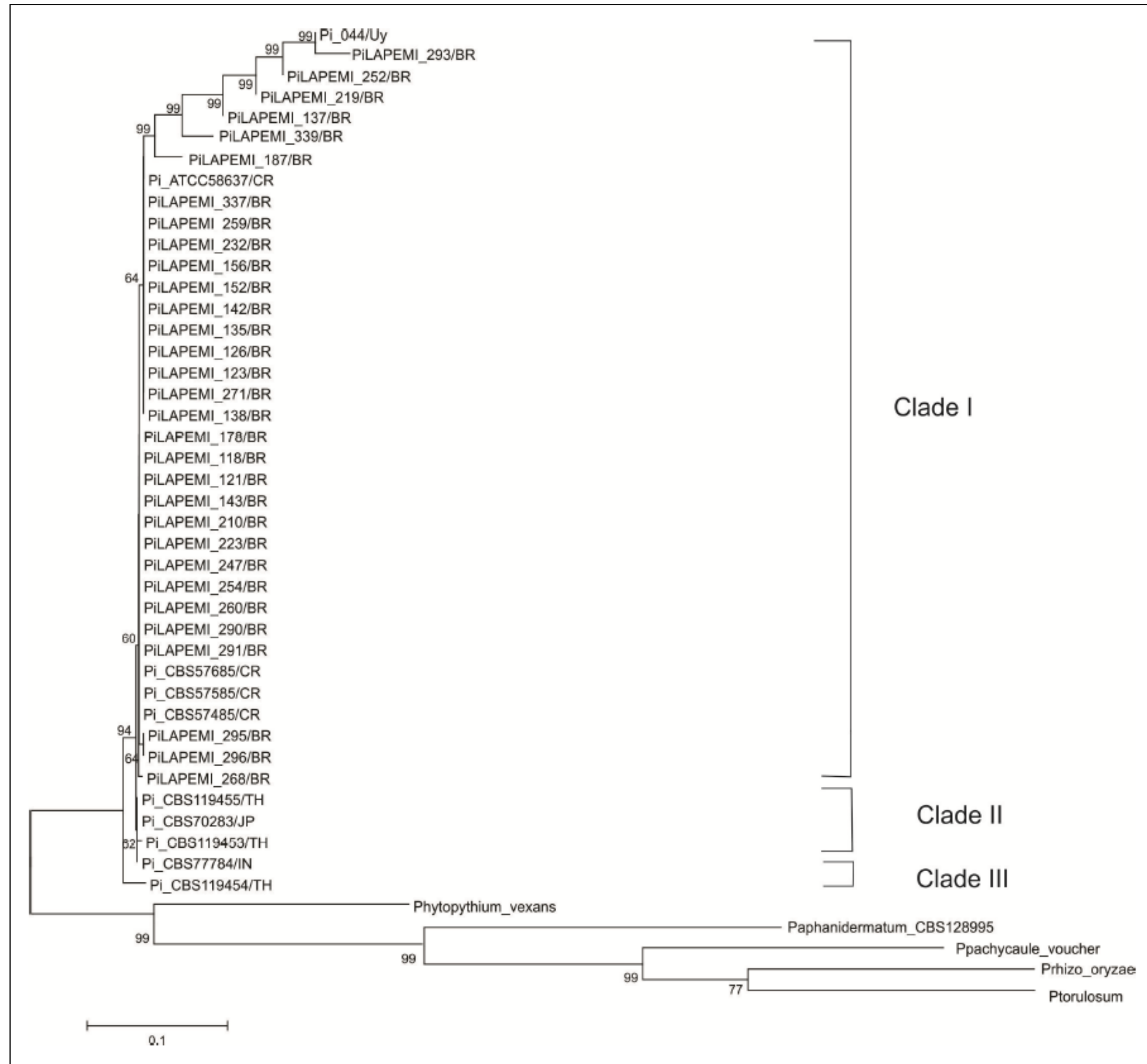

Figure 2 - Neighbor-joining tree based on sequence analysis of the rDNA ITS showing relationships among clinical isolates of P. insidiosum from South America (Brazil (BR) and Uruguay (Uy) and standard strains of Costa Rica (CR), India (IN), Japan (JP) and Thailand (TH), as well as *P. aphanidermatum* and environmental *Pythium* spp. (*P. pachycaule* voucher, *P. rhizo oryzae* and *P. torulosum* isolates). Bootstrap values expressed in percentages based on 10,000 replicates are present at their corresponding clades.

P.insidiosum and other oomycetes since independently assessed genes may not provide much information when compared to genomes. However, genome analyses are still recent, expensive and laborious when compared to the available molecular analyses (RUJIRAWAT et al., 2015; TANGPHATSORNRUANGA et al., 2016).

For the first time, a *P. insidiosum* isolate from Uruguay were included in phylogenetic analysis. A single case of equine pythiosis has been reported by the Laboratorio Regional Este de DILAVE (2012). It is of note there is still little knowledge about pythiosis in Uruguay. However, we are aware of other cases of equine pythiosis in that country (unpublished data). Additionally, MACHADO et al. (2018) suggested that *P. insidiosum* is a generalist pathogen that has the potential to move between the borders of southern Brazil, e.g., RS (the southernmost state in Brazil)

and Uruguay. *P. insidiosum* isolate from Uruguay was grouped in clade I, together with isolates from Brazil. This was evidenced by both multiplex PCR and phylogenetic analysis, proving that the South American isolates are grouped in the same clade, as previously suggested (SCHURKO et al., 2003a,b; KAMMARNJESADAKUL et al., 2011; AZEVEDO et al., 2012; RIBEIRO et al., 2017).

CONCLUSION

The SNP-based multiplex-PCR methodology has benefits (i.e., fast, simple, and low-cost) and was possible to carry out the identification and genotyping of the South American isolates of *P. insidiosum*. For the first time a *P. insidiosum* isolate from equine in Uruguay was identified and genotyped. Furthermore, the American *P. insidiosum* isolates evaluated showed similar genetic characteristics.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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