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Original Research Article

Phylogeny and Genetic Variation of *Paragonimus westermani* (Digenea: *Paragonimidae*) Using rDNA ITS2 from Manipur, India

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Abstract: Paragonimiasis, a crustacean borne trematodiasis caused by lung flukes of the genus *Paragonimus*. Among the species, *Paragonimus westermani* are an important parasite causing clinical manifestation similar to that of pulmonary tuberculosis. So, the knowledge on their phylogenetic analysis, genetic variation, conserved regions within the isolates can bring out information regarding distribution and specific genes responsible for pathogenecity, which can be utilized in control programmes. In Manipur, North East India, freshwater crab (*Barythelphusa lugubris*) has been consumed as a local delicacy by endemic people of the region. They are found harbouring with *P. westermani* metacercaria. The freshwater crab host from the susceptible loci were collected and their genomic DNA was extracted followed with amplification of genetic marker ribosomal DNA ITS2 region. The desired genetic marker were sequenced and analysed for genetic variation, conserved regions and phylogenetic tree construction using various bioinformatics tools of BLAST, ClustalW embedded in Bioedit and MEGA11. The genetic variation analysis revealed the *Paragonimus* metacercaria under studies show 100% sequence identity with the isolates from the India states of Arunachal Pradesh and Assam, with only one conserved region of 338 base lengths. The phylogenetic tree constructed reveals the sequence cladding with the isolates of *P. westermani* from different geographical regions. Among the *P. westermani* isolates, eight conserved regions were depicted, and can be used as a target for drugs in control programme.

Keywords: Metacercaria, *Paragonimus Westermani*, Crabs, rDNA ITS2, India, Manipur.

INTRODUCTION

Among the crustacean borne trematodiasis, Paragonimiasis, an important parasitic zoonosis, is a severe health problem worldwide estimated to infect more than 23 million people. It is endemic in many parts of Asia, South America, Far East, Cameroon and Nigeria of Africa (Kum and Nchinda, 1982; Singh *et al*., 1993, 2012, 2025), with Asia representing ~90% of the case till date (Strobel *et al*., 2005). There are more than 50 species of the genus Paragonimus have been reported, of which around 15 species are reported to infect different mammalian host (Miyazaki I, 1974). In India, human paragonimiasis are known to occur in the north eastern states of Manipur, Arunachal Pradesh, Assam, Meghalaya (co-endemic with tuberculosis), thus emerging as important food-borne trematodiasis disease in India (Singh *et al*., 1986; Narain *et al*., 2003; Razaque *et al*., 1991; Singh *et al*., 1993, 2004, 2012). Recent findings showed the paragonimiaisis cases are increasing in the region and are associated due to consumption of infected crab host (Devi *et al*., 2007; Singh *et al*., 2012; Singh *et al*., 2025). Among the species reported, *P. westermani* have proven to cause human paragonimiasis, confirmed with adult worm recovery from patients (Vanijanonta *et al*., 1981; Singh *et al*., 1982, 1986; Singh *et al*., 2004). This species can undergo development in a wide spectrum of snails and crab species. In Manipur, where the consumption of crustaceans host is a common practise, was recently recognized as endemic area for Paragonimiasis. In the region, the infection with the genus *Paragonimus* has been reported with the species *P. hueit'ungensis*, *P. westermani*, *P. skrjabini*; and *P. heterotremus* (Singh, 2002, Singh *et al*., 2006, 2009; Athokpam and Tandon, 2015, 2024). Human infection occur due to the consumption of raw or improperly

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cooked crabs infected with the metacercarial larval stages (Miyazaki, 1974; Procop, 2009; Sithiathaworn *et al*., 2009). From the focal region, very scanty information are available in the public domain regarding the prevalence of parasite infection in their different intermediate host.

Morphological characterization has been mainly employed for identification of the encysted and excysted metacercaria of *Paragonimus*. Characterization for some species at their larval stages only by morphological data can be difficult and results not satisfactory. So, with the advent of DNA technology, utilizing PCR amplification of rDNA internal transcribed spacers (ITS2) region which occur between the 5.8S and 28S has been widely used for phylogenetic analysis answering various taxonomic questions (Coleman, 2003; Coleman & Vacquier, 2002; Wickramasinghe *et al*., 2009; Sheng *et al*., 2012). The ITS2 rDNA evolves at a fast rate, thus utilizing for phylogenetic analysis at lower taxonomic level (Morgan and Blair, 1995; Rinaldi *et al*., 2005; Goswami *et al*., 2009; Athokpam & Tandon, 2014; Athokpam *et al*., 2016; 2024).). In addition, their genetic variation are associated with ecological interaction, host type, growth and resource requirement of the organisms and the conserve region associating with their virulence (Weider *et a*l. 2005; Li *et al*., 2016). Thus, this marker has proven to be a good candidate for characterizing *Paragonimus* spp (Blair *et al*., 1997; Park *et al*., 2003; Prasad *et al.*, 2011). Genetic variation between the isolates of *Paragonimus westermani* from different geographical regions and with other related species were analysed (Blair *et al*., 1997; Blair & Agatsuma 1997; Herwerden *et al*., 1999; Park *et al*., 2003).

The present study was carried out to utilize rDNA ITS2 of the infected metacercarial larval stage harbouring in the muscle of crab host to construct phylogenetic trees and analyse the intra- and inter-specific genetic variability of the recovered metacercaria with other isolates. Finding conserve sequences among *P. westermani* isolates will help in finding probable sites for drug target. The phylogenetic analysis shows the distribution and relationship of *P. westermani* along with its isolates.

MATERIAL AND METHODS

Parasite Material

Infective metacercariae of *P. westermani* was recovered from the freshwater crab host (*Barythelphusa lugubris masoniana*) collected from the susceptible foci in Manipur, as reported by Athokpam and Tandon, (2015), by artificial digestion technique in which the infected tissues was digested overnight at 37^0C in the in artificial gastric juice [Conc HCl (35-37%): 7-10 ml, distilled water: 1000 ml and pepsin (1:10000): 6 g] (Tandon *et al*., 2007). The metacercariae was recovered with the help of dissecting stereoscopic microscope [MOTIC SMZ-143 SERIES] and was fixed in 70% ethanol for further molecular analysis.

Isolation of Genomic DNA, PCR Amplification

From each 70% alcohol fixed metacercaria, genomic DNA was isolated using QIAamp DNA Mini Kit (50) following manufacturer's instruction (Qiagen, GmbH, Hilden, Germany) with minor modification, and was stored at -20°C until use. Genetic marker comprising rDNA ITS2 was PCR amplified from the extracted genomic DNA using universal primer rDNA ITS2 genetic markers designed based on *Schistosoma* sp. (Bowles *et al*., 1995): 3S(forward):5'-GTACCGGTGGATCACTCGGCTCGTG-3' and A28 (reverse): 5'-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3'. PCR amplification reaction was carried out following the standard protocol of White (1993) with minor modifications. The amplified PCR products were sequenced in both directions using the PCR primer sets 3S - A28 by DNA sequencing services provided by Macrogen, Seoul, Korea. The sequence is submitted to the National Center for Biotechnology information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and their accession numbers acquired.

Phylogenetic Analysis, Sequence Analysis

The sequence generated from the metacercaria was analysed by using various bioinformatics tools including BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI for similarity search; ITS2 database webserver (http://its2.bioapps.biozentrum.uni-wuerzburg.de/) to retrieve the exact ITS2 sequences for further analysis; and Bioedit software for multiple sequence alignment through embedded ClustalW; sequence identities and conserve regions between the inter and intra-species of *P. westermani*.

Phylogenetic analysis was carried out by retrieving the sequence of rDNA ITS2 from the genus *Paragonimu*s from GenBank database and their accession numbers are represented in **Table 1**. Phylogenetic tree was constructed and inter-taxonic relationships were inferred with the use of the following bioinformatics tools. A total of 33 ITS2 sequences were retrieved from the public domain with 24 isolates from neighbouring country and 9 Indian isolates. These sequences were aligned using the Clustal W multiple sequence alignment embedded in Bioedit with default setting, and the resultant aligned fasta format file was entered in MEGA 11 (Tamura *et al*., 2021) for phylogenetic trees construction with distance methods, namely, neighbor-joining. The Boostrap test has been incorporated while phylogenetic tree construction, to

find the percentage of replicates trees in which the associate taxa clustering together. Bootstrapping was done with 1000 replicates and will be shown next to the branches (Felsenstein, 1985; Hillis & Bull, 1993).

Table 1: List of various *Paragonimus* spp (Family Paragonimidae) with corresponding genetic markers ITS2 region used in analysis retrieved from the GenBank

Sl. No.	Name of species	ITS2 Genetic markers	Locality
1	*P. westermani*	JN656182.1	India: Arunachal Pradesh, East Siang"
2	*P. westermani*	PQ510326.1	China: Fujian
3	*P. westermani*	KC417492.1	China: Shanghai
4	*P. westermani*	JN656189.1	India: Assam, Sonitpur
5	*P. westermani*	PQ404924.1	Viet Nam: Quang Tri
6	*P. westermani*	DQ836243.1	India: Meghalaya
7	*P. westermani*	DQ836246.1	India: Arunachal Pradesh"
8	*P. westermani*	AB713404.1	China: Jilin"
9	*P. westermani*	LC144902.1	Viet Nam:Yen Bai
10	*P. westermani*	LC144898.1	Viet Nam:Quang Tri
11	*P. westermani*	DQ351845.1	India: north-east
12	*P. westermani*	AB354217.1	Thailand: Surat Thani
13	*P. westermani*	AB938198.1	India: Manipur
14	*P. westermani*	FJ434982.1	Viet Nam: Quangtri
15	*P. westermani*	AB354214.1	Thailand: Saraburi
16	*P. siamensis*	JQ322636.1	India: Assam
17	*P. westermani*	OQ880562.1	Japan
18	*P. westermani*	AF333277.1	South Korea
19	*P. westermani*	MN069038.1	Russia
20	*P. siamensis*	AB354222.1	Thailand
21	*P. westermani*	AF333277.1	South Korea
22	*P. skrjabini*	KX129924.1	China
23	*P. heterotremus*	DQ836248.1	India: Arunachal Pradesh"
24	*P. heterotremus*	KF781293.1	India: Manipur, Ukhrul
25	*P. bangkokensis*	OM401941.1	China: Hainan
26	*P. xiangshanensis*	OM401942.1	China: Zhejiang
27	*P. kellicotti*	HQ900670.1	USA: Missouri
28	*P. proliferus*	EU401800.1	China: Yunnan Province
29	*P. skrjabini*	AB325516.1	India: Manipur
30	*P. proliferus*	LC360503.1	Viet Nam
31	*P. proliferus*	AB663672.1	Viet Nam
32	*P. miyazakii*	AB629937.1	Japan:Wakayama
33	*P. microrchis*	HM627201.1	China: Yunnan

RESULTS

PCR Amplification of ITS Regions and Its Analysis

The rDNA ITS2 sequence generated from the *P. westermani* metacercaria was deposited in GenBank under accession numbers: KC297494. It was compared with its isolates from different geographical regions retrieved form the public domain. A total of 33 sequences for various *Paragonimus* spp. rDNA ITS2 genetic markers were used during the study (Table 1). The BLAST hit shows the query sequence highly similar to the isolates of *Paragonimus westermani.*

Sequence Analysis

i). Analysis of Pairwise Sequence Identities and Conserved Regions between the Isolates of *P. Westermani*

The analysis had shown that *P. westermani* under study showed 100% sequence similarity with the isolates of Arunachal Pradesh, Assam, respectively. They have shown one conserved region with base length of 338 showing high sequence conservation between these isolates. But the sequence identity with egg's isolate from same state and other north-eastern states of Meghalaya, Arunachal Pradesh showed sequence identity of 99.7% with two conserved regions. The sequence similarity between the isolates from the northeastern regions show in a range of 100 to 97.04%, respectively. When comparison was made with the isolates from other geographical regions especially southeast Asian countries, the sequence similarity show variation in a range of 99.7 to 97.04 %, with highest to china isolates and lowest with Thailand: Saraburi region. The isolates revealed conserved regions in the range of 1 to 9 (Table 2). The genetic variation between the isolates may be attributed to the different host receptivity and adaptation to differential habitats. The conserve sequences can be associated with pathogenecity of the infective metacercarial stage, their transmission and for their survival.

Table 2: Pairwise sequence identities and conserved regions between *P. westermani* at present study with isolates from different geographical regions with respect to ITS2 rDNA genetic markers.

***P.westermani*, isolates**	**Pairwise alignment of ITS2 of *P. westermani*, Manipur isolates with isolates from other geographical regions, (%)**	**Conserved regions**	
		Number	**Conserved Regions length**
P.westermani,India:Arunachal_Pradesh, east Shiang	100	1	338
P.westermani,India:Assam	100	1	338
P.westermani, India:Manipur(egg)	99.7	2	88; 249
P.westermani,China:Fujian	99.7	2	88; 249
P.westermani,China:Shanghai	99.7	2	88; 249
P.westermani, China:Jilin	99.7	2	88; 90
P.westermani, VietNam:YenBai	99.7	2	315; 22
P.westermani, Japan	99.7	2	88; 249
P.westermani _ichunensis,Russia	99.7	2	88; 249
P.westermani,VietNam:Quang_Tri	99.4	3	57; 257; 22
P.westermani, VietNam:Quang_Tri	99.4	3	57; 257; 22
P.westermani,South Korea:Youngam	99.4	3	88; 224; 24
P.westermani, Thailand:Surat_Thani	98.2	6	53; 34; 64; 79; 59; 41
P.westermani,India:Meghalaya	97.6	6	88; 57; 84; 45; 18; 15
P.westermani,India:Arunachal_Pradesh	97.3	7	88; 57; 65; 18; 45; 18; 15
P.westermani, India:north-east	97.04	8	88; 57; 65; 18; 25; 19; 18; 15
P.westermani, Thailand:Saraburi	97.04	9	57; 30; 57; 22; 61; 19; 25; 18; 32

ii). Analysis of Pairwise Conserved Sequences among the *Paragonimus spp*

All the *Paragonimus* spp. rDNA ITS2 sequences retrieve from the GenBank (Table 1) and with our query sequence after aligning by multiple sequence Alignment were studied for conserved regions among them. It was found that among the Genus *Paragonimus*, 8 conserved regions are shown (Fig. 1). The conserved regions being Region 1 staring from 0; with the conserve length ranging from (15 – 57) position. The maximum conserved length of 57 base long fragments present in the (90 – 146) region and the minimum length of 15 at (155 – 169) region. So, the higher conserved regions of length are 57, 53, 42 and 30, respectively. The conserved regions in all the *Paragonimus* species can thus be associated for their transmission, virulence, reproduction, overall pathogenecity.

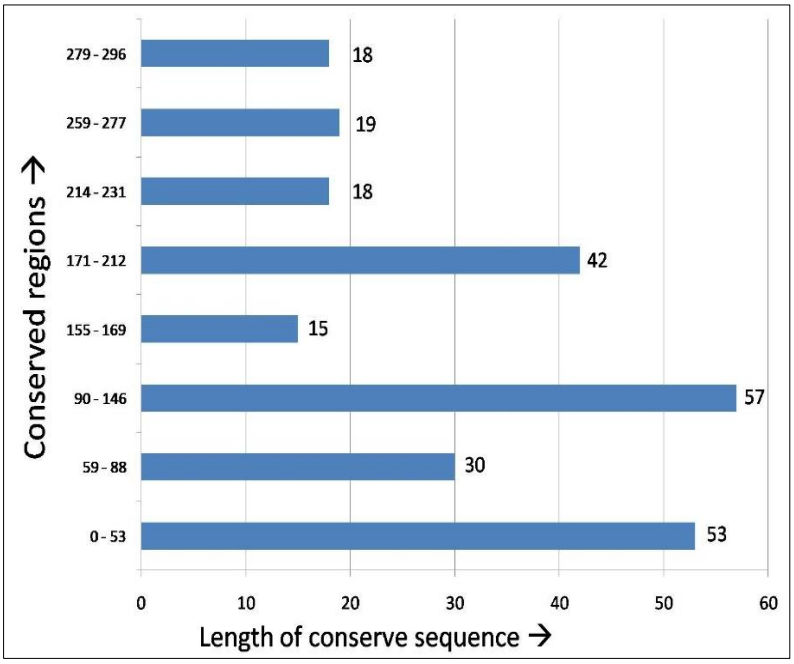

Figure 1: Conserved regions in ITS2 rDNA regions among different species of *Paragonimus* spp

Phylogenetic Study

The phylogenetic trees were constructed using ITS2 sequences of *Paragonimus* spp. from different geographical isolates. The analysis was done using NJ methods depicting the cladding of the present metacercarial form with *P. westermani* isolates from different geographical regions with a significant bootstrap values (Fig. 2). The analysis was carried out using MEGA11.

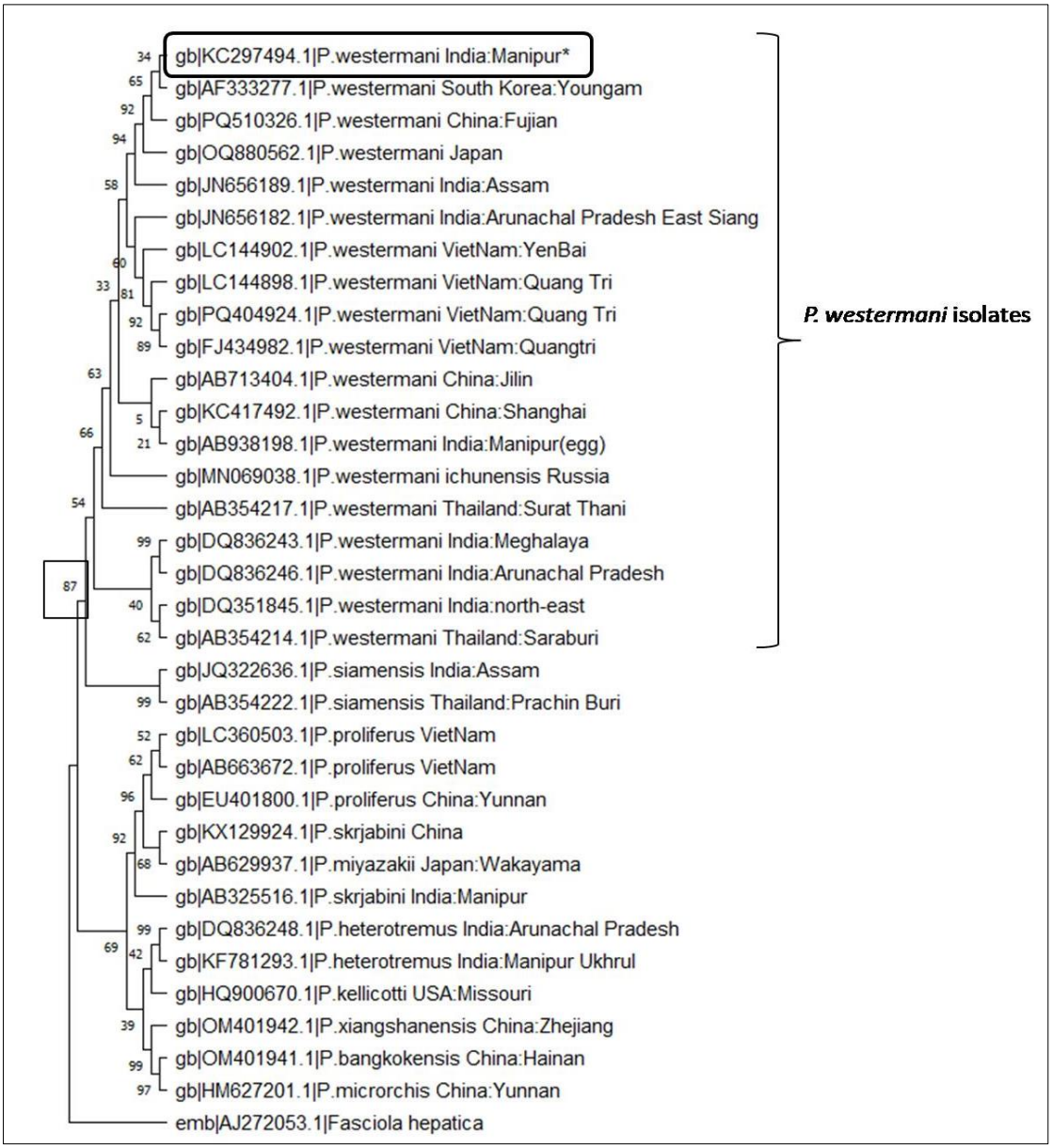

Figure 2: Phylogenetic trees from the ITS2 sequence data of various *Paragonimus* species. The analysis was inferred using Neighbor-Joining method. Bootstrap values are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

DISCUSSION

In phylogenetic analysis of the rDNA ITS2 retrieved from the public domain, the present study revealed the sequence closely claded with *P. westermani* of the family Paragonimidae with a significant boostrap value of more than 70% as generally accepted indicating an authentic phylogenetic analysis (Hillis & Bull, 1993). So, molecular technique has proven the *Paragonimus* metacercaria harbouring the muscle of *Barythelphusa lugubris* from the collected foci has accurately discriminate as *P. westermani* as reported by Athokpam & Tandon., 2015. Ribosomal DNA codes for RNA molecules and their spacers control their transcription process. Both the gene regions and their spacers show high inter- and low intra-variation (Hwang and Kim 1999). Intra-variation among *P. westermani* isolates was found sometimes higher than even inter-variation with its

related species complexes for ITS2 (Blair *et al.*, 1997; Blair & Agatsuma 1997; Herwerden *et al*., 1999), supporting the present study in which *P. westermani* isolates shows the sequence similarities in the range of 100-97.04 % or (0 – 2.96% sequence variation) but for among all *Paragonimus* spp. under study shown a range of 97.04 to 99.7% sequence similarities or (0.3 – 2.96% sequence variation); showing almost comparable inter- and intra- sequence variation. Blair *et al*., (1997) noted the existence of ITS2 genetic variation among *P. westermani* isolates, supporting the present study, and claimed that, *P. westermani* species comprised of many cryptic species.

Li *et al*., (2016) while assembling the complete transcriptomes of *P. westermani*, have annotated various genes responsible for biological pathways, metabolism, secretary protease, molecular mimicking, etc which are ultimately responsible for the pathogenesis of the genus *Paragonimus*. So, the findings of the conserved regions among the isolates of *Paragonimus* from different geographical region may attribute to any of these function responsible for pathogenesis or their virulence nature. Further studies need to be carried out focusing on finding the genes responsible for particular functions. With this, the genetic regions can be used as a probable drug target in the control programme of Paragonimiasis. *Paragonimus* spp exhibit different host specificity and other biological features, that may correlate with rDNA ITS2 intra-variation. Iwagami *et al.*, (2008) define the existence of of sequence variation among different *P. westermani* species in Southeast Asia as well as East Asia. The situation demands to analyse more of sequence similarities, intra-variation, and conserved regions existing among different isolates to help in understanding their evolutionary history and in control programme.

CONCLUSION

The present study provides the molecular characterization of genus *Paragonimus* metacercariae infecting freshwater crab host *Barytelphusa lugubris* in the region. It provides the utility of rDNA ITS2 as molecular tools in taxonomic identification, analysing intra- and inter-variation genetically between different isolates. The findings of conserve regions can be an effective tool in their control measure by especially for drug targets. More studies need to carried out on the exploration of more susceptible intermediate host, understanding their transmission pathways and its pathological effect in the host. Need to analyse more detailed on the conserved sequences with the integration of biochemical and bioinformtic techniques and tools, can enhance the control strategy of Paragonimiasis, a neglected food borne trematodiasis.

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