

## ORIGINAL ARTICLE

# Life without blood: Molecular and functional analysis of hirudins and hirudin-like factors of the Asian non-hematophagous leech *Whitmania pigra*

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**Abstract**

**Background:** Several leech species of the genera *Hirudo*, *Hirudinaria*, and *Whitmania* are widely used in traditional Chinese medicine (TCM) for the oral treatment of disorders associated with blood stasis. Among them, the non-hematophagous leech *Whitmania pigra* expresses a variety of components that have the potential to act on the vertebrate blood coagulation system.

**Objective:** Whether the thrombin inhibitor hirudin, probably the most prominent leech-derived anticoagulant, is actually present in *Whitmania pigra*, is still a matter of debate. To answer that open question was the aim of the study.

**Methods:** We identified several putative hirudin-encoding sequences in transcriptome data of *Whitmania pigra*. Upon gene synthesis and molecular cloning the respective recombinant proteins were expressed in *Escherichia coli*, purified, processed, and eventually functionally characterized for thrombin-inhibitory potencies in coagulation assays.

**Results:** We were successful in the identification and functional characterization of several putative hirudins in *Whitmania pigra*. Some, but not all, of these factors are indeed thrombin inhibitors. *Whitmania pigra* hence expresses both hirudins (factors that inhibit thrombin) and hirudin-like factors (that do not or only very weakly inhibit thrombin). Furthermore, we revealed the exon/intron structures of the corresponding genes. Coding sequences of some putative hirudins of *Whitmania pigra* were present also in transcriptome datasets of *Hirudo nipponia*, a hematophagous leech that is likewise used in TCM.

**Conclusions:** Based on both structural and functional data we provide very strong evidence for the expression of hirudins in *Whitmania pigra*. This is the first description of hirudins in a non-hematophagous leech.

**KEYWORDS**

blood coagulation, hirudin, hirudin-like factors, medicinal leeches, thrombin inhibition

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## 1 | INTRODUCTION

Leeches have been used from ancient times and in many cultures for the treatment of numerous maladies<sup>1</sup> and are part of both the modern academic<sup>2,3</sup> and alternative medicine.<sup>4</sup> The animals (named *shui zhi*) are also part of traditional Chinese medicine (TCM) and are used to treat and cure cardiovascular diseases with a focus on the promotion of blood circulation and the prevention of blood stasis.<sup>5</sup> For application in TCM, the leeches are boiled, subsequently sun dried, and finally orally administered as therapeutic.<sup>6</sup> Several leech species are subsumed under *shui zhi*, but only three of them are listed in the current *Chinese Pharmacopeia*, namely *Hirudo nipponia* (Whitman 1868), *Whitmania acranulata* (Whitman 1886), and *Whitmania pigra* (Whitman 1884).<sup>7</sup> Whereas *Hirudo nipponia* is a hematophagous leech, both *Whitmania acranulata* and *Whitmania pigra* are predators. *Whitmania pigra* feeds on tissues and carrions of invertebrates, especially snails.<sup>8</sup> For that reason, *Whitmania pigra* is used as a biological weapon for efficient pest control in agriculture<sup>9</sup> and can be easily farmed and reared in large quantities for use as *shui zhi* in TCM.<sup>10,11</sup>

Despite its predatory lifestyle, *Whitmania pigra* expresses a spectrum of bioactive molecules that target the vertebrate coagulation cascade.<sup>5,12</sup> Among them, the oligopeptide whitide has been purified and functionally characterized and turned out to be an inhibitor of the extrinsic blood coagulation pathway.<sup>13</sup> In a comparative approach, Khan et al.<sup>14</sup> reported the expression of seven putative anticoagulation factors in the salivary transcriptome of *Whitmania pigra*. Liu et al.<sup>15</sup> described the generation and comparative analysis of transcriptome data sets of three different leeches including *Whitmania pigra*, but the authors addressed ecological questions and did not search for anticoagulants in detail. Hirudin, an inhibitor of thrombin and probably the best known of all leech-derived bioactive factors,<sup>16-18</sup> remained unidentified in all transcriptome datasets.

However, Zhong et al.<sup>19</sup> described the isolation and purification of whitmanin, an anticoagulant whose biochemical data (molecular weight, isoelectric point, amino acid composition including the presence of six cysteine residues) strongly resembles those of hirudins. Further information on the amino acid and/or nucleotide sequences of whitmanin are not available. In 2019, Cheng et al.<sup>20</sup> described hirudin-HN from *Hirudo nipponia* and provided a phylogenetic analysis of various hirudins and hirudin-like factors (HLFs) including a hirudin from *Whitmania pigra* (Wpig\_hirudin). GenBank contains an entry of a partial hirudin mRNA sequence of *Whitmania pigra* (Acc. No. KX768544) that likely represents the respective Wpig\_hirudin. It should be mentioned that the biochemical data of Wpig\_hirudin (MW: 6.28 kDa; pI: 4.42) are different from whitmanin (MW: 6.71 kDa, pI: 4.38). Yao et al.<sup>21</sup> described the antithrombotic effect of an aqueous extract of *Whitmania pigra*, but denied the presence of hirudin. In a yet unpublished manuscript Tong et al.<sup>22</sup> report on a draft genome of *Whitmania pigra* including the identification of two putative hirudin-encoding genes, one complete (hirudin\_1) and the other incomplete (hirudin\_2). Hirudin\_1 is identical to Wpig\_hirudin mentioned above. The data of the draft genome are not yet available for the scientific community. In a very recent publication, Ma et al.<sup>7</sup>

### Essentials

- The expression of hirudins in non-hematophagous leeches is a matter of ongoing debate.
- Several putative hirudins were identified in transcriptome data of *Whitmania pigra*.
- Coagulation assays revealed thrombin-inhibitory potencies of some putative hirudins.
- *Whitmania pigra* has the genetic potential to express both hirudins and hirudin-like factors.

declared that hirudin cannot be isolated from *Whitmania pigra*, despite strong anticoagulant and antithrombotic activities of the saliva.

Nevertheless, it seems not implausible that *Whitmania pigra* may express hirudins or HLFs (factors that share characteristic genetic and structural properties with hirudin but do not or only very weakly inhibit thrombin) in its salivary gland cells. And if this is indeed the case, the number and, even more important, the thrombin-inhibitory potencies, of the respective factors need to be determined.

## 2 | MATERIALS AND METHODS

### 2.1 | Origin and genotyping of animals

The biological material used in this study (dried specimen of *Whitmania pigra*) was purchased from a commercial seller of *shui zhi* in May 2020 and stored at room temperature under dry and dark conditions. Species identity was confirmed by molecular genotyping. In detail, partial sequences of the internal transcribed spacer 2 (ITS2) as nuclear marker and the cytochrome c oxidase subunit I gene (*cox1*) as mitochondrial marker, respectively, were determined and compared to database entries. Cytochrome c oxidase subunit I fragments were amplified and sequenced using the universal primers LCO1490 and HCO2198.<sup>23,24</sup> ITS2 fragments were amplified and sequenced using the universal primers ITS3 and ITS4.<sup>25,26</sup> All sequences were deposited in GenBank and received the accession numbers MW659834–MW659837.

### 2.2 | Tissue preparation

Tissue pieces containing the front suckers and parts of the salivary gland segments were cut off the dried leeches. Total DNA was prepared using the innuPREP DNA Mini kit (Analytik Jena; <https://www.analytik-jena.de/kits/kits-assays-reagenzien/kits-fuer-dnarna-extraktion/innuprep-dna-mini-kit/>) according to the protocol recommended by the manufacturer. Briefly, the dried tissue samples were thoroughly cut into small pieces within the lysis buffer provided with the kit. Samples were digested with proteinase K at 55°C for 3–4 h under constant shaking. All further

steps exactly followed the recommended protocol. Total DNA was eluted using 50 µl of molecular biology grade water in the first step and 24 µl in the second step. Aliquots of the isolated DNA were analyzed on agarose gels and the isolates were stored at -20°C until further use.

### 2.3 | Amplification and cloning of hirudin and HLF genes

For the amplification of hirudin and HLF genes, primers were derived from the respective cDNA sequences that were extracted from the transcriptome database entries (GenBank Acc No SRX2135715<sup>WP</sup>, SRX2451595<sup>WP</sup>, and SRX2451596<sup>WP</sup>). A list of all primers that were used in the study is provided in Table S1 in supporting information. Total DNA of *Whitmania pigra* individuals (Wpig 1 and Wpig 2) was isolated as described above. Polymerase chain reactions to amplify the genes of hirudin and HLFs were performed using *Taq* polymerase (New England Biolabs); fragments of relevant sizes were purified, cloned, and their sequences were determined.

### 2.4 | Gene synthesis

cDNA fragments of putative *Whitmania pigra* hirudin and HLF variants were generated using the gene synthesis service of Synbio Technologies.

### 2.5 | Sequence analysis

Nucleotide and amino acid sequence alignments were generated using the CLS Sequence Viewer software package v8.0 (CLC bio) with the following parameters: gap open cost: 5.0; gap extension cost: 2.0; end gap cost: as any other. Alignments were exported as msf files and further processed using GeneDoc v2.7.<sup>27</sup>

### 2.6 | Expression, purification, processing, and quantification of putative hirudins

The entire protocol to clone cDNAs encoding putative hirudins as well as to express, purify, and quantify the respective proteins was previously described in great detail.<sup>28–30</sup> Briefly, we applied a system developed by QIAGEN. The pQE30Xa vector encodes a factor Xa protease recognition site between the His-tag coding region on the 5' side and the multiple cloning site on the 3' side. Factor Xa protease treatment cleaves off the His-tag and results in a recombinant protein that is free of any vector-derived amino acids at the N-terminus. Molar concentrations of final protein solutions were calculated by dividing the absorbance at 280 nm by the molar absorption coefficient according to the equation  $\epsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$ .<sup>31,32</sup>

## 2.7 | Blood coagulation assays

To verify the thrombin-inhibitory potency of purified hirudins and HLFs, we performed the thrombin time test (TT; reference range 16.8–21.4 s) using a BFT II analyzer (Siemens Healthcare). All steps followed the instructions outlined by the manufacturer. For the coagulation tests, all protein samples were diluted with dialysis buffer to reach final concentrations in the reaction assays of 3.2 µmol/L or 0.32 µmol/L. The desired amount of substrate was directly transferred into the cuvette immediately before the plasma was added. Dade® Ci-Trol® 1 (Siemens Healthcare) was used as standardized human plasma. The incubation of reaction mixtures was carried out at 37.4°C. Measurements that exceeded 300s were stopped and considered complete inhibition of clot formation.

## 3 | RESULTS

The main aim of the present study was to identify and functionally characterize putative hirudins in the non-hematophagous Asian leech *Whitmania pigra*, as well as to reveal the respective gene structures. All factors were tested in their ability to negatively influence thrombin activity in human plasma and hence the blood coagulation cascade. Both naturally occurring as well as genetically modified variants of the respective factors were tested. We furthermore investigated the presence of hirudin and HLF coding sequences in transcriptome datasets of the hematophagous leech *Hirudo nipponia*, a close relative of *Whitmania pigra* that is used in TCM as well. Finally, we tried to resolve uncertainties on the genetic identity of *Whitmania pigra* and *Hirudo nipponia* that arose from the analyses of the respective transcriptome datasets.

### 3.1 | Identification of hirudins and HLFs in transcriptome datasets of *Whitmania pigra*

To date there are three raw transcriptome datasets (Acc. No. SRX2135715<sup>WP</sup>,<sup>15</sup> Acc. No. SRX2451595<sup>WP</sup> and SRX2451596<sup>WP</sup>,<sup>14</sup> all datasets were generated from whole body samples) and one partial sequence of a putative hirudin (Wpig\_hirudin, Acc. No. KX768544<sup>20</sup>) of *Whitmania pigra* deposited in GenBank. We performed repetitive tBLASTn analyses of all datasets with different hirudin and HLF sequences of various origins to identify putative new hirudins and HLFs. In total, we were able to discover cDNA sequences of several yet unknown factors (Wpig\_V1 - V5) and isoforms thereof (V4a/b) as well of the Wpig\_hirudin/hirudin\_1 (Wpig\_V6 in our nomenclature). All putative hirudins or HLFs comprise typical molecular (e.g., the presence and the spacing of six conserved cysteine residues) and biochemical features of hirudins (e.g., the molecular mass of approx. 7 kDa and pI values of approximately 4). The manually assembled sequence data of all putative hirudins were deposited in GenBank and received the accession numbers BK014618–BK014631. Table 1 summarizes the biochemical features, whereas the amino acid sequences of all putative hirudins of *Whitmania pigra* are compared in Figure 1A.

**TABLE 1** Molecular properties of hirudin/hirudin-like factor variants Wpig\_V1-V6 and whitmanin of *Whitmania pigra* as well as Hnip\_V1-V5 of *Hirudo nipponia*

Factor	Length	Acidic/basic	pI value	MW
Wpig_V1	67	14/10	4.61	7.52
Wpig_V2	58	13/7	4.32	6.46
Wpig_V3	67	17/5	3.88	7.40
Wpig_V4a	57	10/7	4.69	6.35
Wpig_V4b	57	11/7	4.50	6.30
Wpig_V5	69	13/10	4.84	7.69
Wpig_V6	57	12/7	4.42	6.28
Whitmanin	63	13/7	4.38	6.71
Hnip_V1a	69	13/10	4.84	7.68
Hnip_V1b	69	13/9	4.71	7.65
Hnip_V2	57	9/7	4.85	6.29
Hnip_V3a	63	8/9	6.73	6.99
Hnip_V3b	64	11/7	4.53	6.97
Hnip_V4	48	4/9	9.08	4.98
Hnip_V5	47	3/9	8.87	4.81

Note: Length indicates the overall length of the respective factor, pI the value of its isoelectric point, and MW the value of its molecular mass. The ratio acidic/basic describes the number of acidic and basic amino acid residues within the molecule. All data refer to the predicted mature secreted factors without their respective signal peptides.

However, there are remarkable differences in the presence of the cDNA sequences of putative hirudins or HLFs among the three transcriptome datasets of *Whitmania pigra*. The datasets SRX2451595<sup>WP</sup> and SRX2451596<sup>WP14</sup> contain the sequences of all putative factors. In contrast, the dataset SRX2135715<sup>WP15</sup> contains the sequence information for Wpig\_V1-3 and V6 only, but lacks the information for Wpig\_V4 and V5 (Table 2).

### 3.2 | Identification and molecular characterization of hirudin and HLF genes in *Whitmania pigra*

To evaluate whether the cDNA sequences of hirudins or HLFs were correctly predicted, we analyzed total DNA isolated from dried individuals of *Whitmania pigra* for the presence of the corresponding genes. The genes for Wpig\_V1, V2, V3, and V6 could be successfully identified in two different specimens and their sequences were determined. All sequence data were deposited in GenBank and received the accession numbers MW659823–MW659833. Despite intensive efforts (altogether 30 specimens of *Whitmania pigra* were screened as individuals or in pools) we failed to identify the genes of Wpig\_V4 and V5. All genes comprise the typical structure of hirudin/HLF genes: four exons separated by three introns (see Figure 2).

#### (A)

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Hmed HV1: VVYTDCTETGQNL-CLCEDSNVCGQGKNCILGNSG-EKNQCVTG-E-GTPK-PQSHND--GD-FEEIP-EEYL--Q
Wpig_V1 : LHYTDCTETGQNL-CLCEGDNACVVRGNRCILGSTK-KDNKCPG-Y-GKAM-HQNKPD--EE-SEQFSYDDDD-DK
Wpig_V2 : QSFTACTRSNPNTF-CLCEGDDLCGEDKICKLGDDPSK-NECVER-E--TAK-Y-GG----F--E-F--IDYD--K
Wpig_V3 : QSFTACTRSNPNTF-CLCEGDDLCGEDKICKELGDDPSK-NECVEGISPGTQK-IDSNEE--YD-AE-R--VNYDY-D
Wpig_V4a: QRFKECSENNP-TPCLCENGLNCTSGNTCDLGP-P-K--KCIVK-Q--SS--ISENKE--SK-SD-Y--DEYD---
Wpig_V4b: QRFKECSENNP-TPCLCENGLNCTSGNTCDLGP-P-K--KCIVK-Q--PS--ISENKE--SD-SF-D--DDYD---
Wpig_V5 : NRYSVCTETGQNL-CLCEGSDLCSLDNHCEIGSNG-K-NRCVKG-E-GKPKKQNSDLSEEKYEPI SFEDYD--K
Wpig_V6 : QSFTACTRSNPNTF-CLCEGDDLCGEDKICKELGDDPSN-NECVER-E--TAK-Y-GG----F-----L--HRVD--K

```

C                    C C                    C                    C                    C

#### (B)

```

Hnip_V1a: ---NRYSVCTETGQN-LCLCEGSDLCSLDNHCEIGS-NGKNRCVKGEGKP--KKPQNSDLPEEKYEPIPIEDYD-K
Hnip_V1b: ---NRYSVCTETGQN-LCLCEGSDLCSLGNRCEIGS-NGENRCVKGEGKP--KKPQNSDLSEEKYEPI SFEDYD-K
Wpig_V5 : ---NRYSVCTETGQN-LCLCEGSDLCSLDNHCEIGS-NGKNRCVKGEGKP--KKPQNSDLSEEKYEPI SFEDYD-K
Hnip_V2 : ---QRFKECSENNP-TPCLCENGLNCTSGNTCDLGP-PKK--CIVKQPSISENKG-----SKSDYDEYD--
Wpig_V4a: ---QRFKECSENNP-TPCLCENGLNCTSGNTCDLGP-PKK--CIVKQSSISENKE-----SKSDYDEYD--
Hnip_V3a: ---QRFKDCSESNP-TPCLCENSNLCAFGNTCDLGP-PKK--CIIVKVSPPPTSEKEKNNKNG----SKSDYDY--
Hnip_V3b: ---QHFTECSESNP-TPCLCENGLNCAFGNSCDLGP-PKK--CIIVKSSSSTSEEEKNNKNG----SKSDYDEYD--
Hnip_V4 : NKAELLAACGKRV-TLCACNGVEFCGRGKCKRFGSTPAGNKCVCV-KDDSP-----
Hnip_V5 : HMSGNLIACKGDHV-TACVCEGTAICGKGGKCTILGSTVKENKCV-KNTG-----

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C                    C C                    C                    C                    C

**FIGURE 1** Multiple sequence alignment of hirudin/hirudin-like factor (HLF) variants Wpig\_V1-V6 of *Whitmania pigra*, hirudin variant HV1 of *Hirudo medicinalis* (A), and Hnip\_V1-V5 of *Hirudo nipponia* (B). The alignments were generated using the CLS Sequence Viewer software package v8.0 (CLC bio). Black background indicates conserved residues; gray background indicates similar residues. The six conserved cysteine residues giving rise to the three-dimensional structure of the archetype hirudin HV1 are marked in bold. Abbreviations are used according to the International Union of Pure and Applied Chemistry code.

TABLE 2 Presence of hirudin/hirudin-like factor coding sequences in the genome (our observations) and transcriptome data sets of *Whitmania pigra* (SRX2135715<sup>WP</sup>, SRX2451595<sup>WP</sup>, and SRX2451596<sup>WP</sup>) and *Hirudo nipponia* genotype 1 (SRX4283435<sup>HN</sup>) and genotype 2 (SRX3466461<sup>HN</sup>)

faktor	genome	SRX2135715 <sup>WP</sup>	SRX2451595 <sup>WP</sup>	SRX2451596 <sup>WP</sup>	SRX4283435 <sup>HN</sup>	SRX3466461 <sup>HN</sup>
Wpig_V1	+	+	+	-	-	-
Wpig_V2	+	+	+	+	-	-
Wpig_V3	+	+	+	+	-	-
Wpig_V4a/b	-	-	+	+	+	-
Wpig_V5	-	-	+	+	+	-
Wpig_V6	+	+	-	+	-	-
Hnip_V1a/b	-	-	+	+	+	-
Hnip_V2	-	-	+	+	+	-
Hnip_V3a/b	-	-	-	-	-	+
Hnip_V4	-	-	-	-	+	-
Hnip_V5	-	-	-	-	-	+

Note: + indicates detection, - indicates lack of detection. Boxes highlight the different distribution patterns of sequences in *Whitmania pigra* (solid line) and *Hirudo nipponia* (dotted line). The dashed line box indicates the similar distribution pattern of the respective factors in *Whitmania pigra* SRX2451595<sup>WP</sup> and SRX2451596<sup>WP</sup> only and in *Hirudo nipponia* genotype 1 (SRX4283435<sup>HN</sup>).

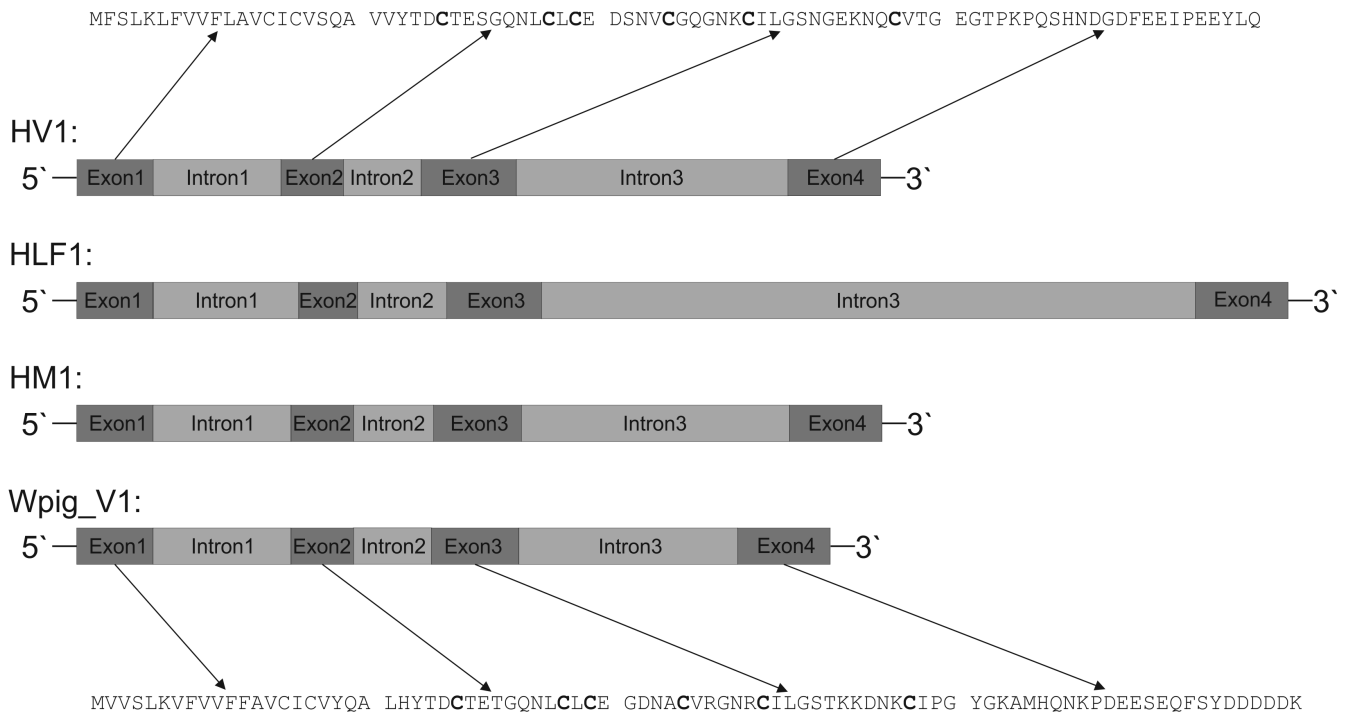


FIGURE 2 Schematic representation of hirudin and hirudin-like factor (HLF) gene structures. Exons are labeled in dark gray, and introns are labeled in light gray. Sizes are adjusted relative to the size of exon1 of hirudin HV1 of *Hirudo medicinalis*. Arrows connect the respective exon and the corresponding partial sequence of either HV1 (upper lane) or Wpig\_V1 (lower lane). HV1: hirudin variant 1 (VV) of *Hirudo medicinalis*; HLF1: hirudin-like factor of *Hirudo medicinalis*; HM1: hirudin variant 1 of *Hirudinaria manillensis*<sup>33</sup>; Wpig\_V1: hirudin variant Wpig\_V1 of *Whitmania pigra*.

Strikingly, the introns are located at the very same positions (with respect to the derived amino acid sequences) as in all other hirudin/HLF genes that were characterized so far. In addition, corresponding

exons of different genes only marginally differ in length, and the same can be observed for introns 1 and 2 (see also Table S2 in supporting information). The combined exon sequences were in every

case almost identical to the predicted cDNA sequences (100% degree of sequence identity for Wpig\_V1 and V6 and 96% for Wpig\_V2 and V3, respectively).

### 3.3 | Functional analyses of hirudin variants of *Whitmania pigra*

The molecular and biochemical properties of all putative hirudins (or HLFs) of *Whitmania pigra* (see Figure 1 and Table 1) are in good agreement with the respective properties of “true” hirudins (factors that inhibit thrombin, comprise an anti-coagulatory potency, and hence prolong the coagulation time in appropriate coagulation assays). To test whether the factors Wpig\_V1-V6 of *Whitmania pigra* are indeed hirudins, we expressed, purified, and functionally tested them. The results of the thrombin time assays are summarized in Figure 3. Whereas the factors Wpig\_V1, V4a/b, and V5 markedly prolonged the coagulation time above control level and hence clearly displayed an inhibitory effect on thrombin, the factors Wpig\_V2, V3, and V6 did not. Consequently, *Whitmania pigra* expresses both hirudins (Wpig\_V1, V4a/b, and V5) and HLFs (Wpig\_V2, V3, and V6).

### 3.4 | Identification of hirudins and HLFs in transcriptome datasets of *Hirudo nipponia*

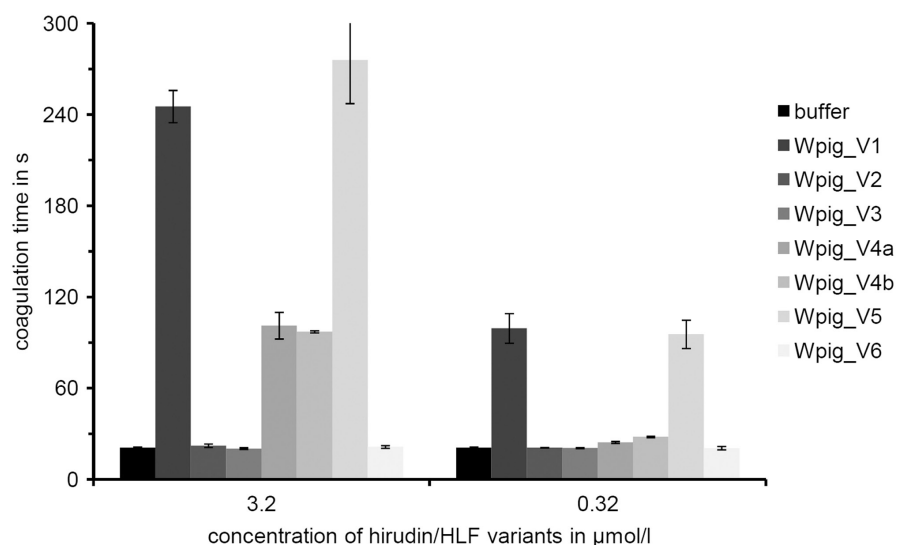
Like *Whitmania pigra*, *Hirudo nipponia* is considered *shui zhi* in TCM. But in contrast to *Whitmania pigra*, *Hirudo nipponia* is a hematophagous leech. Interestingly, hirudin-HN of *Hirudo nipponia* (GenBank Acc. No. MK947218.1<sup>20</sup>) is almost identical to the hirudin Wpig\_V5 (with a degree of 95% sequence identity), whereas a second putative hirudin that was identified in *Hirudo nipponia* (GenBank Acc. No. MN116511.1 and QIA62024, unpublished) has no direct counterpart in *Whitmania pigra*. At the moment, there are two raw transcriptome datasets of *Hirudo nipponia* deposited in GenBank: SRX4283435<sup>HN20</sup> and SRX3466461<sup>HN34</sup>; both datasets were generated from salivary gland

tissue. To evaluate whether these datasets contain cDNA sequences of additional putative hirudins or HLFs, we performed tBLASTn analyses as already described above for *Whitmania pigra*. In total, we were able to identify cDNA sequences of three yet unknown factors (Hnip\_V2, V4, and V5) and isoforms of hirudin-HN (Hnip\_V1a/b) and the second hirudin (Hnip\_V3a/b in our nomenclature). Hnip\_V2 is almost identical to Wpig\_V4a (with a degree of 96% sequence identity). In contrast, Hnip\_V3, V4, and V5 do not have a counterpart in *Whitmania pigra*. The putative hirudins Hnip\_V1-V3 comprise the typical molecular and biochemical features of hirudins, while the factors Hnip\_V4 and V5 are clearly different. Both factors have a pI value of around 9, lack the elongated C-terminal tail, and comprise an N-terminal end of eight amino acid residues in length. The manually assembled sequence data of all putative hirudins of *Hirudo nipponia* were deposited in GenBank and received the accession numbers BK014610–BK014617. The amino acid sequences are given in Figure 1B; their biochemical features are summarized in Table 1 (see section 3.1).

Interestingly, no evidence could be found for the presence of homologues of Wpig\_V1-3 and \_V6 in both transcriptome datasets of *Hirudo nipponia*. In addition, sequences encoding the almost identical factors Wpig\_V5 and Hnip\_V1a/b as well as the likewise almost identical factors Wpig\_V4a/b and Hnip\_V3 were identified in the dataset SRX4283435<sup>HN</sup> only, but not in the dataset SRX3466461<sup>HN</sup>. In contrast, the sequence of Hnip\_V3a/b was exclusive to the dataset SRX3466461<sup>HN</sup> and did not occur in the dataset SRX4283435<sup>HN</sup>. The sequence of Hnip\_V4 was present only in dataset SRX4283435<sup>HN</sup>, whereas Hnip\_V5 was present only in dataset SRX3466461<sup>HN</sup>. Table 2 (see section 3.1) summarizes the presence of all hirudin variants in the different transcriptome datasets of *Hirudo nipponia*.

### 3.5 | Genetic identity of *Whitmania pigra* and *Hirudo nipponia*

The two specimens of *Whitmania pigra* that were analyzed in the study were almost identical (655 out of 658 bp) in a partial



**FIGURE 3** Standard blood coagulation assays using the thrombin time test assay (TT) of hirudin/ hirudin-like factor (HLF) variants Wpig\_V1-V6 of *Whitmania pigra*.  $n = 3-4$ , error bars indicate standard deviation.

sequence of the mitochondrial cytochrome c oxidase subunit I gene (*cox1*), a marker that is commonly used for DNA barcoding and genotyping.<sup>35</sup> Comparison to the complete mitochondrial reference genome of *Whitmania pigra* (GenBank Acc. No. NC\_013569.1<sup>36</sup>) revealed a comparably high degree of sequence similarity of about 98.5% (649/650 out of 658 bp). The respective sequences of *Whitmania acranulata* (GenBank Acc. No. MK347500.1<sup>37</sup>) and *Whitmania laevis* (Baird 1869; GenBank Acc. No. KC688269.1<sup>38</sup>) displayed a degree of only 88 to 89% sequence similarity (Table 3). These data clearly proved that the two specimens used in our study indeed belonged to the species *Whitmania pigra*. For *Hirudo nipponia*, there are two different entries present in GenBank: KC667144.1 (genotype 1, Hnip1<sup>39</sup>) and GQ368749.1 (genotype 2, Hnip2<sup>40</sup>). Whereas the first entry revealed a degree of 95% sequence similarity, the second entry was identical in only 85% of all nucleotide positions to the respective *cox1* sequences of *Whitmania pigra* (Table 3). This observation raised the question on the genetic background of the biological samples that were used to generate the transcriptome datasets for both *Whitmania pigra* and *Hirudo nipponia* reported above.

To analyze the raw transcriptome datasets with average read lengths 100 bp or 150 bp we defined a highly variable 130 bp subsequence of the 658 bp partial *cox1* sequence (position 508–637) as a probe to differentiate among Wpig, Hnip1, and Hnip2 (see Figure 4). All available transcriptome datasets for *Whitmania pigra* and *Hirudo nipponia* were screened and the best hits are summarized in Table 4.

TABLE 3 Molecular genotyping of the *Whitmania pigra* specimen used in this study and degree of similarity to reference sequences

Species	degree <i>cox1</i>	degree ITS2
<i>Whitmania pigra</i>	98%	99%
<i>Hirudo nipponia_V1</i>	95%	99%
<i>Whitmania laevis</i>	89%	97%
<i>Hirudo nipponia_V2</i>	85%	92%
<i>Hirudo medicinalis</i>	81%	83%
<i>Hirudinaria manillensis</i>	77%	76%

Abbreviations: *cox1*, partial sequence of the cytochrome c oxidase subunit I gene; ITS2, internal transcribed spacer 2

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Wpig : TTGGTCAGTTGTTATTACTACTATTTTATTATTGTTATCATTACCAGTTT TAGCAGCAGCTATTA
Hnip1 : ATGGTCAGTAATTATTACTACTATTTCTTCTACTTTTATCATTACCTGTTTTAGCTGCAGCAATTA
Hnip2 : TTGATCTGTAATTATTACTACTATTTTACTATTATTATCATTACCAGTTCTAGCAGCAGCTATTA

Wpig : CAATATTACTTTACAGATCGAAATTTAAATACCTTTCTTTGACCCTATAGGAGGGGGGGATCCT
Hnip1 : CTATATTATTAACAGATCGAAATCTAAATACCTCTTTTGGATCCTATAGGAGGGGGGAGACCCA
Hnip2 : CAATACTTTTAACGATCGAAACCTAAATACCTATTCTTTGACCCTATAGGTGGTGGAGACCC

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FIGURE 4 Sequence alignment of a highly variable subsequence of the partial *cox1* sequence derived from the mitochondrial genomes of *Whitmania pigra* (Wpig), *Hirudo nipponia* genotype 1 (Hnip1) and genotype 2 (Hnip2). Background indicates conserved residues in all three (black) or in two (gray) sequences. Abbreviations are used according to the International Union of Pure and Applied Chemistry code.

Our data clearly indicate that SRX2135715<sup>WP15</sup> contains sequences of *Whitmania pigra* only. SRX4283435<sup>HN20</sup> contains sequences of *Hirudo nipponia* genotype 1 and SRX3466461<sup>HN34</sup> contains sequences of *Hirudo nipponia* genotype 2 only. The *Whitmania pigra* datasets SRX2451595<sup>WP</sup> and SRX2451596<sup>WP14</sup> however, contain sequences of both *Whitmania pigra* and *Hirudo nipponia* genotype 1. These results almost perfectly match to the distribution pattern of hirudin and HLF sequences reported above (see section 3.1, Table 2).

## 4 | DISCUSSION

Despite being a non-hematophagous leech, *Whitmania pigra* is traditionally used as *shui zhi* in TCM for the treatment of maladies and diseases that are associated with disturbances of blood flow or hemostasis.<sup>5</sup> Several putative anticoagulants have been described in *Whitmania pigra*, including whitmanin,<sup>19</sup> whitide,<sup>13</sup> and others.<sup>5,12,14</sup> The therapeutic potential of *Whitmania pigra* as *shui zhi* can hence already almost be fully explained by the complex pharmacological effects of the mentioned constituents that are likely present not only in the whole leech, but also in extracts from it. However, the presence of hirudin, the most effective thrombin inhibitor described so far, remained unclear. In the present study we describe the screening of three accessible transcriptome datasets of *Whitmania pigra* for the presence of coding sequences of putative hirudins and/or HLFs. We identified and subsequently functionally characterized six variants of putative hirudins/HLFs: Wpig\_V1–V6. The variant Wpig\_V6 is almost identical to hirudin\_1 in the draft genome of *Whitmania pigra*<sup>22</sup> and to Wpig\_hirudin<sup>20</sup>; GenBank Acc. No. KX768544), whereas the variant Wpig\_V3 resembles hirudin\_2 in the draft genome. The variants Wpig\_V1, V2, V4a/4b,, and V5 have not yet been described in *Whitmania pigra*.

The functional characterization of all factors revealed clear differences in terms of thrombin-inhibitory potency. Wpig\_V1 and V5 are thrombin inhibitors with inhibitory potencies that are comparable to those of HLF5 and HLF8 of *Hirudinaria (Poecilobdella) manillensis* (Lesson 1842)<sup>41</sup> and of the hirudin of *Macrobodella decora* (Say 1824),<sup>42</sup> but lower compared to that of the archetype hirudin

**TABLE 4** Degree of similarity of a species- and genotype-specific probe derived from the partial sequence of the cytochrome c oxidase subunit I gene (*cox1*) compared to best hits of BLAST searches within transcriptome datasets of *Whitmania pigra* (SRX2135715<sup>WP</sup>, SRX2451595<sup>WP</sup>, and SRX2451596<sup>WP</sup>), *Hirudo nipponia* genotype 1 (SRX4283435<sup>HN</sup>), and *Hirudo nipponia* genotype 2 (SRX3466461<sup>HN</sup>)

probe	SRX2135715 <sup>WP</sup>	SRX2451595 <sup>WP</sup>	SRX2451596 <sup>WP</sup>	SRX4283435 <sup>HN</sup>	SRX3466461 <sup>HN</sup>
Wpig	99	100	100	88	87
Hnip1	85	99	100		84
Hnip2	91	87	86	85	100

Note: The box highlights the presence of different mitochondrial *cox1* sequences in one biological sample.

variant HV1 as well as of HLF1V and HLF1long of *Hirudo medicinalis* (Linnaeus 1758).<sup>29</sup> The variant Wpig\_V4a/b has an even lower thrombin-inhibitory potency, whereas the variants Wpig\_V2, V3, and V6 are almost inactive even at the highest test concentration of 3.2 μmol/L (see Figure 3). Compared to hirudin V1 and Wpig\_V1, V4a/b, and V5, the central globular domains of Wpig\_V2, V3, and V6 contain a higher number of acidic amino acid residues and are hence more negatively charged. In previous investigations we have worked out how important the central globular domain of hirudins and HLFs is for thrombin inhibition of the respective factors.<sup>29,30</sup> It seems plausible that presence and distribution of the acidic amino acid residues in the central globular domains of Wpig\_V2, V3, and V6 are responsible for the lack of thrombin-inhibitory potency, but this hypothesis remains to be proven. It is noteworthy that none of the hirudin and HLF variants that we have identified in *Whitmania pigra* exactly matches the biochemical data of whitmanin (see Table 1). The expression of additional hirudins in *Whitmania pigra* remains possible. It is also important to emphasize that we did not prove the actual presence of hirudins and HLFs in the saliva or in body extracts of *Whitmania pigra*. However, with the knowledge on the exact molecular nature of the respective factors it may now be possible to specifically evaluate their actual presence in *Whitmania pigra*, especially in the light of all the conflicting results that we mentioned in the Introduction section.

For Wpig\_V1, V2, V3, and V6 the respective genes could be identified in genomic DNA preparations of individual specimen of *Whitmania pigra*. All genes display the typical structure of hirudin and HLF genes<sup>28,43</sup> indicating a common evolutionary origin. Despite intensive efforts we failed to identify the genes for Wpig\_V4a/b and V5. Wpig\_V5 is almost identical to hirudin-HN (GenBank Acc. No. MK947218.1), a hirudin identified in the hematophagous leech *Hirudo nipponia*.<sup>20</sup> We hence additionally screened two accessible transcriptome data sets of *Hirudo nipponia* and revealed evidence for the expression of several additional hirudins/HLFs (Hnip\_V1-V5, see Figure 1B). Of those, Hnip\_V1 is equivalent to hirudin-HN<sup>20</sup> and Wpig\_V5, whereas Hnip\_V3 is equivalent to a second hirudin from *Hirudo nipponia* (GenBank Acc. No. MN116511.1, unpublished). To our surprise, Hnip\_V2 is almost identical to Wpig\_V4a/b. Given the high degree of sequence similarity, both Hnip\_V1 and V2 are very likely thrombin inhibitors. Whether the same holds true for Hnip\_V3 remains to be proven. Hnip\_V4 and Hnip\_V5 are clearly different in structure (see Figure 1B) and net charges (pI values of about 9). Both

features strongly point against thrombin-inhibitory potencies of these particular factors.

The coding sequences for the hirudins/HLFs of *Whitmania pigra* and *Hirudo nipponia* were not equally distributed in the respective transcriptome datasets. Instead, there exists a distinct distribution pattern (see Table 2 and Table S3 in supporting information). Strikingly, a comparable pattern among the data sets can be observed for the presence of species- and genotype-specific *cox1* sequences (see Table 4). These remarkable differences raise serious concerns about the genetic homogeneity and identity of *Whitmania pigra* and *Hirudo nipponia*.

We propose the following scenario to explain our observations.

- Hirudo nipponia* genotype 2 (transcriptome data set SRX3466461<sup>HN34</sup>) represents *Hirudo nipponia* sensu stricto.
- The transcriptome data set SRX2135715<sup>WP15</sup> was derived from specimen of *Whitmania pigra* sensu stricto.
- Hirudo nipponia* genotype 1 (transcriptome data set SRX4283435<sup>HN20</sup>) does not represent *Hirudo nipponia* sensu stricto, but rather is a close relative of *Whitmania pigra* and very likely belongs to the genus *Whitmania* instead the genus *Hirudo*

Our point of view is strongly supported by both Ye et al.<sup>38</sup> and Ocegüera-Figueroa et al.<sup>44</sup> The authors state that a mitochondrial genome sequence (GenBank Acc. No. KC667144) that is claimed to be of *Hirudo nipponia* should belong to the genus *Whitmania* instead. A partial *cox1* sequence derived from KC667144 perfectly matches with SRX4283435<sup>HN</sup> (100% degree of sequence identity), but not as perfectly with SRX3466461<sup>HN</sup> (90% degree of sequence identity). The degree of identity of this sequence with the respective sequence of the mitochondrial genome of *Whitmania pigra*<sup>36</sup> is 96%.

d) The transcriptome data sets SRX2451595<sup>WP</sup> and SRX2451596<sup>WP14</sup> were derived either from a mixture of specimen representing both *Whitmania pigra* sensu stricto and *Hirudo nipponia* genotype 1 or from hybrids between these two species.

The hybrid theory is supported by the observation that the sequence of Hnip\_V4 is present in *Hirudo nipponia* genotype 1, but not in the datasets SRX2451595<sup>WP</sup> and SRX2451596<sup>WP</sup> of *Whitmania pigra* (see Table 2). Gene loss<sup>45</sup> and mitochondrial heteroplasmy (presence of different mitochondrial genomes within one individual, see Table 4)<sup>46</sup> are often observed as an outcome of hybridization. The sample mixture theory on the other hand is supported by the fact that the transcriptome data sets SRX2451595<sup>WP</sup> and



SRX2451596<sup>WP</sup> were each generated from a pool of three leech individuals.<sup>14</sup> Hence it seems not impossible that mixtures of specimens representing both *Whitmania pigra* sensu stricto and *Hirudo nipponia* genotype 1 (as already pointed out very likely a yet unrecognized *Whitmania* species) were unintentionally used as the source for RNA extraction in both cases. *Whitmania pigra* and *Hirudo nipponia* (sensu stricto, genotype 2) can be easily distinguished by their feeding strategies. It would be of great interest and value to evaluate the respective strategies of *Hirudo nipponia* genotype 1 (and of the hypothetical hybrid) as well. Such investigations would certainly help to better understand the molecular, physiological, and behavioral processes behind gain and loss of hematophagy in leeches in general.

## 5 | CONCLUSIONS

Our data and results provide very strong evidence for the presence and expression of several hirudins and hirudin-like factors in the non-hematophagous Asian leech *Whitmania pigra*. Given the high stability of hirudin molecules it is plausible to assume that active hirudin (in terms of thrombin inhibition as the pharmacological mode of action) is present also in *shu zhi*, the dried and processed bodies of leeches (including *Whitmania pigra* and *Hirudo nipponia*) that are used in TCM for the prevention and treatment of various disorders of blood circulation.

### AUTHOR CONTRIBUTIONS

CM conceived the ideas and designed the methodology; ZW prepared and analyzed genomic DNA; MH and DS purified leech factors and measured their thrombin-inhibitory potencies; CM analyzed the data and drafted the manuscript; JPH supervised the practical and theoretical works. All authors contributed to the preparation of the final manuscript and gave approval for publication.

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### CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

### ETHICAL APPROVAL

We declare that the experiments described in this paper comply with the current laws in Germany. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

### INFORMED CONSENT

All authors gave approval for publication. The work presented here did not include research on humans or with human-derived material.

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