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ORIGINAL ARTICLE

# Identifying Intraspecific Variation in Venom Yield of Chinese Cobra (*Naja atra*) from Ten Populations in Mainland China

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**Abstract** Detailed information on venom yield is helpful in preparing antivenoms and treating snakebites, but such information is lacking for many species of venomous snakes. The Chinese cobra (*Naja atra*) is a large sized, venomous snake commonly found in southeastern China, where it causes a heavy burden of snakebites. To examine the effects of various factors (morphology, sex, age, season, and geographical origin) on the venom yield in this snake, we collected venom samples of 446 individuals (426 adults and 20 neonates) from 10 populations of *N. atra* over an eight-year period. We used two variables, lyophilized venom mass (venom yield) and solid content of venom (% solids), to quantify the venom yield. We used linear regression analysis to check if venom yield was related to morphological factors, one-way ANOVA and one-way ANCOVA to detect the sexual, ontogenetic, and geographic variation in venom yield, and repeated-measures ANOVA to examine seasonal shifts in venom yield. Our results indicate that venom yield of *N. atra* is positively related to the morphological traits examined, with male snakes expelling more venom than females. Venom yield in *N. atra* was age-related, with elder snakes always expelling more venom than younger ones. Geographic variation in venom yield was also observed, while seasonal variation was not. The solid content of venom was lower in males than in females, but this was not related to morphology, season, age, or geography. Our findings suggest that venom yield in *N. atra* is influenced by multiple factors, as well as by the interactions among these factors.

**Keywords** *Naja atra*, venom yield, intraspecific variation, snakebite

## 1. Introduction

Venom is a powerful weapon for venomous snakes to subdue and digest their prey and defend themselves against predators. The venom reserve accounts for only a very small proportion of the body mass and the secretion of venom comes at a cost in terms of metabolism and time (McCue and Mason, 2006; Morgenstern and King, 2013; Pintor *et al.*, 2010). Therefore, venom is a precious resource and snakes should use it to get maximum benefit. Venom yield, the amount of venom a snake secretes from its venom gland, is affected by many factors including taxonomy, morphology, age, sex, body condition, fang separation distance, season, temperature, habitat, diet, population origin, and frequency, and number of milkings (Cascardi *et al.*, 1999; Daltry *et al.*, 1996; de Roodt *et al.*, 2016; Dissanayake *et al.*, 2015; Mackessy *et al.*, 2006; Mirtschin *et al.*, 2002). In general, venom yield influences the quantity of venom injected into prey or victims and hence leading to various degree of envenoming severity; it also influences the production of snake venom suppliers for commercial distribution. Elucidating the variation in venom yield and related factors would be helpful to understand the predation strategy of venomous snakes, as well as to establish schemes for the treatment of snakebite and for venom extraction.

There are some 60 species of venomous snakes in China, where the burden of snakebites, especially those

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caused by the Chinese cobra (*Naja atra*), is relatively heavy (Qin, 1998; Shan *et al.*, 2016). This large sized venomous snake is found in southeastern China through Vietnam and occupies a variety of habitats including grassland plains, jungle and riverbanks; it feeds on a variety of prey items, including rodents, birds, fishes, frogs, toads, lizards and other snakes (Zhao, 2006). In the past four decades, commercial antiserum raised specially against *N. atra* venom has been produced and widely used to treat *N. atra* envenomation. However, this antiserum in treating patients envenomed by *N. atra* from different regions remain unknown. Presumably due to the possible intraspecific variation in venom composition, that will lead to different clinical pathophysiology of envenomation. It has been known that venom composition and yield is the proximate cause for the diversity of venom function, symptoms and severity induced by snakebites (Gao *et al.*, 2011, 2014; Gibbs *et al.*, 2011; Oh *et al.*, 2017; Queiroz *et al.*, 2008; Reeks *et al.*, 2016; Sintiprungrat *et al.*, 2016), and previous studies of *N. atra* venom have uncovered geographical and age-related variations in its proteome and enzymatic and toxicological activities (He *et al.*, 2014; Huang *et al.*, 2015). However, virtually no attention has been paid to systematically detecting intraspecific variation in venom yield of *N. atra*. To bridge this gap, *N. atra* specimens collected from ten populations in mainland China were studied for its venom yield, and its correlation with morphology, sex, age, season, or geography.

## 2. Materials and Methods

**2.1. Animals** A total of 426 adult *N. atra* were collected from local snake catchers from ten localities (populations) between 2008 and 2015, covering almost the snake's entire range in mainland China: Baise (BS, $N = 36$; Guangxi), Chenzhou (CZ, $N = 24$; Hunan), Huaihua (HH, $N = 25$; Hunan), Ji'an (JA, $N = 13$; Jiangxi), Jianyang (JY, $N = 69$; Fujian), Lishui (LS, $N = 42$; Zhejiang), Quanzhou (QZ, $N = 162$; Guangxi), Wuzhishan (WZS, $N = 32$; Hainan), Yongzhou (YZ, $N = 14$; Hunan) and Zhoushan (ZS, $N = 9$; Zhejiang) (Figure 1). All snakes were maintained in our laboratory at Hangzhou Normal University; temperatures were controlled at 26 °C ± 2 °C and a cycle of 12 h light and 12 h dark was maintained with fluorescent tubes. Excess food [mice (*Mus musculus*), common toads (*Bufo gargarizans*), and Chinese loaches (*Paramisgumus dabryanus*)] were provided at seven-day intervals, and water enriched with vitamins and minerals was always available. Twenty eggs laid by females from the ZS population were collected in early July 2011 and incubated at 26 °C ± 2 °C; all these eggs hatched in late August. Newly hatched snakes were fed with minced Chinese loach and six months later they were fed with mice and common toads. Of these 20 snakes, two died at nine months of age, one at 12 months of age, four during the period of months 18–21, and the remaining thirteen were released to the wild in June 2013 soon after the end of the experiment. Morphological measurements taken for each snake included body mass, snout-vent length (SVL), head width and head length.

**2.2. Venom collection** After transfered from the wild to the laboratory and before venom extraction, snakes of all populations were fasted for two weeks and provided only with water. Venom of juvenile and adult snakes was extracted by letting them bite on parafilm-wrapped jars, massaging the venom glands by hand; venom of snakes at 1–6 months of age was collected using 100-μl plastic pipette micro tips according to Mirtschin *et al.* (2006); this reduces the loss of venom and allows the maximum amount of venom yield to be detected. Fresh venom was centrifuged to remove impurities for 15 min at 10 000 *g*, 4 °C, and then lyophilized. Both the lyophilized venom and fresh venom with impurities removed were weighed to assess their respective masses.

**2.3. Statistical analysis** Two variables related to the profiles of venom yield, the lyophilized venom mass (expressed as venom yield in the following) and solid content of venom (% solids, calculated from the lyophilized venom mass divided by the fresh venom mass × 100), were analyzed in this study. Statistical analyses were performed with Statistica 8.0. Linear regression was used to analyze the morphological correlates of venom yield and % solids. Joining analysis (tree clustering) was used to construct the relationships between the ten populations according to the Complete Linkage rule based on the Euclidean distance between venom yields. We used one-way ANOVA and one-way ANCOVA to examine the sexual, ontogenetic, and geographic dependence of venom yield, and repeated-measures ANOVA to examine seasonal shifts in venom yield and % solids. The significance level was set at $\alpha = 0.05$.

## 3. Results

Data pooled for all sampling localities showed that venom yield of *N. atra* ($r^2 = 0.28$, $F_{1, 424} = 165.47$, $P < 0.0001$) was positively related to SVL, and such a positive relationship was also true when data for each locality

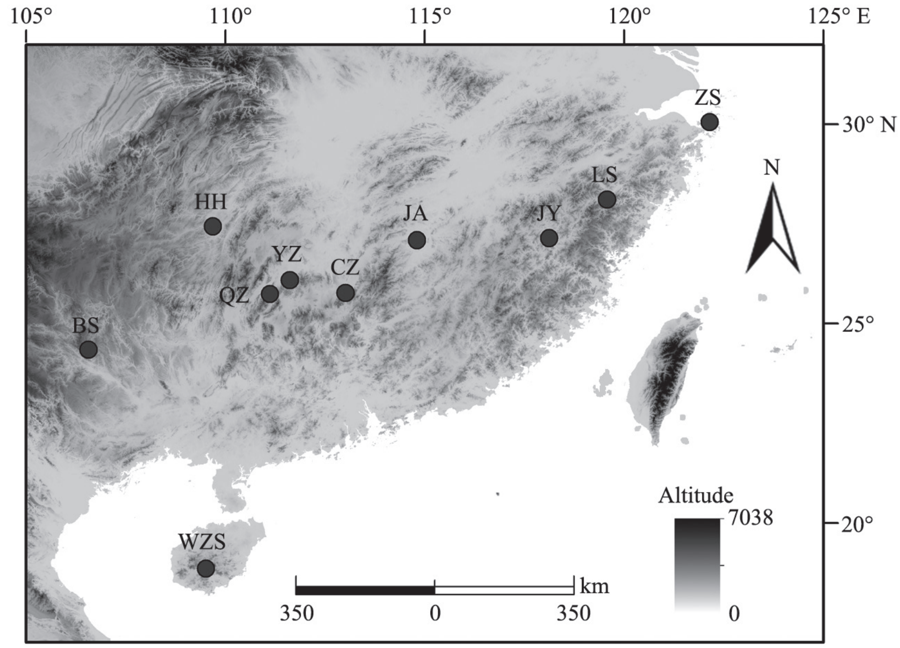

**Figure 1** Sampling localities of *Naja atra* in this study. WZS: Wuzhishan; BS: Baise; QZ: Quanzhou; CZ: Chenzhou; YZ: Yongzhou; HH: Huaihua; JA: Ji'an; JY: Jianyang; LS: Lishui; ZS: Zhoushan.

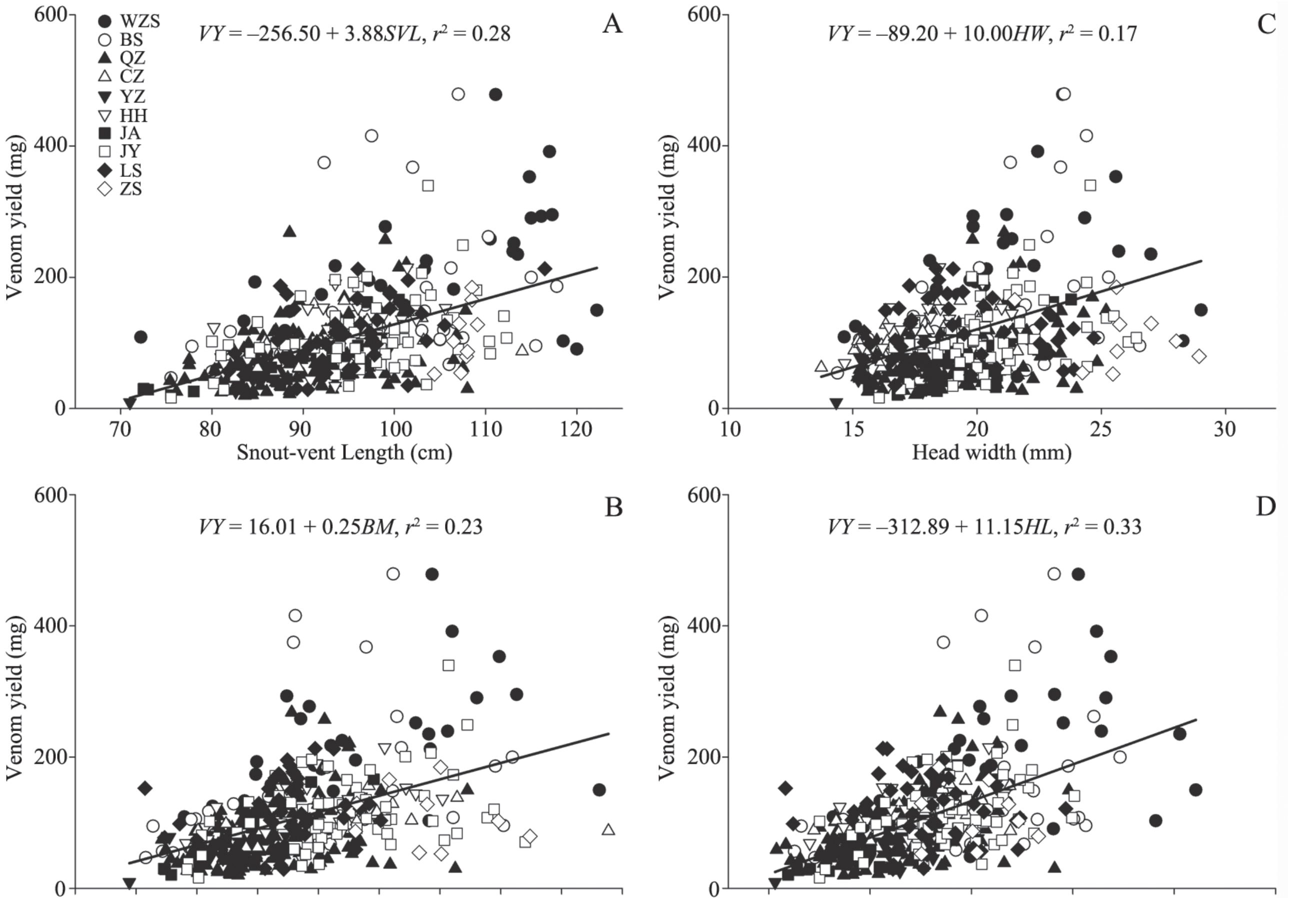

**Figure 2** Morphological dependence of venom yield in *N. atra* from different localities. See Figure 1 for definitions for WZS, BS, QZ, CZ, YZ, HH, JA, JY, LS and ZS. VY: venom yield; SVL: Snout-vent length; HW: Head width; HL: Head length; BM: Body mass.

were analyzed separately (all $P < 0.05$; Figure 2). Data pooled for all localities or analyzed separately for each locality showed that % solids was not related to SVL (all $P > 0.16$).

Venom yield also increased with the head width ($r^2 = 0.17$, $F_{1, 424} = 88.72$, $P < 0.0001$), head length ($r^2 = 0.33$, $F_{1, 424} = 205.38$, $P < 0.0001$) and body mass ($r^2 = 0.23$, $F_{1, 424} = 126.67$, $P < 0.0001$) of *N. atra*. Nevertheless, the % solids showed no relationship with the above three morphological characteristics (all $r^2 < 0.01$, all $P > 0.06$). Considering these characteristics were significantly correlated with SVL (all $r^2 > 0.44$, all $P < 0.0001$), we used relative head width, relative head length and relative body mass to analyze the relationship between venom yield and morphological characteristics. The relative head width, relative head length and relative body mass were expressed as the residual scores from the general linear regression of head width, head length and body mass, respectively, vs SVL. The results revealed that venom yield was not related to relative head width ($r^2 < 0.01$, $F_{1, 424} = 3.18$, $P = 0.08$) or relative body mass ($r^2 < 0.01$, $F_{1, 424} = 1.12$, $P = 0.29$), but did show a positive relationship with relative head length ($r^2 = 0.05$, $F_{1, 424} = 22.48$, $P < 0.0001$).

One-way ANOVA showed that male *N. atra* on average expelled a higher amount of venom than did females ($F_{1, 424} = 16.01$, $P < 0.0001$; Figure 3), but males had a lower % solids than females ($F_{1, 424} = 8.55$, $P = 0.004$; 28.7% in females and 27.3% in males). In the present study, males and females did not differ significantly in mean SVL (93.2 cm in females and 93.8 cm in males) or body mass (363.7 g in females and 359.2 g in males), but males on average had a larger head width (20.7 vs 18.6 mm; $F_{1, 424} = 64.14$, $P < 0.0001$) and length (38.8 vs 36.6 mm; $F_{1, 424} = 47.52$, $P < 0.0001$) than females. Venom yield was significantly related to these four morphological characteristics, suggesting that the potential effects of these variables should be factored out when explaining the sexual variation in venom yield. One-way ANCOVA revealed that males expelled a higher amount of venom than females of the same SVL ($F_{1, 423} = 18.86$, $P < 0.0001$) or body mass ($F_{1, 423} = 22.93$, $P < 0.0001$), but both sexes did not differ significantly in the amount of venom after accounting for head width ($F_{1, 423} = 0.94$, $P = 0.33$) or length ($F_{1, 423} = 0.06$, $P = 0.80$).

Ontogenetic shifts in venom yield were observed in *N. atra* from the ZS population ($F_{6, 102} = 36.71$, $P < 0.0001$). Venom yield increased nonlinearly with increasing age (Figure 4A and B) as well as increased exponentially with increasing body size ($r^2 = 0.78$, $F_{1, 99} = 360.6$, $P < 0.0001$) (Figure 4B). The difference in % solids of venom was slightly significant ($F_{6, 102} = 2.56$, $P = 0.02$): the percentage of solids was 33.7% in six-month-old snakes, which was relatively higher than that of one-month-old (29.6%), twelve-month-old (29.4%) and adult (28.4%) snakes.

Repeated-measures ANOVA revealed that *N. atra* from the ZS population did not show seasonal shifts in venom yield ($F_{3, 24} = 2.61$, $P = 0.07$) (Figure 5) or % solids ($F_{3, 24} = 2.78$, $P = 0.06$; spring: 26.7%, summer: 27.0%, autumn: 28.7%, winter: 27.5%).Geographical variation in venom yield was also observed in *N. atra* ($F_{9, 416} = 18.44$, $P < 0.0001$). Tukey's *post hoc* test showed that venom yield was highest in the WZS population and lowest in the QZ, JA and YZ populations, with the BS, CZ, HH, JY, LS and ZS populations in between (Figure 6A). Joining analysis revealed these populations could be divided into three clusters: cluster 1, WZS and BS; cluster 2, JY, ZS, CZ, HH and LS; and cluster 3, JA, YZ and QZ (Figure 6B). Moreover, our results indicate that the % solids does not vary geographically ($F_{9, 416} = 1.77$, $P = 0.07$). Venom yield was positively related to the morphological traits examined, and each trait varied significantly among these populations (all $P < 0.0001$). One-way ANCOVA also showed that venom yield varied among populations after accounting for SVL ($F_{9, 415} = 9.97$, $P < 0.0001$), head width ($F_{9, 415} = 17.25$, $P < 0.0001$), head length ($F_{9, 415} = 7.35$, $P < 0.0001$), or body mass ($F_{9, 415} = 13.80$, $P < 0.0001$).

## 4. Discussion

The following three methods are conventionally used to extract venom from *N. atra*. Method 1: a snake is forced to bite on the edge of a glass dish. Method 2: a snake is decapitated and then its venom glands are removed for extraction. Method 3: a snake is forced to bite on the edge of a glass dish, massaging the venom glands by hand. Venom yields from these methods were found to increase in the order 1 < 2 < 3 (Li and Zhong, 1983). Methods 1 and 3 cannot prevent the venom being contaminated by impurities from the mouth, and it is forbidden to use Method 2 due to violation of the principles of animal welfare. To extract venom from adult *N. atra*, we followed the common method where snakes were forced to bite on the parafilm-wrapped jars with the hand massaging the venom gland, and this method could minimize the potential contamination to venom and reduce the damage to the venomous tooth while comparing with the previous Methods 1 and 3. We used plastic pipette tip to collect venom from newly hatched young, and this could reduce

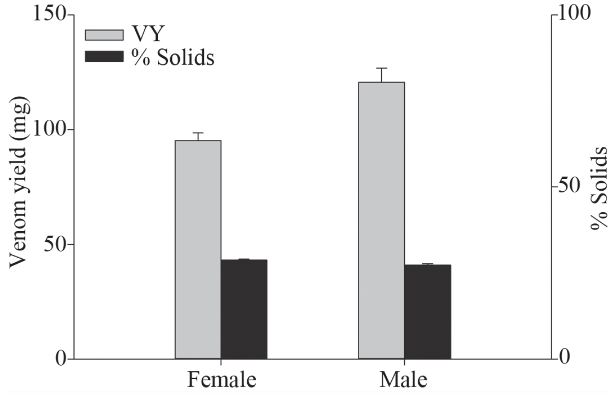

**Figure 3** Sexual differences in venom yield. Data expressed as mean + standard error (SE). VY: venom yield.

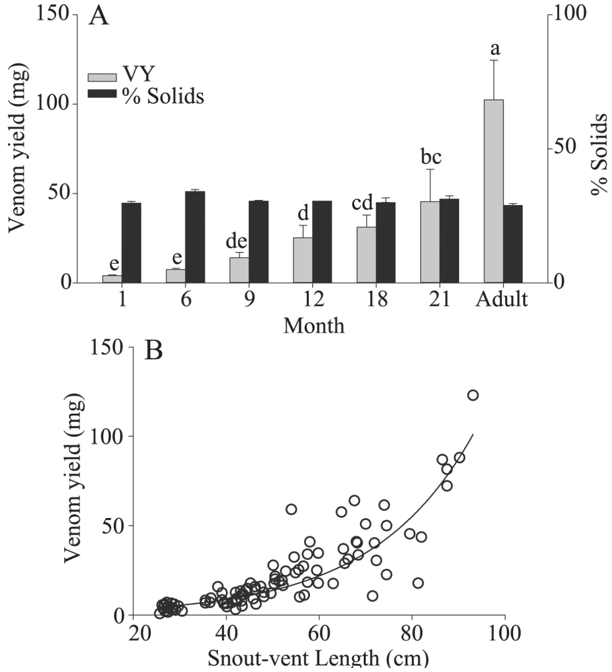

**Figure 4** Ontogenetic shifts in venom yield. Data expressed as mean + SE. Means with different letters differ significantly (Tukey's *post hoc* test, α = 0.05; a > b > c > d > e).

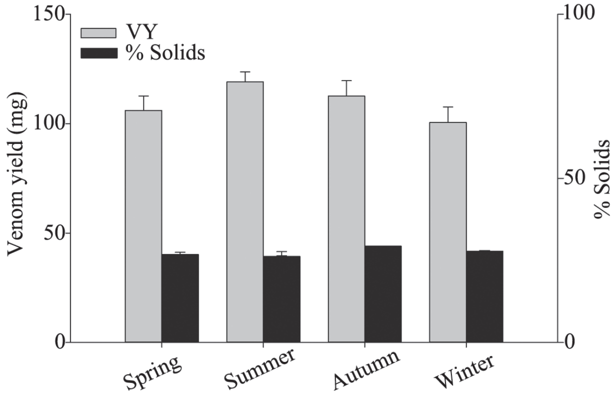

**Figure 5** Venom yields in four seasons. Data expressed as mean + SE.

the loss of venom and harvest venom yield with the maximum amount.

In most venomous snakes, body size is considered as an important source of the variation in venom yield and the venom yield shows either an allometric/exponential or an isometric/linear relationship with body size (Abdel-Aal and Abdel-Baset, 2010; de Roodt *et al.*, 2016; McCleary and Heard, 2010; Mirtschin *et al.*, 2002). It has been observed that the venom yield is even more correlated to body size than to sex or other morphological characters of *Naja naja* (Dissanayake *et al.*, 2015). Such a relationship has been found both during ontogeny (Mackessy *et al.*, 2006) and in snakes with a relatively broad range of body sizes (Gao, 2010), which consistent with the current study in *N. atra*. Our results reveal that venom yield increases with SVL in adult *N. atra*, but not in a significant allometric/exponential way (Figure 2A). Largely because of the relatively narrow range of adult SVLs (71.0–122.2 cm), thus a linear relationship between venom yield and SVL appears to be much more suitable for snakes used in this study. In addition, venom yield is influenced by body mass, with heavier snakes always expelling a greater amount of venom (de Roodt *et al.*, 1998). Removing the effect of body size (i.e. SVL in this study), we found that venom yield was not affected by relative body mass in *N. atra*. This suggests that the observed effect of body mass on venom yield is a secondary consequence of variation in body linear size. As in *Pseudonaja textilis*, venom yield is affected by relative head length instead of relative head width in *N. atra*. It might be inaccessible that a low $r^2$ value indicates significant correlation between venom yield and morphological characteristics in the present study. This discrepancy is attributed to the large sample size and high dispersion in our data (Figure 2), which a positive linear correlation can still be confirmed by the high $F$ value. The positive relationship between body size and head size (and thus, venom gland capacity) is complex (Mirtschin *et al.*, 2002), and maybe the effects of body size on different cranial structures within and between species are variable due to the different evolutionary forces on diet selection and other adaptations.

Body size is also one of the factors that can affect the severity of snakebites caused by the same snake species: larger snakes might inject more venom into their victims (Janes *et al.*, 2010). In China, victims of severe envenomation caused by *N. atra* are always taken to hospital and injected with monovalent commercial antiserum (*N. atra* snake antivenins, raised against *N. atra* venom; Shanghai Serum Biological Technology Co., Ltd.,

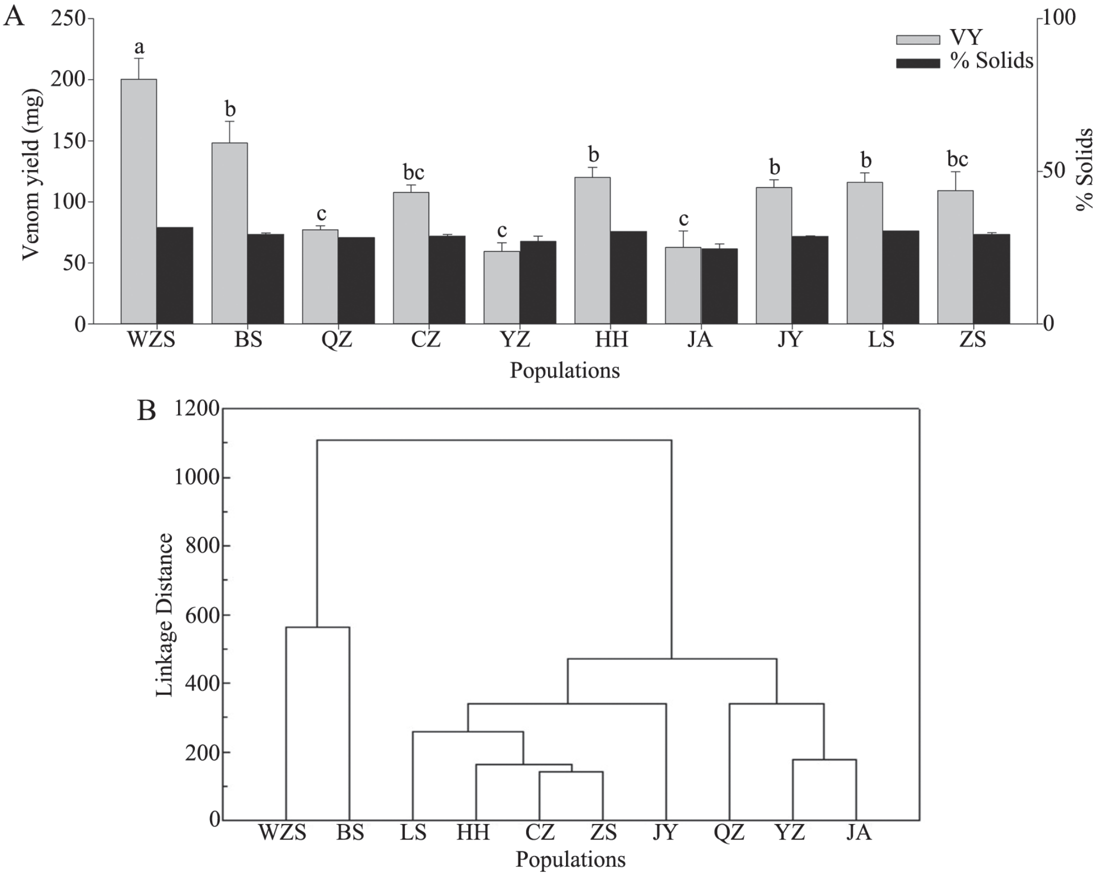

**Figure 6** Geographical variation in venom yield. Data expressed as mean + SE. Means with different letters differ significantly (Tukey's *post hoc* test, α = 0.05; a > b > c).

Shanghai, China); those with mild envenomation or bitten by juvenile *N. atra* are sometimes not taken to hospital but just treated with Chinese traditional medicines by a village doctor, especially in remote rural and mountainous areas. Actually, it would not be clinically reliable when treatment of *N. atra* envenomation is only based on the body size of the snake, because the severity of snakebites is mainly related to variability in venom composition, as well as the venom amount injected in each snakebite incidence. In some instances, smaller snakes may induce more severe snakebites than larger ones, because the venom composition, and toxicity may change during the development of some venomous snakes (Gao *et al.*, 2014; He *et al.*, 2014; Mackessy *et al.*, 2006).

Reports on the relation between sex and venom yield in snakes vary: in some, females are reported to expel a larger amount of venom than do males (de Roodt *et al.*, 1998; Furtado *et al.*, 2006), while in others females expel a smaller amount of venom than do males (Mirtschin *et al.*, 2002) or both sexes expel the same amount (Dissanayake *et al.*, 2015; Mirtschin *et al.*, 2002). The sexual variation in venom yield may just be a secondary consequence of the sexual difference in body size (Mirtschin *et al.*, 2002). However, this explanation does not apply to all venomous snakes. In *Bothrops jararaca*, for instance, sexual variation in venom yield cannot be eliminated thoroughly even if the effect of body size is removed (Furtado *et al.*, 2006). Sex-based variation in venom yield and composition may supposedly be driven by a potential divergence in diet between both sexes, especially in snakes with extreme sexual dimorphism (Furtado *et al.*, 2006; Tan *et al.*, 2017). Here, we found that male *N. atra* produce a relatively higher amount of venom than do females after accounting for SVL or body mass. It seems likely that sexual variation in venom yield of *N. atra* largely results from the sex-related difference in head size (head width and head length), and might be driven by the potential requirement of larger venom expenditure in snakes with larger head sizes, which denote they capture bigger prey.

Less attention has been paid to seasonal shifts in venom yield in snakes. The only two studies conducted so far addressing seasonal shifts in venom yield consistently show that venom yield is higher in summer and lower in winter (Mirtschin *et al.*, 2002; Xiong *et al.*, 1992). An increased venom yield in summer has been supposed to result from the higher foraging frequency of snakes during that time, forcing them to expel venom more frequently than in other seasons (Xiong *et al.*, 1992). In the present study, seasonal shifts in venom yield of *N. atra* corresponded to the absolute amount of venom expelled, but were only close to the level of statistical significance ($P$ = 0.07). Compared with snakes in previous studies, *N. atra* in our study were maintained at a relatively stable temperature and fed all year. Our snakes should therefore maintain an almost unchanged venom supply during the four seasons.

Geographical variation in venom profiles has been greatly elaborated in many studies; however, most of them focus on venom composition and only a few address venom yield (Chanhome *et al.*, 2009; Mirtschin *et al.*, 2002, 2006; Tan *et al.*, 2015, 2018; Wong *et al.*, 2018). Given that *N. atra* is widely distributed and results in a heavy burden of snakebites in China, the profiles of venom yield from different populations need to be elucidated. It has been reported that the venom yield of *N. atra* is greater in wild-caught individuals than in captive ones, and could represent the genuine profile of venom yield of snakes in the wild (Cheng, 1989); thus, we used the data on venom yield of *N. atra* newly captured from the wild for analysis in the present study. As expected, geographical variation is evident in *N. atra*, and the ten populations can be clustered into three groups in terms of venom yield (Figure 6). Similarly, our previous study showed that these populations are grouped into three clades phylogenetically: clade 1, HN (WZS in this study) and BS; clade 2, ZS, LI (LS in this study), JY and JA; clade 3, QZ, CZ, YZ and ZJ (HH in this study) (Lin *et al.*, 2014). The geographical variation in venom yield has been inferred to be independently related to genetic and environmental causes (Mirtschin *et al.*, 2002). In the present study, it seems likely that the venom yield of *N. atra* among the ten populations is correlated with the phylogeney within the same species, but not exactly. Thus, it is suggested that the genetic differences and environmental changes may play an integrative role in the geographic variation in venom yield of *N. atra*.

In China, according to the drug label, it is recommended to inject patients envenomed by *N. atra* with 2–4 vials of commercial monovalent antiserum; sometimes the real dosage injected into the patients is different and subjected to optimization due to the varying severity of snakebite upon administered to hospital. Generally, snake venoms from different populations always appear to covary in venom composition with geography, and the venom variation gives rise to regionally varying utilization strategies of antiserum in the treatment of snakebites (Sintiprungrat *et al.*, 2016; Wong *et al.*, 2016). Here, we also suppose that the venom yield effect might be a secondary source of functional variation in *N. atra* venom; thus, the venom yield profiles from different populations should be referred when treating snakebites caused by *N. atra*.

The solid content of venom is supposed to be a trade-off between the amount of dry venom expelled per unit volume and the venom viscosity: the higher the amount of dry venom, the more viscous the venom, and therefore the diffusion velocity of venom through the tissue of the bitten prey is reduced (Mirtschin *et al.*, 2002). We found that younger *N. atra* displayed a relatively higher solid content of their venom than older individuals, and thus suppose that younger snakes have to increase the amount of dry venom per unit volume to ensure a high success rate of predation, because the volume of venom secreted and expelled by these snakes was considerably lower than that by older specimens. However, further study is required to verify the evolutionary implications and the relationship with the variation in solid content of *N. atra* venom.

## 5. Conclusions

Intraspecific variation in venom yield implicates evolutionary significance; it is also medically important for the preparation of antivenoms and the treatment of snakebites, especially for the widespread venomous snakes. Our findings indicate that the venom yield of wild captured *N. atra* is morphology-, sex- and age-related and varies geographically. This can be treated as a source to refer during snakebite treatment, as well as in the production of venom, and also in understanding the evolutionary adaption in morphology-, sex-, age-, origin- and season-related variation of venom yield in *N. atra*. To elucidate the evolutionary implications of variation in the venom yield of *N. atra* more thoroughly, more causal factors of variation in venom yield should be investigated, such as body condition, diet and venom milking frequency, which is a limitation of the current study. Furthermore, it is suggested that the venom yield-related effect can serve as a secondary source for the functional variation in venom, and diversity of venom function or severity is mainly related to venom composition variability driven by multiple factors, as well as by the interactions among these factors. Thus, to understand the evolutionary implications of *N. atra* venom and snakebite management in mainland China populations comprehensively, *N. atra* venom profiles in terms of composition, pharmacokinetics and the correlation to the pathophysiology of envenomation as well as the relationship with factors that potentially have an effect (sex, age, morphology, season, etc.), should be clarified in a further investigation.

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