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Genetic diversity and phylogenetic relationships among and within populations of *Whitmania pigra* and *Hirudo nipponica* based on ISSR and SRAP markers

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ABSTRACT

The objective of this study was to obtain an overview of the genetic relationships among and within *Whitmania pigra* and *Hirudo nipponica* using the ISSR and SRAP markers that were derived from related accessions, 11 *W. pigra* and 4 *H. nipponica*, from 8 provinces in China. All populations were uniquely fingerprinted by two markers. Mean genetic similarities were estimated at 0.76 and 0.79 using the ISSRs and SRAPs. Two main clusters that separated the *W. pigra* and *H. nipponica* populations were produced using UPGMA analysis. The Mantel test resulted in a good fit (SRAP = 0.80609) to the fit (ISSR = 0.76006) of the cophenetic values. Comparing the two marker systems, the ISSR and SRAP similarity indices were correlated ($r = 0.8564$). Finally, an appropriate strategy for conserving the *Hirudo* germplasm is proposed.

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1. Introduction

Hirudo live naturally in moist places on the aquatic plants or algae of many rivers and lakes. Their geographical distribution is throughout the world; however, *Whitmania pigra* and *Hirudo nipponica* are Chinese species that have commonly been known as traditional Chinese anticoagulant medicines for thousands of years, including *W. pigra* Whitman, *H. nipponica* Whitman and *Whitmania acranulata* Whitman; in clinical practice, these species have been used for promoting blood circulation and stasis relief (Chinese Pharmacopoeia Editorial Committee, 2010). As an anticoagulant medicine, they are also widely applied in the United States and Europe. It is currently believed that cardiovascular disease, a serious threat to human health (Ozono, 2006), could be cured by proprietary Chinese traditional medicines and health products using *Hirudo* as a major component. *H. nipponica* (Nikonov et al., 1999), *W. pigra* (Jin and Zhang, 2002; Shen et al., 2002; Shi et al., 2006, 2007; Guo et al., 2006; Liu et al., 2010, 2011), *Hirudinaria manillensis* (Zhang et al., 2008) and *Hirudo medicinalis* (Kasparek et al., 2000) have been studied extensively for decades; however, knowledge of their phylogenetic relationships and of the distribution and status between and within *Hirudo* is currently limited.

Molecular markers could be an appropriate tool for identifying the species. Moreover, molecular markers are effective for DNA fingerprinting, genetic diversity analyses and germplasm evaluation. Sequence-related amplified polymorphism (SRAP)

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(Li and Quiros, 2001) has been recognised as a new and useful molecular marker system in buffalograss (Budak et al., 2004) and medicinal *Chrysanthemum morifolium* (Shao et al., 2010). The genetic characteristics of *W. pigra* and *H. nipponica* are currently largely unknown.

W. pigra and *H. nipponica* are breeds of *Hirudo* that are listed in the <The Pharmacopoeia of the People's Republic of China> (Chinese Pharmacopoeia Editorial Committee, 2010). The efficacies of the two species are similar, but they belong to *Haemopidae*, *Whitmania* and *Hirudinidae*, *Hirudo*, respectively. To the best of such knowledge, no study has examined the application of ISSR or SRAP markers to the genetic diversity of the *W. pigra* and *H. nipponica* programme. The present study was conducted to understand the genetic diversity and genetic relationships of the various accessions sampled from 15 representative populations (225 individuals) using ISSR and SRAP markers. The objectives are to (1) more clearly define the genetic similarity among and within populations of *W. pigra* and *H. nipponica* in China and (2) compare different marker techniques. This study will aid in the long-term objective of identifying genetic diversity analyses among and within populations of *W. pigra* and *H. nipponica* using molecular markers.

2. Materials and methods

2.1. Animal materials

The animal materials used in this investigation were from 15 populations and represented almost all of the natural distribution areas of *Hirudo* in China. These populations can be grouped into two species, i.e., *W. pigra* and *H. nipponica*. The population from the three regions, i.e., South China (GL, GZ, LA, DL), East China (SY, JH, DF, TXC, TXW, JR, SQ, NJC, MAS, LY) and North China (HS), includes two artificial breeding varieties (NJC, TXC) and the remainder of the population contains wild species (Fig. 1 and Table 1). A total of 225 individuals from the 15 populations were included in this study. Fresh abdominal muscle from each animal was collected and immediately dried with silica gel. All samples were stored at −70 °C until processing.

2.2. DNA extraction, primers, PCR conditions, and gel electrophoresis

Total genomic DNA was extracted using the protocol established by Sambrook and Russell (2001). The quality and quantity of the DNA were determined using 0.8% agarose gel electrophoresis. DNA samples were diluted to 20 ng/ml with 1× TE buffer and stored at −20 °C prior to ISSR and SRAP analyses.

The ISSR and SRAP primers employed are listed in Table 2 [Invitrogen Biotech (Shanghai) Co., China]. For all methods, the polymerase chain reaction (PCR) mixtures and electrophoresis conditions were performed as described by Budak et al. (2004). Amplifications were performed using a thermocycler PTC 200™ Programmable Thermal Controller (Bio-Rad, USA) for one cycle of 4 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 50 °C for the ISSR analysis; and 1 min at 72 °C, followed by a final extension step at 72 °C for 5 min before cooling the reaction mixture to 10 °C. Based on the expected numbers and the sizes of the restriction fragments that were visualised on the 2.5% agarose gels stained with ethidium bromide, ten microlitres

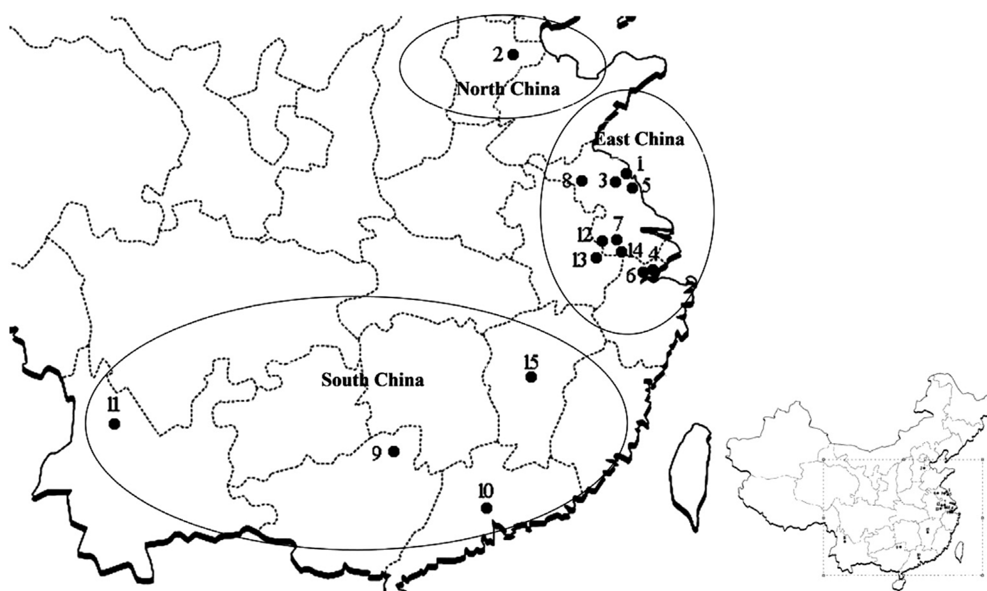

Fig. 1. The locations of populations sampled in this study were assigned to three geographical regions as described in Table 1.

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