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Discovery of the Fuyan teeth: challenging or complementing the out-of-Africa scenario?

Yu-Chun LI, Jiao-Yang TIAN, Qing-Peng KONG

Although it is widely accepted that modern humans (*Homo sapiens sapiens*) can trace their African origins to 150-200 kilo years ago (kya) (recent African origin model; Henn et al, 2012; Ingman et al, 2000; Poznik et al, 2013; Weaver, 2012), an alternative model suggests that the diverse populations of our species evolved separately on different continents from archaic human forms (multiregional origin model; Wolpoff et al, 2000; Wu, 2006). The recent discovery of 47 teeth from a Fuyan cave in southern China (Liu et al, 2015) indicated the presence of *H. s. sapiens* in eastern Eurasia during the early Late Pleistocene. Since the age of the Fuyan teeth (80-120 kya) predates the previously assumed out-of-Africa exodus (60 kya) by at least 20 kya, this inconsistency provides some support for the multiregional origin model, and thus may challenge the recent African origin hypothesis.

If the Fuyan cave individuals were derived from archaic humans in eastern Eurasia and evolved into the contemporary modern populations that resided in the region, as suggested by the multiregional origin model, a closer morphological relationship or even successive morphological characteristics between the Fuyan teeth and *Homo erectus* from eastern Eurasia should be observed. Unfortunately, metric assessment of the teeth samples shows that Fuyan individuals differ morphologically from the Asian *H. erectus* (Liu et a, 2015), and therefore it is unlikely that they evolved from the local *H. erectus* populations (Dennell, 2015). Instead, the close affinity between Fuyan teeth and European Late Pleistocene samples and contemporary humans indicates that Fuyan man derived from common ancestors of modern humans, thus lending further support to the recent African origin model.

Nevertheless, the early Late Pleistocene occupation of Fuyan man in eastern Eurasia raises another question on how early our ancestors dispersed from Africa and successfully colonized eastern Eurasia. Based on the ages of modern human fossils in the Middle East (Skhul and Qafzeh in Israel, about 100 kya; McDermott et al, 1993; Millard, 2008; Smith et al, 2010), it is believed that the exodus from Africa started about 100 kya and reached eastern Eurasia about 74 kya before the eruption of the Toba volcano (Petraglia et al, 2007). However, genetic evidence (mainly from mitochondrial genomes) suggests that the initial settlers left Africa approximately 60 kya and then rapidly dispersed into eastern Eurasia *via* a southern coastal route about 40-60 kya (Macaulay et al, 2005; Sun et al, 2006). The lack of human fossils dating earlier than 70 kya in eastern Eurasia implies that the out-of-Africa immigrants around 100 kya likely failed to expand further east (Shea, 2008). Consistent with this notion, the Late Pleistocene hominid records previously found in eastern Eurasia have been dated to only 40-70 kya, including the Liujiang man (67 kya; Shen et al, 2002) and Tianyuan man (40 kya; Fu et al, 2013b; Shang et al, 2007) in China, the Mungo Man in Australia (40-60 kya; Bowler et al, 1972), the Niah Cave skull from Borneo (40 kya; Barker et al, 2007) and the Tam Pa Ling cave man in Laos (46-51 kya; Demeter et al, 2012). Furthermore, although the Zhiren cave man (~110 kya) in southern China was suggested to represent the earliest Late Pleistocene hominid in this region (Liu et al, 2010), later research indicated it would be more appropriately assigned to *H. erectus* (Dennell, 2010). In this regard, the evident *H. s. sapiens* morphological characteristics of the Fuyan teeth provide strong evidence supporting that the occupation of early modern humans in eastern Eurasia could be traced back to the early Late Pleistocene. [1]

One explanation for this early colonization could be that our ancestors dispersed out of Africa more than once. Hitherto, the issue on how many successful dispersals occurred from which our ancestors colonized eastern Eurasia is still contentious. Previous evidence from mitochondrial DNA (mtDNA) and archaeological studies suggests that modern humans dispersed into eastern Eurasia very rapidly *via* the coastal route only once (Kivisild et al, 2006; Macaulay et al, 2005; Mellars, 2006; Watson et al, 1997). This “single dispersal model” has been challenged by the “multiple dispersal model” proposed by whole nuclear genome study on the aboriginal man from southern Western Australia (Rasmussen et al, 2011). Since the upper boundary of the first dispersal time (75 kya; Rasmussen et al, 2011) is very close to the estimated date of Fuyan man, it is likely that Fuyan man were from the first settlers on the eastern Eurasian continent. Additionally, the gap of 20 000 years or

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more between the Fuyan teeth and the potential second dispersal based on the estimated divergence of European and East Asian lineages (60-25 kya; Soares et al, 2009; Rasmussen et al, 2011; Behar et al, 2012) seems to favor at least two separate out-of-Africa dispersals of early modern humans.

One should be extremely cautious, however, as the dating gap could also be attributable to the lack of human records in that time frame or uncertainties in mutation rates of the genetic markers under study or methodologies adopted in time estimation (Fu et al, 2013a; Rieux et al, 2014; Scally & Durbin, 2012). In fact, with the revised mutation rates, recent studies have proposed that the out-of-Africa exodus likely started at approximately 62-130 kya (Fu et al, 2013a; Rieux et al, 2014; Scally & Durbin, 2012), while the European and Asian split occurred at about 40-93 kya (Fu et al, 2013a; Scally & Durbin, 2012), much earlier than the previously estimated dates. More intensive studies on the mutation rates of both nuclear genomes and mtDNA are crucial to resolve these inconsistencies. It is noteworthy that even if modern humans migrated out of Africa more than once, whether the Fuyan population genetically contributed to the contemporary eastern Eurasians, or whether later immigrants from the second dispersal completely replaced the Fuyan individuals remains to be investigated. Information from the ancient DNA buried in the teeth samples will be of great help to answer these questions and then shed more light on the prehistory of our species.

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Physiological approaches to understanding molecular actions on dorsolateral prefrontal cortical neurons underlying higher cognitive processing

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ABSTRACT

Revealing how molecular mechanisms influence higher brain circuits in primates will be essential for understanding how genetic insults lead to increased risk of cognitive disorders. Traditionally, modulatory influences on higher cortical circuits have been examined using lesion techniques, where a brain region is depleted of a particular transmitter to determine how its loss impacts cognitive function. For example, depletion of catecholamines or acetylcholine from the dorsolateral prefrontal cortex produces striking deficits in working memory abilities. More directed techniques have utilized direct infusions of drug into a specific cortical site to try to circumvent compensatory changes that are common following transmitter depletion. The effects of drug on neuronal firing patterns are often studied using iontophoresis, where a minute amount of drug is moved into the brain using a tiny electrical current, thus minimizing the fluid flow that generally disrupts neuronal recordings. All of these approaches can be compared to systemic drug administration, which remains a key arena for the development of effective therapeutics for human cognitive disorders. Most recently, viral techniques are being developed to be able to manipulate proteins for which there is no developed pharmacology, and to allow optogenetic manipulations in primate cortex. As the association cortices greatly expand in brain evolution, research in nonhuman primates is particularly important for understanding the modulatory regulation of our highest order cognitive operations.

Keywords: Lesion; Microinfusion; Iontophoresis; Viral manipulations; Systemic administration

INTRODUCTION

A great challenge for this century is to discover how molecular mechanisms influence brain circuits, so that we can understand how genetic insults lead to symptoms of disease. This goal is particularly important for the higher cognitive functions of the primate association cortex, which are the target of so many devastating disorders. For example, the layer III pyramidal cells circuits in the dorsolateral prefrontal cortex (dlPFC) generate the mental representations that are the foundation of abstract thought, yet these circuits weaken with normal aging, and degenerate in schizophrenia and Alzheimer's disease. Thus, it is particularly important to understand the molecular regulation of these newly evolved circuits. The arousal systems (e.g., norepinephrine (NE), dopamine (DA), acetylcholine (Ach), serotonin, orexins, and histamine) project to the cortex from the brainstem and basal forebrain, and release transmitter based on waking/sleep state, and the brain's own interpretation of environmental events. These neuromodulators alter information processing in the brain, determining the strength of memories and the state of conscious awareness. Recent data indicate that dlPFC circuits in primates are particularly sensitive to changes in these neuromodulatory actions, and that they are regulated at the intracellular level differently than classic synapses in rodents, with mechanisms that are sometimes opposite to those seen in sensory cortical and hippocampal circuits. For example, they are rapidly taken "off-line" by exposure to even quite mild, uncontrollable stress through activation of cAMP signaling, conditions which strengthen many subcortical functions (Arnsten, 2009; Arnsten et al, 2015). As the association cortices greatly expand in brain evolution, many of these questions can only be addressed in nonhuman primates. The following review summarizes some of the approaches to study modulatory and molecular influences on primate cortical function. [1]

Lesions and depletion of neuromodulators

Lesions remain a key research tool, as they can reveal what is necessary for function. The earliest studies of neuromodulatory

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influences on higher cortical function in monkeys examined the effects of depleting catecholamines from the dlPFC on the performance of a spatial working memory task (Brozoski et al, 1979). This pioneering study compared the effects of the neurotoxin 6-OHDA (with or without treatment to try to protect noradrenergic terminals) to that of dlPFC cortical tissue ablation in rhesus monkeys. Ablation of the dlPFC produced a dramatic and permanent deficit on performance of the working memory task. Remarkably, the 6-OHDA lesions that produced large depletions of both DA and NE produced deficits in working memory performance as severe as those caused by tissue ablation. In contrast, depletions of serotonin from the dlPFC had little effect. This study was the first indication that the correct modulatory state is essential for the functioning of the dlPFC. The study was replicated in marmoset monkeys, and extended to studies of the orbital PFC, where both serotonin and catecholamines were found to be important for function, with qualitative differences in the errors made depending on which monoamines were lesioned (Roberts, 2011; Walker et al, 2009). More recently, research in rhesus monkeys has focused on cholinergic mechanisms, showing that destruction of Ach terminals in the dlPFC also produces significant deficits in working memory performance (Croxson et al, 2011). These lesion studies revealed the importance of catecholamines and Ach to dlPFC function, and inspired further studies of the receptor and intracellular mechanisms underlying their critical actions.

There are several advantages to the lesion approach. As described above, the lesion method is one of the few that can reveal whether a mechanism is necessary for function and the consequences of its removal to cognitive function. Depletion of a transmitter can also help to dissociate drug actions at pre- *v.s.* post-synaptic receptors, as drugs acting at presynaptic receptors lose their efficacy when the substrate is depleted or destroyed, while post-synaptic actions remain and are often magnified due to post-synaptic super-sensitivity. An example of this is the work showing that clonidine's beneficial effects on working memory occur at post-synaptic sites in the dlPFC. Clonidine is an alpha-2 adrenoceptor agonist, and initial studies of alpha-2 receptors focused on their presynaptic location (Langer, 1978). As shown in Figure 1, research in rhesus monkeys showed that clonidine's beneficial effects on cognition were magnified in response to NE depletion in the dlPFC (Arnsten & Goldman-Rakic, 1985), or by more global monoamine depletion with systemic reserpine (Arnsten & Cai, 1993; Cai et al, 1993). Demonstration of a post-synaptic site of action was key for discovering the importance of NE actions on dlPFC neurons.

Lesion studies also have many disadvantages. There are few neurotoxins available for this purpose, and those that do exist are often not very selective and/or effective. For example, the effective 6-OHDA lesion in Brozoski et al (1979) produced a 87% depletion of DA and a 76% depletion of NE, even though treatments were given to try to protect NE terminals. Another major disadvantage of lesions is that it takes time for the depletion to occur, and there are usually compensatory actions that can mask the effects of the lesion. Thus, negative effects are hard to interpret. Nonetheless, they have been foundational to the field, and will remain a bench post for identifying the most important modulatory influences on cortical function.

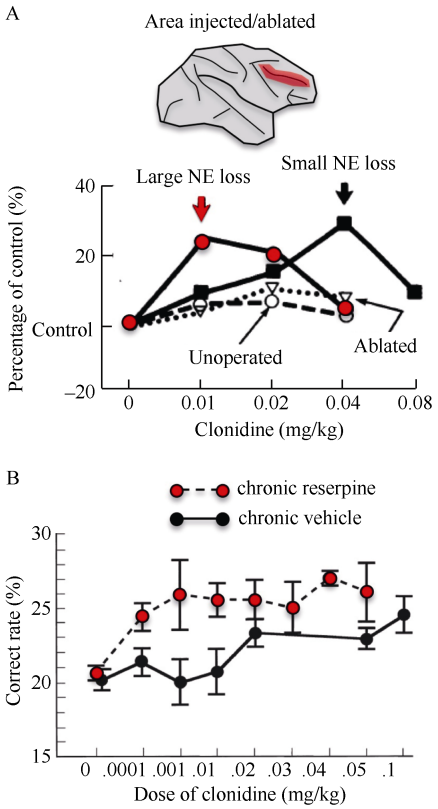

Figure 1 Lesion studies demonstrated that stimulation post-synaptic alpha-2 receptors in dlPFC improves working memory function

A: Rhesus monkeys with 6-OHDA lesions or ablations of the dlPFC (red area) were treated with the alpha-2 agonist, clonidine, prior to performing a spatial working memory task. Clonidine's potency related to the degree of NE depletion from dlPFC, consistent with actions at post-synaptic alpha-2 receptors in this region (Adapted from Arnsten & Goldman-Rakic, 1985); B: Rhesus monkeys were treated with chronic reserpine to deplete monoamines globally, a classic test for pre- *v.s.* post-synaptic drug actions. Clonidine's beneficial effects on spatial working memory were enhanced following reserpine treatment, consistent with a post-synaptic site of drug action (Adapted from Cai et al, 1993).

Microinfusions of drug

A highly effective tool for examining the contribution of not only a neuromodulator, but its receptors, is the ability to infuse drug into cortex to observe immediate effects on cognitive behavior. For example, blocking alpha-2 adrenoceptors by infusions of yohimbine into the rhesus monkey dlPFC revealed the critical importance of endogenous NE stimulation of these receptors. Yohimbine infusions impaired working memory (Li & Mei, 1994), weakened impulse control (Ma et al, 2003), and induced locomotor hyperactivity (Ma et al, 2005), producing a profile of deficits similar to Attention Deficit Hyperactivity Disorder (Figure 2). Infusions into primate dlPFC have also helped to illuminate how high levels of catecholamine release during stress impair

working memory function, as the loss of working memory function can be mimicked by the infusion of NE alpha-1 (Arnsten et al, 1999) or DA D1 (Gamo et al, 2015) receptor agonists into rhesus monkey dlPFC. Conversely, infusions of a D1 receptor antagonist similarly impaired working memory (Sawaguchi & Goldman-Rakic, 1991), consistent with the D1 receptor inverted U dose response (Zahrt et al, 1997). More recently, impairments in associative learning of visual features were observed with D1 receptor antagonist infusions into the ventrolateral PFC in rhesus monkey (Puig & Miller, 2012).

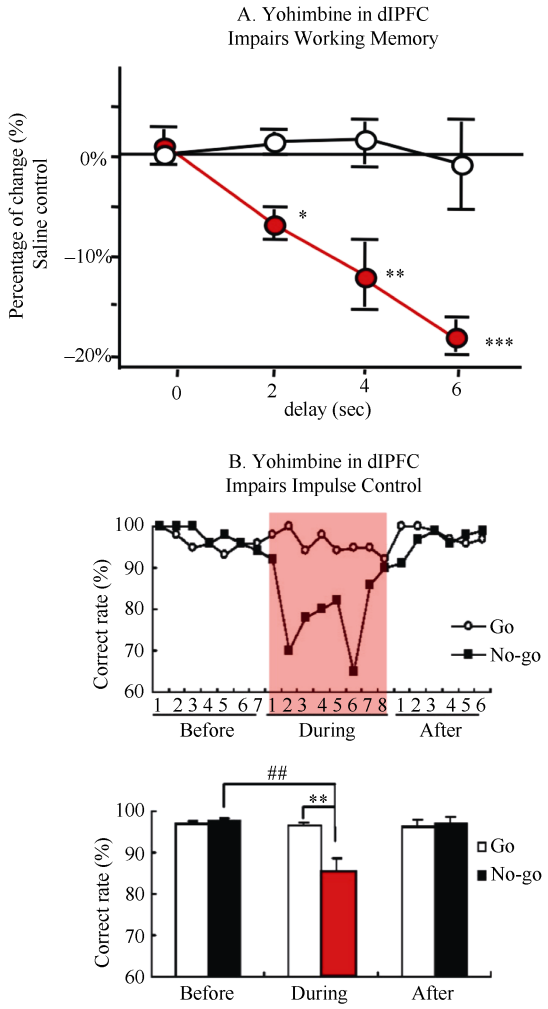

Figure 2 Local infusions of the alpha-2 receptor antagonist, yohimbine, into the dlPFC of rhesus monkeys impaired cognitive function and top-down control of behavior (The red areas represent yohimbine infusion period)

A: Infusions of yohimbine into the rhesus monkey dlPFC produced profound impairments in working memory performance. In contrast, comparable infusions of the alpha-1 receptor antagonist, prazosin, or the beta receptor antagonist, propranolol, had no effect (Adapted from Li & Mei, 1994); B: Infusions of yohimbine into the rhesus monkey dlPFC impaired impulse control on a Go/No-Go task. Monkeys still performed well on Go trials, but could not inhibit behavior on No-Go trials (Adapted from Ma et al, 2003).

There are many advantages to the infusion technique. Infusions can be large enough to influence a large volume of cortex, and thus sufficient to have consequences for behavior (in contrast to iontophoretic application of tiny amounts of drug; see below). The infusion technique can be used with a wide variety of pharmacological compounds, irrespective of whether they cross the blood brain barrier, and thus one can see the functional consequences of blocking *v.s.* stimulating receptors, enzymes, transporters and/or intracellular signaling pathways. As the drug acts immediately, there is no time for compensatory brain actions, and one is more likely to see the functional ramifications of drug actions.

There are also several important disadvantages of the infusion technique. Infusions, even if done slowly, induce extensive gliosis that damages the site for future research. Thus, the extensive work done to prepare the experiment (e.g., training the animal, doing sterile surgical implantations of cannulae), yields only a few data points. This is particularly problematic under conditions were there are limited numbers of subjects. A second major problem is that infusions are generally incompatible with neuronal recordings, as the fluid ejection moves the neurons. Thus, investigations at the cellular level require iontophoretic application of drug.

Iontophoretic application of drug

Iontophoresis uses a very small (nA) electrical current to move minute amounts of an electrically charged molecule out of a micropipette and into the brain. As there is no fluid movement, nearby neurons do not move, and one can continue with stable neuronal recordings. This technique has been essential in revealing the cellular basis for catecholamine and cholinergic actions in the primate dlPFC, including intracellular and ionic influences on neuronal physiology during higher cognitive processing. In monkeys performing a spatial working memory task, there are neurons that are able to maintain spatially tuned firing across a delay period when there is no sensory stimulation, i.e., so called Delay cells. Delay cells are thought to reside in deep layer III of the primate dlPFC, where recurrent excitation through NMDA receptor synapses allows them to maintain firing in the absence of sensory stimulation. Immunoelectron microscopy (immunoEM) revealed that alpha-2A adrenoceptors were localized immediately next to HCN channels on dendritic spines in layer III of dlPFC, suggesting that these molecules might interact (Wang et al, 2007). As the open state of HCN channels is increased by cAMP signaling, while alpha-2A adrenoceptors inhibit cAMP production, iontophoretic studies tested for functional interactions. These studies found that blocking alpha-2 receptors by iontophoresis of yohimbine markedly reduced the firing of Delay cells, while co-iontophoresis of ZD7288 to block HCN channels restored firing (Wang et al, 2007) (Figure 3). Thus, the physiological interaction was confirmed.

The major advantage of iontophoresis is that it is the only technique that allows the reliable observation of drug effects on neuronal firing in cognitively-engaged monkeys. As the drug has immediate actions, there is no time for the brain to mount compensatory counteractions, and a mechanism can be probed

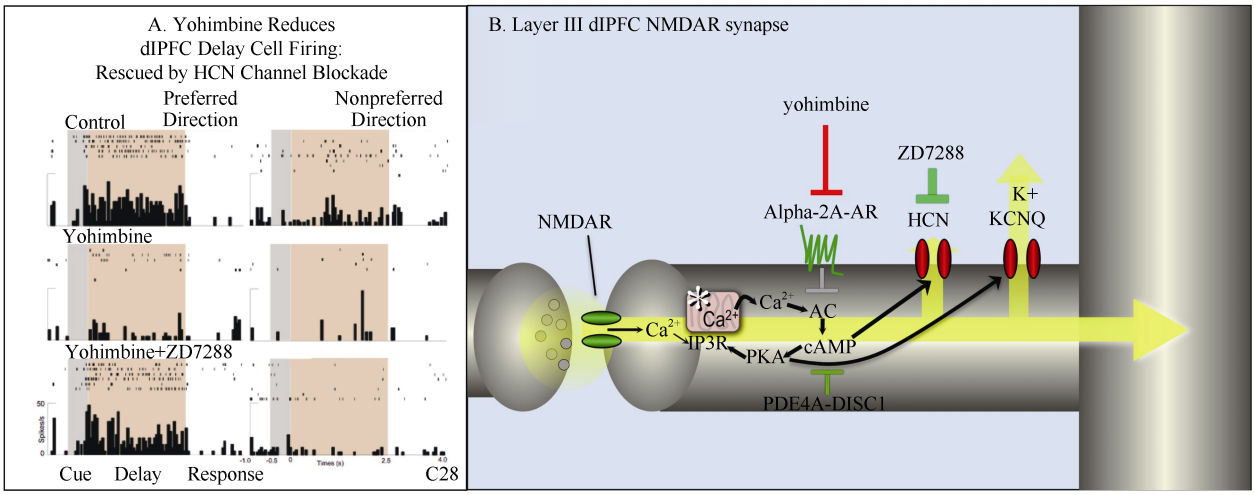

Figure 3 Iontophoresis reveals the cellular actions of alpha-2 receptors in the primate dlPFC

A: Iontophoresis of the alpha-2 receptor antagonist, yohimbine, onto a Delay cell in the dlPFC reduced persistent firing in monkeys performing a spatial working memory task. Firing was restored by co-iontohoresis of the HCN channel blocker, ZD7288 (Adapted from Wang et al, 2007); B: Schematic illustration of the key role of alpha-2A adrenoceptors in inhibiting feedforward, cAMP-calcium-K^+ channel signaling in layer III of the primate dlPFC. Yohimbine blocks these receptors, opening K^+ channels, weakening synaptic efficacy, and reducing task-related neuronal firing needed for working memory.

effectively. Washout periods can be used to see if neuronal firing returns to normal (although some second messenger actions have long-lasting effects whereby neuronal firing does not normalize within the confines of a testing session). The ability to iontophorese multiple compounds also allows for testing of physiological interactions not possible with other techniques. The amount of drug released in brain with iontophoresis is sufficient to influence a group of cells, e.g., a minicolumn or local microcircuit, but usually not adequate to influence behavior. These features can be both a strength and/or a weakness. The absence of changes in behavior allows for easier interpretation of the data (changes in neuronal firing are not simply due to behavioral changes), and one can observe the changes in neuronal firing in the context of its normal circuitry. However, there are times when one would like to manipulate a single neuron independent of its microenvironment, and this is not possible with this technique.

The major disadvantage of iontophoresis is that it requires the use of electrically charged compounds. As drugs are purposefully synthesized to be lipophilic to promote brain penetration, this can limit the pharmacological arsenal available for this technique. The absence of behavioral changes can also be problematic if one wants to know how the manipulation would alter cognitive performance.

Viral manipulations

Viral manipulations allow the genetic manipulation of protein expression, including proteins for which there is no developed pharmacology. These powerful genetic methods have been tremendously successful in rodents, but are just beginning to be applied in primates. The current focus of the field is on the development of genetic manipulations to allow optogenetic manipulations in monkeys, e.g., to selectively activate excitatory neurons in the primary visual cortex (Nassi et al, 2015). However, there is a great need to extend this technology to be able to knockdown or overexpress signaling proteins for which there are currently no pharmacological tools, e.g. as has been done in rodent PFC. For example, viral knockdown of DISC1 (Disrupted In SChizophrenia) in rat medial PFC lowers the threshold for stress-induced PFC dysfunction, possibly by unanchoring PDE4A and dysregulating cAMP signaling (Gamo et al, 2013).

There will be enormous advantages to the application of these techniques in primate brain, including the opportunity to manipulate a wide universe of molecules with immediate relevance to genetic insults in human cognitive disorders. As higher cortical circuits are often regulated differently than circuits in rodents, this will be especially important for understanding how genetic insults lead to symptoms of higher cognitive dysfunction in established brain circuits.

However, the current disadvantages of this technology are daunting. Applying these methods to methods is still in its infancy, and there is a critical need to verify efficacy and selectivity, as methods that work in rodent neurons can fail to have the same effect in primate tissue. This process is very expensive, and has been particularly challenging in the current NIH funding environment. Even when successful, the method will have its challenges, as the genetic changes take days/weeks to express, and thus the brain has a chance to undergo compensatory/reactive changes. For example, one cannot perform the rapid before vs. after methods that are so powerful for single unit recordings with iontophoresis. Nonetheless, this arena represents a critical direction for future research.

Comparison to systemically administered compounds

Finally, it can be important to examine changes in neuronal firing following systemic administration of drug, as this has

direct relevance to human patients taking medications. Some types of agents have the same effect whether they are administered systemically, or by local iontophoresis. For example, systemic administration of the alpha-2 agonist, clonidine, enhances the firing of dlPFC Delay cells (Li et al, 1999), similar to the enhancing effects of iontophoretically applied guanfacine, a more selective alpha-2A-adrenoceptor agonist (Wang et al, 2007). However, this correspondence between systemic and local application is not always seen, e.g. systemic administration of the NMDAR blocker, ketamine, increases the firing of dlPFC Response cells, while local NMDAR blockade reduces Response cell firing (Wang et al, 2013).

The great advantage of systemic administration of drug is that it allows direct comparison to human medications. For example, clonidine and guanfacine are both now approved for the treatment of ADHD, and their beneficial effects on dlPFC neuronal firing likely contribute to their therapeutic actions (Arnsten & Jin, 2012). Systemic administration also becomes necessary when drugs cannot be iontophoresed due to lack of electric charge. However, global actions throughout brain and body provide very little mechanistic information unless coupled with some of the techniques described above.

Summary

In summary, we have made great progress in understanding the molecular needs of the primate dlPFC, using a variety of complementary approaches to reveal intracellular mechanisms and create new treatments for human cognitive disorders. However, we will need to adapt viral technology to bridge our understanding of primate cortex with the genetic revolution.

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Editor's comments

This review article entitled "Physiological approaches to understanding molecular actions on dorsolateral prefrontal cortical neurons underlying higher cognitive processing" examines the relationship between neurotransmitters and prefrontal cognitive function. The authors of this article are leading the field in such research.

When reviewing the article, we were highly impressed by the focused and detailed experimental design. In the field of modern brain science and experimental psychology, it is very difficult to acquire objective results from a single point of view, or through only one (or two) experiment(s). In the presented article, the authors emphasize an unsurprising, but important fact that scientific investigation requires the integration of multidisciplinary levels of study, including, for example, from molecular bases to behavioral representations. Romantically, instead of just being part of the research methods, we should consider experimental design as an elegant art. The present review article is highly recommended because it a fine example of how to systematically conduct experimental design based on a particular scientific issue.

This highlighted paper also compels a strong appreciation for the central role that dopamine (DA) plays among neurotransmitters; much like Shakespeare's drama, without Romeo or Juliet, there can be no Montague or Capulet family, no Mercutio, Benvolio, Tybalt or Count Paris… And, In 2000, following confirmation that DA is a neurotransmitter, Swedish scientist Arvid Carlsson was awarded the Nobel Prize in physiology and medicine.

Dopamine is mainly responsible for information processing of positive emotions such as love and reward, as well as playing a role in negative issues such as addiction. Furthermore, many neurological diseases are related to abnormalities in the DA system. In Parkinson's disease, for example, DA neurons in the substantia nigra are degenerative, which leads to basal ganglion dysfunction and muscle tension. In infantile chorea disease, DA hyperfunction and acetylcholine hypofunction result in muscle softness and weakness. In addition, schizophrenia is also believed to be caused by overactive DAD2 receptors.

Even in the digestive system, DA and acetylcholine balance is important to maintain normal digestion, with hyperfunction of acetylcholine and DA accelerating gastrointestinal peristalsis and weakening digestive function, respectively. Interestingly, domperidone, a familiar drug, can strengthen digestive function due to its role as a DA receptor antagonist, whereby it inhibits the function of DA to enhance gastrointestinal digestive function indirectly. In addition to acetylcholine, DA also has close relationships with the glutamatergic, gamma-aminobutyric acid (GABA) and norepinephrinergic systems, and is a precursor to norepinephrine synthesis in vivo.

In addition to the existing research technology mentioned by the authors in the discussed article, new technologies have been developed in recent years, including optogenetics and Transcription Activator-Like Effector Nucleases (TALEN), a novel gene editing tool. We believe that with classic and modern technology and expanding expertise, Nonhuman primate brain research will continue to make great progress in the future.

Accelerated evolution of constraint elements for hematophagic adaptation in mosquitoes

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ABSTRACT

Comparative genomics is a powerful approach that comprehensively interprets the genome. Herein, we performed whole genome comparative analysis of 16 Diptera genomes, including four mosquitoes and 12 Drosophilae. We found more than 540 000 constraint elements (CEs) in the Diptera genome, with the majority found in the intergenic, coding and intronic regions. Accelerated elements (AEs) identified in mosquitoes were mostly in the protein-coding regions (>93%), which differs from vertebrates in genomic distribution. Some genes functionally enriched in blood digestion, body temperature regulation and insecticide resistance showed rapid evolution not only in the lineage of the recent common ancestor of mosquitoes (RCAM), but also in some mosquito lineages. This may be associated with lineage-specific traits and/or adaptations in comparison with other insects. Our findings revealed that although universally fast evolution acted on biological systems in RCAM, such as hematophagy, same adaptations also appear to have occurred through distinct degrees of evolution in different mosquito species, enabling them to be successful blood feeders in different environments.

Keywords: Mosquitoes; Constraint elements; Accelerated elements; Hematophagy

INTRODUCTION

Recent advances in DNA sequencing technology and comparative genomic analysis have unlocked many mysteries of genetic variation in phenotype determination and have greatly changed the landscape of biological research (Chen, 2015; Kim et al, 2011; Li et al, 2010). With the application of next-generation sequencing (NGS), whole genome sequencing of organisms of interest has become less time and cost consumptive. Draft genomes of many non-model species have been completed in the past five years. Comparative analysis of related whole genomes is a useful approach in genome interpretation (Chen, 2015; Zeng et al, 2013). This has facilitated the discovery of functionally important genomic elements responsible for specific phenotypes and identification of adaptive genomic imprints that occurred during evolution (Lindblad-Toh et al, 2011). [1]

Mosquitoes are found within the dipteran flies (Culicidae), and feed on vertebrate blood (such as human, bird, rat and livestock). Many mosquito-borne pathogens, such as malaria, lymphatic filariasis and dengue fever, can be transmitted between humans and other vertebrates during blood feeding, causing a large number of deaths worldwide annually, particularly in Africa (Tsuji et al, 1990a). This demonstrates the extent of the threat on global health imposed by mosquitoes. Blood nutrients such as proteins and iron are essential for female mosquitoes to lay eggs and complete the reproductive process, even though there are variations in reproductive strategies between autogenous and anautogenous mosquitoes (Tsuji et al, 1990b). Mosquitoes are capable of ingesting a large volume of blood in excess of their body weight during a single meal (Friend et al, 1965), exposing them to high temperature and osmotic pressure and oxygen toxicity from heme, iron and ROS, which came from the processes of blood digestion and mitochondrial metabolism.

In addition, due to their long divergence time and various habitats, different mosquitoes have evolved variation in phenotype, feeding behavior and host preference. A good understanding of the genome components and their role in mosquito species [0] may help determine the mechanisms of

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specific traits (i.e., hematophagy), and provide a potential starting point to ascertain the driving forces of disease transmission and develop measures to effectively control mosquito-borne diseases.

Constraint elements (CEs) are a group of genomic regions that evolved under restriction across different species over a long period of evolutionary time, and have been widely studied in vertebrate, insect, worm and yeast genomes (Lindblad-Toh et al, 2011; Siepel et al, 2005; Tian et al, 2009; Woolfe et al, 2005). Constraint in a genome sequence implies that it is conserved and plays an important role in biological function. Recent and rapid evolution in a group of CEs in a lineage (hereafter called accelerated elements (AEs)) implies that adaptive evolution may have occurred (Amemiya et al, 2013; Holloway et al, 2008; Lindblad-Toh et al, 2011). Comprehensive investigation of CEs and AEs at the genomic-wide scale has emerged as a useful research tool in evolutionary study and has provided directions to test hypotheses and design experiments (Amemiya et al, 2013; Lindblad-Toh et al, 2011). With the development of whole genome sequencing technologies, the genomes of four blood-feeding insects (*Aedes aegypti* (Aa) (Nene et al, 2007), *Anopheles darling* (Ad) (Marinotti et al, 2013), *Anopheles gambia* (Ag) (Holt et al, 2002) and *Culex quinquefasciatus* (Cq) (Arensburger et al, 2010), along with 12 previously published (Clark et al, 2007) *Drosophila* genomes (*Drosophila melanogaster, D. pseudoobscura, D. ananassae, D. erecta, D. grimshawi, D. mojavensis, D. persimilis, D. sechellia, D. simulans, D. virilis, D. willistoni,* and *D. yakuba*) were retrieved from the UCSC Genome Brower and the Ensembl Metazoa database (version 20). Whole genome comparative analysis of these genomes provides the opportunity to comprehensively interpret the genomic evolution of Diptera and the molecular mechanism of hematophagy adaptation in mosquitoes. We carried out a comprehensive search for CEs through genome-wide multiple alignments of the Diptera genomes (four mosquitoes and 12 Drosophilae) to predict the AE divergence of constraint genomic regions in specific mosquito lineages.

MATERIALS AND METHODS

Whole genome alignments

Genomes of 12 *Drosophila*, including *D. melanogaster (dm3), D. pseudoobscura (dp4), D. ananassae (droAna3), D. erecta (droEre2), D. grimshawi (droGri2), D. mojavensis (droMoj3), D. persimilis (droPer1), D. sechellia (droSec1), D. simulans (droSim1), D. virilis (droVir3), D. willistoni (droWil1),* and *D. yakuba (droYak2)*, and four mosquito species (*Aedes aegypti, Anopheles darling, Anopheles gambia* and *Culex quinquefasciatus*) were download from the UCSC Genome Brower and Ensembl Metazoa database (version 20). With the exception of *Aedes aegypti, Anopheles darling, Anopheles gambia, Culex quinquefasciatus* and *D. melanogaster*, reference base pair wise alignments for all species were download from the UCSC Genome Brower. Using the *D. melanogaster* (BDGP assembly release 5) genome as a reference, pair wise alignments were performed using the LASTZ (http://www.bx.psu.edu/miller_lab/) program with parameters set to "E=30 O=400 K=3000 L=2200 M=50 --format=axt". To reduce single coverage with respect to the reference genome and obtain long-linked and longer syntenic contiguous alignments, the alignment blocks were passed through "chaining" and "netting" processes using axtChain, ChainNet and netSyntenic from UCSC tools, as described previously (Kent et al, 2003). Sixteen *D. melanogaster*-centric whole genome alignments were generated using MULTIZ (Blanchette et al, 2004) v012109/roast and graded by the phylogenic topology tree in Figure 1A.

Evolutionary constraint elements detection

To measure conservation tracks of alignment, four-fold degenerate sites were extracted from chromosomes (2R, 2L, 3R, 3L) of MUTIZ alignments using msa_view software from the PHAST (Siepel et al, 2005) package based on a gff format gene annotation file, and a neutral model (non-conserved model) was then generated using PhyloFit. Conservation scores and conserved sequences were predicted using PhastCons. Running parameters were set with an average conserved sequence length of 45 bp ("--expected-length 45"), target coverage of input alignments was set to 0.3 ("--target-coverage 0.3"), and scaling factor for non-conserved model was set to 0.3 ("--rho 0.3"). Elements with length <30 bp were discarded. The non-conserved phylogenies were estimated by PhyloFit using four-fold degenerate sites, as shown in Figure 1A.

Lineage-specific accelerated elements identification

Lineage-specific accelerated elements were identified by first defining candidate genomic regions across all Diptera, excluding the lineage of interest. Conserved regions were identified using the PhastCons program, as mentioned above, and the pipeline parameters were set to "--expected-length 45 --target-coverage 0.3 --rho 0.3".[0] Lineage-specific accelerated elements were identified by likelihood ratio tests (Holloway et al, 2008) implemented using PhyloP software from the PHAST package by comparing conserved region substitution rate scores between lineages of interest and the rest of the subtree. A neutral model for inference was created by the above mentioned methods using PhyloFit. PhyloP was run with the scoring system set to "CONACC". Lineages used in accelerated element detection (lineage of interest) included *Aedes aegypti, Anopheles darling, Anopheles gambia, Culex quinquefasciatus* and the common ancestor of mosquitoes (RCAM). The element with "alt_subscale >1" showed the region that evolved faster than the remaining part of the tree, thereby providing evidence of acceleration. A *P*-value of <0.01 was used as the cutoff (FDR-adjusted).

Element genomic location and functional category enriched analysis

All CEs and AEs were annotated with *D. melanogaster* ENSEMBLE gene annotations using ANNOVAR (Wang et al, 2010), and the genomic regions were classified as down- and up-stream, exonic, intergenic, untranslated and intronic regions. Functional enrichment and pathway annotation clustering were performed using the DAVID (Database for Annotation Visualization and Integrated Discovery) tool (Huang et al, 2008).

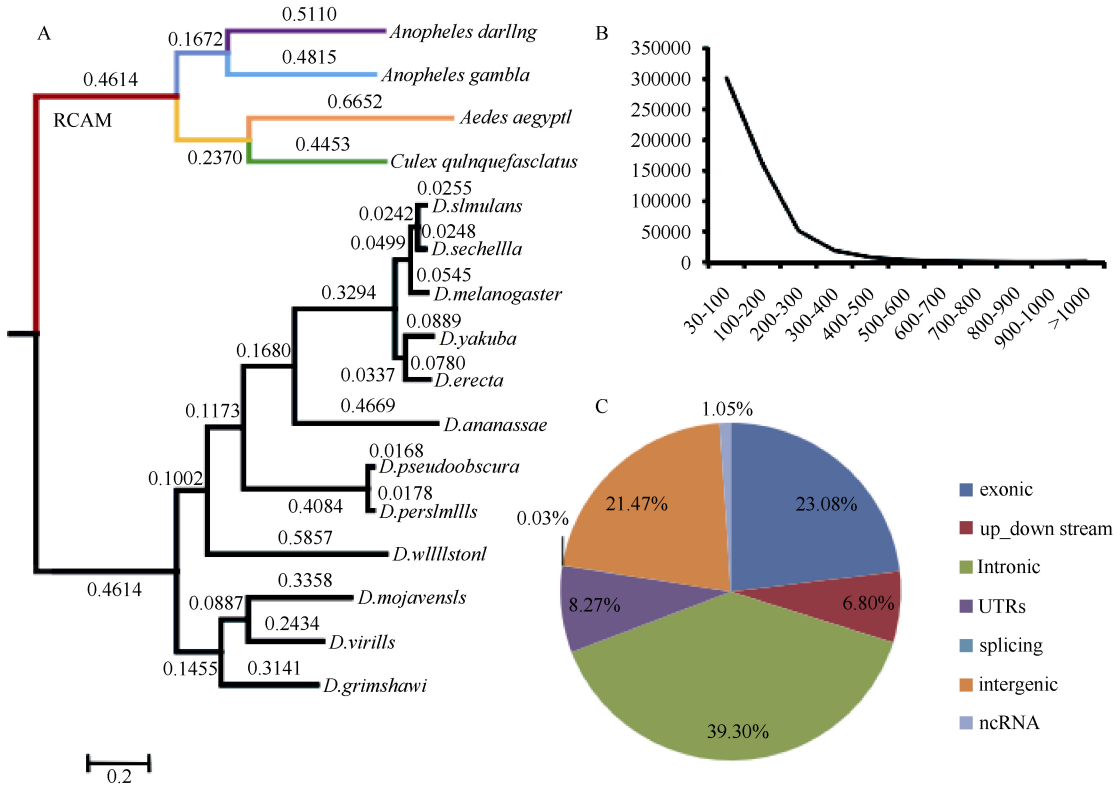

Figure 1 Distribution of constraint elements in 16 Diptera genomes

A: Non-conserved phylogenies estimated by PhyloFit using four-fold degenerate sites. B: Length distribution of constraint elements. C: Distribution of constraint elements by annotation type.

RESULTS

Evolutionary constraint elements (CEs) in Diptera

Genome-wide CEs in Diptera (four mosquitoes and 12 *Drosophila*) were retrieved using PhastCons (see Materials and Methods). A total of 547 726 CEs with length ranging from 30 bp to 6 500 bp (mean size 125.6 bp) were identified (Figure 1B), covering 40.8% of the *D. melanogaster* genome. About 38% of CEs were located in the protein-coding gene regions, which included 23.10% in the coding region, 8.27% in the untranslated regions (3'UTR and 5'UTRs) and 6.80% within 1 kb of the upstream and downstream regions. A large proportion of CEs were found in the intronic and intergenic regions (39.30% and 21.47%, respectively) (Figure 1C). The distribution pattern in the dipteran genome was similar to that of vertebrate genomes, indicating the potentially important role of non-coding regions in both vertebrates and dipterans. However, the proportion of the dipteran genome covered by CEs was higher than that in mammals (~4.5% of mammal genome) (Lindblad-Toh et al, 2011), which may relate to the different properties of the insect genome compared with the mammal genome, such as higher gene density and more compact gene distribution (Bird et al, 2007; Holloway et al, 2008; Lindblad-Toh et al, 2011). The constraint element results in our study were in accordance with previous identification in the four insects (*Anopheles gambia, D. melanogaster, D. yakuba* and *D. pseudoobscura*), except for a slight difference in the proportion of constraint element composition, which may be due to an increased number of higher quality genome sequences used in whole genome alignment (Siepel et al, 2005).

Accelerated conserved elements in mosquitoes Identification of lineage-specific accelerated elements by likelihood ratio tests

We carried out a series of likelihood ratio tests to retrieve elements showing rapid evolution of the constraint regions in each mosquito lineage using PhyloP software. Constraint genomic regions used for acceleration detection were obtained using the same method as described above by excluding the lineage of interest across all 16 dipteran genome alignments (see Materials and Methods). We retrieved elements showing a divergence of constraint regions in RCAM, Aa, Ad, Ag and Cq lineages, respectively.

In total, 1 463, 199, 954, 660 and 779 AEs in RCAM, Aa, Ad, Ag and Cq lineages were identified, respectively (Figure 2A). These AEs showed a similar length distribution pattern (Figure 2B). The Phylop scores for AEs were negatively correlated with their lengths (P<0.01) (Figure 2C). The majority of AEs were assigned to the protein-coding genomic region, but a few were found in the non-coding region (Figure 2D). This distribution pattern was similar to the AEs identified in *D. melanogaster*, but different to human and primate lineages with a large number of

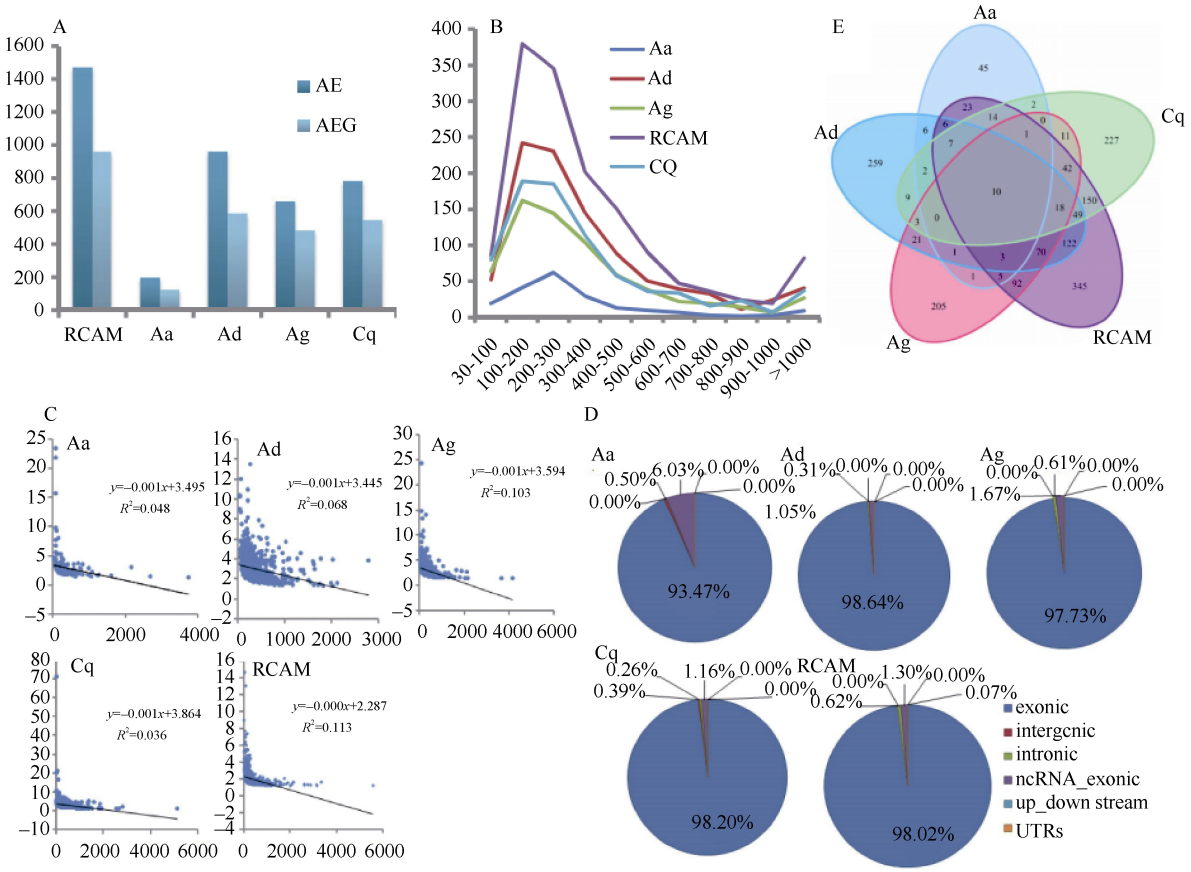

Figure 2 Landscape of CEs, AEs and AEGs detected in mosquitoes

A: Number of CEs and AEGs detected in Aa, Ad, Ag, Cq and RCAM. B: Length distribution of accelerated elements in Aa, Ad, Ag, Cq and RCAM. C: Correlation between Phylop score and length of AEs. D: Distribution of AEs in specific genomic regions. E: Venn diagram shows overlap between AEGs detected in Aa, Ad, Ag, Cq and RCAM.

elements in the non-coding regions (Lindblad-Toh et al, 2011). These findings may be the result of the higher density and compactness of gene distribution in insect genomes compared with that of vertebrate genomes (Bird et al, 2007; Holloway et al, 2008; Lindblad-Toh et al, 2011).

Functional enrichment analysis of AEs

The AEs were annotated with *D. melanogaster* ENSEMBLE gene annotations using ANNOVAR (Wang et al, 2010), and functional enrichment analysis of protein-codon genes covered by AEs (here after referred to as AEGs) were performed using DAVID, including KEGG pathways and GO categories. We found that these AEGs were enriched in a wide variety of functional categories in different lineages. In total, 957, 126, 586, 483 and 545 AGEs were identified in RCAM, Aa, Ad, Ag and Cq lineages, respectively (Figure 2A). Of these, only 10 AEGs were shared by RCAM and the other lineages (Figure 2E).

We found that AEGs in RCAM showed divergence from CEs, and these AEGs were enriched in various functional categories. However, some were over-presented in metabolic-related GO categories, including iron transport/binding, metal ion transport/binding, potassium ion transport/binding, lipid metabolic process, iron, metal ion and potassium ion channel activity (P<0.05) (Supplementary Table 1). This finding was not unexpected because compared with *Drosophila*, mosquitoes have the ability to handle high levels of iron, lipids and heme after ingesting a large volume of blood (Graca-Souza et al, 2006). In addition, some genes participated in exopeptidase, metalloexopeptidase, metallocarboxypeptidase, metallopeptidase and hydrolase activities (P<0.05) (Supplementary Table 1). For instance, exopeptidases, one of the two major classes of secreted proteases in the midgut, function as carboxypeptidases, while amino peptides degrade polypeptides from the terminal ends into exopeptides (Isoe et al, 2009). Therefore, the rapid evolution of these genes in mosquitoes may explain their high efficiency in blood feeding and digestion. Imbibing a large volume of blood from a warm-blooded, vertebrate host not only quickly generates osmotic stress, which requires an efficient excretory system for rapid removal of excess water, but also increases the mosquito's body temperature as blood enters the gut (Benoit et al, 2011). Interestingly, we found heat shock protein 26 (Hsp26) with rapid evolution in RCAM, which has

been reported with increased expression when mosquitoes are exposed to high temperature (42°C) (Zhao et al, 2010).

Due to the long-term and constant use of insecticides, mosquitoes have shown partial tolerance or resistance to insecticides such as DTT, permethrin and malathion. Certain genes or mutations may have contributed to this drug tolerance or resistance in mosquitoes (You et al, 2013). As expected, we found many genes showing evolutionary acceleration enrichment in detoxification and drug metabolism GO categories and pathways, such as response to toxic substances, response to arsenic-containing substances, and detoxification of arsenic-containing substances (P<0.05) (Supplementary Table 1). Three genes, *GSTD1, GSTS1* and *GSTD9*, belong to the glutathione s-transferase (GST) family of proteins, which are involved in the detoxification of a wide range of xenobiotics and protection from oxidative damage, as well as the intracellular transport of hormones, endogenous metabolites and exogenous chemicals including insecticides [0](Sanil et al, 2014). Our findings also revealed that *CYP4C3*, *CYP4D8* and *CYP4P1*, members of the cytochrome P450 monooxygenases (CYPs), showed rapid evolution. These genes play vital roles in insecticide resistance and enhance higher rates of insecticide metabolism (Chandor-Proust et al, 2013).

Via analysis of the AEGs detected in the studied lineages, we discovered many genes showed rapid evolution, which were functionally associated with various ion, ATP, lipid and glucose metabolic processes in Aa, Ad, Ag and Cq (Supplementary Table 2-5), and with hydrolase activity in Ad, Ag and [0]Cq (Supplementary Table 3-5). In Cq, more genes and GO categories related to blood feeding showed rapid evolution compared with the other lineages (Figure 2A), including ion, ATP, lipid hydrolase activity and glucose metabolism categories, exopeptidase, metalloexopeptidase, metallocarboxypeptidase and metallopeptidase activities, but few blood feeding related genes and terms were found in Aa, Ad and Ag (Supplementary Table 2-5). More genes showing CE acceleration in Cq may help explain how these diverse blood feeders effectively and successfully digest blood from multiple hosts, including birds, humans and livestock. In Ag, Ad and Cq, we also found several genes enriched in the functional categories of xenobiotic transporter activity, drug transporter activity and xenobiotic-transporting ATPase activity, and most were members of CYPs and GSTs, such as GSTD4, GSTD9, CYP4D8, and CYP6A9 (Supplementary Table 3-5).

DISCUSSION

We identified more than 540 000 genomic regions (>=30 bp) that evolved under constraint over several million years in Diptera flies by comparative analysis of multiple alignments of 12 Drosophilae and four mosquito genomes. Most CEs were categorized into intergenic, intronic and exonic regions, similar in proportion with that of vertebrates and previous reports (Holloway et al, 2008; Siepel et al, 2005). A higher fraction of CEs (covering 40.8% of the *D. melanogaster* genome) were identified in the dipteran genome than that identified in mammals (~4.5% of the mammal genome) (Lindblad-Toh et al, 2011), which may be due to the higher density and compactness of gene distribution in the insect genome compared with that of the vertebrate genome (Bird et al, 2007; Holloway et al, 2008; Lindblad-Toh et al, 2011).

Being conserved in a genome sequence implies constraint in function. Genomic elements in a lineage that have been under constraint over long periods of evolutionary time, but which have experienced recent and rapid evolutionary expansion, suggest adaptive evolution in those lineages (Holloway et al, 2008). We identified AEs in RCAM, Aa, Ad, Ag and Cq lineages, respectively. Contrary to CEs, the majority of AEs were found in the protein-coding genomic region, with a small proportion located in the non-coding region. This distribution pattern is similar to the AEs identified in the *D. melanogaster* lineage reported previously (Holloway et al, 2008), but different from human and primate lineages in which a large number of elements are assigned into the non-coding regions (Lindblad-Toh et al, 2011). Due to the larger genome size and general biological complexity of vertebrates compared with insects, greater fractions of AEs are found in the non-coding genomic regions in vertebrates. This is likely a reflection of the important role of other non-coding sequences (i.e., lncRNA and circ-RNA) in eukaryotes, which serve as important regulatory factors to regulate gene expression (Lindblad-Toh et al, 2011; Siepel et al, 2005), as verified in recent studies (Iyer et al, 2015; Zhang et al, 2013).

Compared with Drosophila, blood feeding is an intrinsic characteristic of mosquitoes and nutrients in blood are essential for completing reproductive processes. Mosquitoes suffer osmotic and temperature stress after ingesting a large volume of blood, but can deal with it effectively. Comparison of the mosquito genome with that of Drosophila may provide insight into the factors responsible for the above traits. As expected, we discovered some genes related to the transportation and binding of iron, lipids and heme and the digestion of blood essential exopeptidase. We also found hydrolase-related genes, which showed divergence from the CEs in the mosquito lineages and which may contribute to the evolution of blood feeding habits after the divergence from Drosophilae. [0] In addition, some of the detoxification and insecticide resistance-related genes discovered (i.e., glutathione s-transferases (GSTs) and cytochrome P450s) showed rapid evolution in mosquitoes, which may be an adaptive change due to the constant use of chemical insecticides. Genes related to blood feeding adaptation and insecticide resistance also showed rapid evolution in RCAM. While this further evolved in certain mosquitoes (Ag, Aa, Ad and Cq lineages), few genes were shared by RCAM and the other lineages (Figure 2E). This implies that mosquitoes probably possess an independent mechanism and/or different ability to adapt to blood meals or drug resistance. Earlier evidence showed that mosquitoes exhibit differences in host preference (Takken & Verhulst, 2013). For instance, Cq is a diverse blood feeder that can effectively and successfully digest blood from multiple hosts, including birds, humans and livestock. In Cq, we discovered more genes that displayed rapid evolution (Figure 2) and more GO categories related to blood feeding adaptation than any other

lineage. This finding indicates that although the same biological systems were universally fast-evolving, adaptations occurred through somewhat distinct degrees of evolution in different mosquito clades (Lindblad-Toh et al, 2011).

Our study had a number of limitations. First, some of the genomes used in our comparison are recently drafted genomes with potential sequencing and assembly errors, which may lead to bias. In addition, multiple-species, whole genome comparisons are a complex and tedious task, and might led to alignment errors (incorrect alignment blocks or alignment failures) resulting from the program used. Conserved elements and lineage-specific elements estimated here were more or less constrained and fast-evolved than in reality. Our analysis focused on AEGs and did not assign a gene or mutation to specific traits, while recent studies have shown that lineage-specific accelerated elements (LAEs) have important roles in biological processes (Iyer et al, 2015). Improving genome accuracy, alignment quality and functional characterization of accelerated elements in mosquitoes, including LAEs, are important for future study, and may improve understanding of the biological, especially lineage-specific traits of mosquitoes.

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Morphometric variability of *Arctodiaptomus salinus* (Copepoda) in the Mediterranean-Black Sea region

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ABSTRACT

Inter-species variability in morphological traits creates a need to know the range of variability of characteristics in the species for taxonomic and ecological tasks. Copepoda *Arctodiaptomus salinus*, which inhabits water bodies across Eurasia and North Africa, plays a dominant role in plankton of different water bodies-from fresh to hypersaline. This work assesses the intra- and inter-population morphometric variability of *A. salinus* in the Mediterranean-Black Sea region and discusses some observed regularities. The variability of linear body parameters and proportions was studied. The impacts of salinity, temperature, and population density on morphological characteristics and their variability can manifest themselves in different ways at the intra- and inter-population levels. A significant effect of salinity, pH and temperature on the body proportions was not found. Their intra-population variability is dependent on temperature and salinity. Sexual dimorphism of *A. salinus* manifests in different linear parameters, proportions, and their variability. There were no effects of temperature, pH and salinity on the female/male parameter ratio. There were significant differences in the body proportions of males and females in different populations. The influence of temperature, salinity, and population density can be attributed to 80%-90% of intra-population variability of *A. salinus*. However, these factors can explain less than 40% of inter-population differences. Significant differences in the body proportions of males and females from different populations may suggest that some local populations of *A. salinus* in the Mediterranean-Black Sea region are in the initial stages of differentiation.

Keywords: Crustacea; Differentiation; Morphology; Sex differences; Environmental factors

INTRODUCTION

Intra-species variability, an inherent property of living organisms allowing species to exist in a changing environment, is primary raw material for the evolutionary process. This variability, however, creates the need to know the range of variability of important morphological characteristics of the species (Elgmork & Halvorsen, 1998; Gaviria & Forró, 2000). The size of copepods is affected by a number of environmental factors and varies widely (Deevey, 1948). The length and other linear body dimensions of them therefore, are not always a good identification of the copepod species; the proportions between the body parameters may play a more important role in taxonomy (Elgmork & Halvorsen, 1998; Isinibilir et al, 2009). Phenotypic variability and intra-species diversification of copepods were shown (Matthews et al, 2011) to modulate the availability of resources to other species (ecosystem engineering) and shape selection pressures on other organisms (niche construction). There is growing recognition that both inter-population and intra-population variation can have significant effects on population, community, and ecosystem dynamics (Matthews et al, 2014). However, these issues are poorly understood yet. Changes in intra-population diversity may indicate a destabilization of populations (Knyazeva, 2010; Scheffer et al, 2009; Shadrin, 2012; Williamson, 1981). For some copepod species discordance between the rates of morphological differentiation, molecular evolution, and reproductive isolation has been shown (Burton, 1998; Lee & Frost, 2002). Therefore, the studies of a morphological variability for taxonomic, ecological and evolutionary tasks continue to be useful. [1]

The eurythermal and euryhaline copepod species *Arctodiaptomus salinus* Daday, 1885, which has resting eggs and inhabits the water bodies across Eurasia and North Africa, often plays a dominant role in plankton in different types of water bodies (Folijan, 1966; Krupa et al, 2008; Marrone, 2006; Rokneddine, 2004). The population size structures of *A. salinus* in different types of water bodies varies; the average size of females varies from 1.00 mm to 2.38 mm (Folijan, 1966; Rokneddine, 2004; Anufriieva & Shadrin, 2014). Average body

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mass of *A. salinus* in different populations varies at 11.4 times and consequently individual metabolic activity at 6.2 times (Anufriieva & Shadrin, 2014). Two size groups of *A. salinus* in the Crimean and the Siberian lakes were observed, the presence of which do not relate to temperature, salinity, and pH (Anufriieva & Shadrin, 2014). The high ecological and morphological plasticity of *A. salinus* allowed the authors to use it as a good model subject to study different aspects of phenotypic variability in populations of copepods.

The aim of this work is to assess the intra- and inter-population morphometric variability of *A. salinus* in the Mediterranean-Black Sea region, the factors influencing it, and to discuss some observed regularities. The authors suppose that there are significant inter-population differences in morphometric traits, which cannot be explained by environmental peculiarities only.

MATERIALS AND METHODS

Study area and sample collection

Zooplankton samples were collected in the salt waters of the Crimea (largest peninsula in the Black Sea) from 2009 to 2012. One more sample was taken in Tambukan salt lake (July 2012) in the North Caucasus (Russia). On each sampling occasion 50-100 L of water was filtered through 110 μm mesh-size plankton net and the resulting sample was immediately preserved with a 4% buffered formalin solution. *In situ* salinity, temperature and pH were measured at the time of sampling using a portable hand-held salinity refractometer (Kelilong WZ212) and a portable temperature/pH meter (PHH-830). Copepods abundance was determined by direct counting using an Olympus SZ-ST stereo microscope with subsequent conversion to volumetric values based on the volume of filtered water. Several samples of zooplankton from various places in Italy, Spain, and Tunisia with accompanying information were kindly provided to us by Dr. F Marrone. As a rule, 30 adult individuals of each sex from every sample were measured under a light Carl Zeiss Axio Scope A1 microscope at 20-40× magnifications; the measured linear parameters: TL-total length; WC-width of cephalothorax; LA-length of the abdomen; LC-length of the cephalothorax.

Statistical analysis

Variability of parameters in the samples was characterized by the coefficient of variability-CV (CV=standard deviation of a parameter in a sample divided by the mean parameter value). For each sample the average coefficients of variation were calculated separately for the linear parameters (CVl) and proportions of the body (CVp) as follows: the sum of the coefficients of variation for different traits divided by the number of the particular coefficients of variation. The level of sexual dimorphism (for studied morphometrical parameters) was evaluated as female/male ratio. All the data were subjected to a statistical processing (STATISTICA software package, version 6.0, Statsoft, Inc.). The significance of the differences of the average values was evaluated using the Student's *t*-test. To test the homogeneity and normality of the general data sets of the studied parameters we used the probability paper method in the analysis of size frequency distributions (Cassie, 1954), and then tested it in STATISTICA. STATISTICA was also utilized to calculate Euclidean distances between stations and to make tree clustering. Selection of the best approximated equations was made from those available in Excel, according to the highest R^2. Information on sampling sites and samples is presented in Table 1.

RESULTS

Variability of the linear parameters

From sample to sample, average values of all linear parameters varied over a wide range, and a level of their variability (CV) was also high (Table 2). Changes of all linear parameters correlated with each other; the correlation coefficients were significant (P=0.001) in all cases. Previous work (Anufriieva & Shadrin, 2014) had analysed the influence of different factors on body length, so did not need to repeat that analysis. For this reason only the variability of different linear parameters was analysed in this study.

CV of total body length of males ranged from 3.42% to 12.23% in different populations; of females it ranged from 3.32% to 17.96%. The variability of other linear dimensions was slightly higher (Table 2). The correlation coefficients (R) between CV of different linear parameters of the females ranged from 0.686 to 0.810, and each of them was significant (P=0.005-0.001). In males CV of the different characteristics also correlated; the correlation coefficients ranged from 0.366 to 0.895 (P=0.05-0.001). The average CV of linear parameters (CVl) was calculated for all samples (Table 3). CVl in males and females correlated significantly with each other (R=0.612; P=0.05).

The environmental factors affected CV of the studied traits. The average CVl of females in the general data set increased with temperature (R=0.35; P=0.06). In males the trend was not significant. The trend for the Crimean population was not clear and was not significant for males or females. The average CVl significantly increased with increasing salinity. The dependence for males of the general sample set can be approximated by the equation (R=0.441; P=0.02):

$$CVlm=5.478\ S^{0.10} \quad (1)$$

where CVlm-the average CVl in males, S-salinity, ppt.

For females the dependence is approximated by the equation (R=0.705; P=0.001):

$$CVlf=4.814\ S^{0.19} \quad (2)$$

where CVlf-the average CVl in females.

Separately for the Crimean population we observed an insignificant positive relationship in females. In males it is significant (R=0.602; P=0.05) and is given by:

$$CVlm=4.303\ S^{0.19} \quad (3)$$

At salinities of 15-20 ppt there is a maximum of variability of linear parameters. CV of body size of females in the Crimean lakes demonstrates the significant positive linear dependence on the population density (R=0.82; P=0.025). This dependence for males does not demonstrate such a relationship, and was closer to a dome-shaped dependence. There was no pH influence on CV.

Table 1 List of the sampled water bodies with *A. salinus* presence

№	Sampling date	Site name-country	Coordinate	Kind of environment	Altitude (m)	Area (km^2)	Max depth (m)	S (ppt)	T (°C)	N(ind./m^3)	n_f	n_m
1	17.06.05	Lake Banyoles-Spain	N42°07′- E02°45′	Lake, permanent	172	1.18	62.4	0.7-2*	7-26*	-	20	17
2	21.04.05	Pantano Grande di Vendicari-Italy	N40°44′- E33°30′	Swamp, temporary	0	-	-	19.6	15.4	-	30	30
3	15.02.06	Sebkha El Ariana- Tunisia	N40°53′-E32°37′	Sebkha, temporary	1	-	-	2.2	19.2	-	30	30
4	25.02.05	Stagno 4 di Isola Longa-Italy	N41°57′-E33°17′	Pond, temporary	2	-	-	3.0	13.7	-	17	30
5	30.07.02	Lago di Pergusa- Italy (Sicily)	N41°31′- E33°20′	Lake, unstable	667	1.83	12	5.0	25.6	-	7	13
6	11.07.12	Lake Tambukan-Russia (Caucasus)	N43°58′- E43°10′	Lake, unstable	548	1.87	10	21	22	600 160	30	30
7	2.06.12	Lake Yanyshskoe- Russia (Crimea)	N45°07'- E36°24'	Lake, unstable	0	0.2	1	25	24	93 120	30	30
8	1.05.09	Lake Takilskoe-Russia (Crimea)	N45°07'- E36°24'	Lake, unstable	0	0.2	1	16	13	45 600	25	25
9	1.05.09	Lake Yanyshskoe-Russia (Crimea)	N45°07'- E36°24'	Lake, unstable	0	0.2	1	16	13	305 400	30	30
10	13.08.09	Lake Yanyshskoe- Russia (Crimea)	N45°07'- E36°24'	Lake, unstable	0	0.2	1	74	24.5	2 880	30	30
11	06.08.12	Lake Aktashskoye-Russia (Crimea)	N45°22'- E35°49'	Lake, unstable	0.5	26.8	2	60	32	6 460	30	30
12	17.08.10	Lake Tobechik- Russia (Crimea)	N45°10'- E36°21'	Pond, permanent	0.3	18.7	1.2	35	26	7 320	30	30

№: number of sample; S: salinity; T: temperature; N: population density; n_f and n_m: number of female and male specimens studied; *: salinity and temperature ranges, data for sampling time are not available.

Table 2 Average values of the linear parameters of *A. salinus* and their variability in the studied samples (average value/CV)

No.	TL		WC		LA		LC	
	f	m	f	m	f	m	f	m
1	1.42/3.32	1.26/5.00	0.36/5.03	0.25/16.29	0.42/5.67	0.46/8.35	1.04/4.09	0.83/3.98
2	1.55/17.96	1.48/6.68	0.38/6.04	0.36/5.95	0.46/9.48	0.61/9.55	1.13/3.94	0.91/5.38
3	2.08/7.25	2.02/3.42	0.61/8.29	0.42/7.73	0.66/9.16	0.88/4.17	1.47/6.79	1.18/3.90
4	1.45/7.52	1.41/5.18	0.37/6.85	0.33/8.43	0.52/9.10	0.60/6.54	0.95/8.46	0.83/7.00
5	1.72/8.44	1.65/4.04	0.42/8.26	0.40/8.29	0.64/14.51	0.70/6.68	1.11/4.84	0.97/3.06
6	1.24/5.19	1.15/7.24	0.32/10.75	0.28/11.29	0.31/15.02	0.40/15.33	0.91/7.14	0.76/9.39
7	1.44/10.22	1.44/12.23	0.41/14.06	0.38/15.19	0.39/19.04	0.52/21.76	1.06/12.38	0.91/11.01
8	2.29/5.58	2.02/3.83	0.61/9.84	0.46/4.96	0.74/9.17	0.89/4.20	1.63/5.91	1.20/3.77
9	2.08/11.46	1.94/8.69	0.61/15.51	0.46/8.38	0.61/14.94	0.80/11.14	1.50/10.87	1.16/8.68
10	1.62/6.57	1.45/5.58	0.46/13.55	0.35/13.18	0.47/11.12	0.56/9.19	1.18/6.87	0.92/6.35
11	1.41/5.45	1.26/5.58	0.40/8.46	0.30/13.30	0.44/10.06	0.49/12.08	0.99/6.20	0.80/5.81
12	1.49/6.26	1.32/4.95	0.38/8.28	0.32/7.16	0.51/9.85	0.50/11.25	1.02/7.70	0.85/5.43
Average values	1.65/20.89	1.53/20.40	0.45/25.65	0.36/19.88	0.50/26.50	0.61/28.02	1.17/7.10	0.94/6.15

No.: number of sample from Table 1; TL: total length; WC: width of cephalothorax; LA: length of the abdomen; LC: length of the cephalothorax; f: female; m: male.

Table 3 Average variability in different samples of *A. salinus*

No.	Female		Male	
	CVp	CVl	CVp	CVl
1	4.53	5.35	7.91	8.40
2	5.99	6.33	6.67	6.89
3	7.87	4.57	4.86	4.80
4	7.98	7.16	6.42	6.79
5	9.01	6.04	5.28	5.52
6	9.53	12.77	12.83	10.81
7	13.92	13.34	11.11	15.05
8	7.63	6.02	3.95	4.19
9	13.02	9.52	7.06	9.22
10	9.52	10.62	11.19	8.58
11	7.54	9.15	12.89	9.19
12	8.02	8.01	9.15	7.02
Xav	8.71	8.24	8.28	7.29
SD	2.63	2.88	3.11	2.00
CV, %	30.22	34.93	37.56	27.26

No.: number of sample from table 1; CV: coefficient of variation; CVp: average CV of proportions; CVl: average CV of linear traits; Xav: average value; *SD*: standard deviation.

Variability in body proportions

The probability paper was used to analyze the general sets of the different body proportions. Distributions of all proportions were unimodal and very close to a normal distribution, indicating that the studied proportions of “big” and “small” forms (Anufriieva & Shadrin, 2014) were practically identical. Mean proportions and their CVp are given in Table 4. CVp values were close to that for the linear parameters, and ranged from 5.27% to 13.79%. TL/LC was the least changeable on average proportion in populations. A significant effect of salinity, pH and temperature on the body proportions was not found. The levels of their intra-population variability are dependent on temperature and salinity. A significant increase of CVp was observed with increasing temperature (*P*=0.05-0.001). In males, this dependence is stronger. Increasing salinity also leads to significant changes in CVp of some proportions. For example, in females CVp of TL/WC increased with increasing salinity in the range from 2.2 to 74 ppt (R=0.646; *P*=0.005); dependence can be approximated by the equation:

$$CVpf=0.083\ S+5.119 \quad (4)$$

where CVpf-CVp of TL/WC proportion in females.

In males, the dependence of the variability of this proportion on salinity is expressed even more strongly (R=0.898; *P*=0.001), and the dependence can be approximated by the equation:

$$CVpm=0.123\ S+5.248 \quad (5)$$

where CVpm-CVp of TL/WC proportion in males.

The increase in variability of TL/LA was not observed in the entire range of salinity in males and females. Using Crimean samples the dependence of the proportions and their variability levels on the density of the population were analyzed; the dependence was absent. CVp of the different proportions were significantly correlated with each other (R=0.450-0.700; *P*=0.05-0.0005) in males and females separately, and the average CVp for males and females correlated also (R=0.758; *P*=0.001). CVl positively related with CVp (for females R=0.716; *P*=0.0005; for males R=0.634; *P*=0.005). The dependence of the average CVp with salinity in males can be approximated for the general totality of samples by the equation (R=0.589; *P*=0.001):

$$CVpm=4.882\ S^{0.18} \quad (6)$$

where CVpm-the average CVp in males.

In females this index is slightly but significantly positively

Table 4 Average values of body proportions of *A. salinus* and their variability in the studied samples (average value/CV)

№	TL/WC		TL/LC		TL/LA		LC / WC		LC/LA		WC/LA	
	f	m	f	m	f	m	f	m	f	m	f	m
1	4.00/4.71	5.10/1.53	1.37/2.52	1.52/3.21	3.44/5.04	2.76/4.74	2.92/6.16	3.37/16.54	2.51/7.27	1.83/7.43	0.86/6.44	0.55/14.01
2	4.02/17.95	4.11/6.80	1.36/17.57	1.63/3.73	3.36/18.71	2.45/5.14	2.96/5.48	2.53/6.32	2.46/9.25	1.51/8.47	0.83/9.47	0.60/9.54
3	3.41/5.10	4.85/6.99	1.42/2.00	1.72/1.99	3.17/3.91	2.28/2.17	2.41/5.81	2.83/2.17	2.24/5.65	1.33/3.90	0.93/4.96	0.47/6.66
4	3.91/6.67	4.24/6.12	1.58/3.45	1.70/3.65	2.82/6.19	2.34/5.02	2.56/7.36	2.49/5.23	1.85/9.68	1.38/8.45	0.72/9.6	0.56/10.05
5	4.12/3.17	4.16/5.78	1.55/3.77	1.70/2.53	2.73/6.55	2.36/3.85	2.67/4.58	2.44/7.26	1.77/10.45	1.39/6.55	0.66/7.71	0.57/5.69
6	3.88/10.31	4.22/9.83	1.36/5.86	1.52/6.77	4.01/13.20	2.96/12.99	2.86/8.85	2.78/10.54	2.98/17.98	1.96/19.07	1.05/20.36	0.71/17.79
7	3.51/8.84	3.80/8.14	1.36/7.10	1.58/7.24	3.81/15.31	2.84/11.68	2.58/9.27	2.41/9.19	2.83/20.2	1.81/17.00	1.10/19.31	0.75/13.38
8	3.79/5.71	4.38/4.07	1.41/2.77	1.69/1.84	3.11/5.92	2.27/4.00	2.69/5.92	2.58/3.49	2.21/8.16	1.34/4.51	0.82/7.65	0.52/5.77
9	3.44/8.89	4.25/7.45	1.39/2.96	1.67/3.77	3.45/9.37	2.44/6.43	2.48/8.95	2.55/7.45	2.49/12.07	1.47/9.91	1.01/14.87	0.58/10.61
10	3.60/13.21	4.22/14.06	1.38/3.45	1.58/3.82	3.44/7.88	2.62/7.57	2.61/11.99	2.67/13.47	2.50/11.02	1.66/11.27	0.97/16.18	0.63/16.93
11	3.56/7.90	4.22/13.88	1.42/ 3.69	1.57/ 5.79	3.26/9.01	2.62/9.98	2.50/7.71	2.69/14.19	2.30/12.47	1.68/15.61	0.92/14.09	0.63/17.88
12	3.90/5.30	4.10/8.70	1.47/4.83	1.56/4.93	2.98/8.30	2.68/8.20	2.65/6.42	2.63/6.34	2.03/12.89	1.73/12.73	0.77/10.86	0.66/13.98
Average values	3.73/10.11	4.28/11.92	1.41/5.27	1.62/6.11	3.37/13.79	2.56/11.82	2.66/7.38	2.66/8.52	2.35/11.42	1.59/10.41	0.89/11.79	0.60/11.86

№: number of sample from table 1; TL: total length; WC: width of cephalothorax; LA: length of the abdomen; LC: length of the cephalothorax; f: female; m: male.

correlated also with salinity (R=0.418; *P*=0.01); the dependence may be approximated by the equation:

$$CVpf=6.50\ S^{0.10} \quad (7)$$

where CVpf-the average CVp in females.

In the Crimean population a significant correlation of CVp with salinity in females was not observed; but in the males it was significant (R=0.771; *P*=0.005) and is approximated by the equation:

$$CVpm=1.459\ S^{0.51} \quad (8)$$

Temperature also significantly affected CVp in all samples. Dependence in males can be approximated by the following equation (R=0.664; *P*=0.0005):

$$CVpm=1.244+0.340\ T \quad (9)$$

where T- temperature (°C).

In the Crimean population the dependence in males can be approximated (R=0.903; *P*=0.0001):

$$CVpm=0.644+0.389\ T, \quad (10)$$

Population density does not affect CVp of males, but in females CVp significantly increased with an increase of population density (R=0.694; *P*=0.005); the dependence is not strong linear.

Sexual dimorphism in body proportions was expressed in the same degree as in the linear parameters (Table 5). The exception is the LC/WC, which is almost identical in males and females. The level of inter-population variability in the expression of sexual dimorphism in body proportions was rather high; the coefficients of variation for different proportions varied from 4.8% to 15.4%. There were no effects of temperature, pH and salinity on the female/male parameter ratio. The significant differences in the influence of salinity and temperature on variability in the proportions of males and females that were revealed are as above.

Table 5 Sexual dimorphism of *A. salinus* in body proportions in different samples

No.	TL/WC		TL/LC		TL/LA		LC/ WC		LC/ LA		WC/ LA	
	Xav	CV	Xav	CV	Xav	CV	Xav	CV	Xav	CV	Xav	CV
1	0.78	3.07	0.90	0.78	1.24	1.06	0.87	0.37	1.38	0.98	1.56	0.46
2	0.98	2.64	0.84	4.72	1.37	3.64	1.17	0.87	1.63	1.09	1.39	0.99
3	0.70	0.73	0.83	1.00	1.39	1.80	0.85	0.78	1.68	1.45	1.97	0.74
4	0.92	1.09	0.93	0.94	1.20	1.23	1.03	1.41	1.34	1.15	1.30	0.96
5	0.99	0.55	0.91	1.49	1.16	1.70	1.09	0.63	1.28	1.60	1.17	1.36
6	0.92	1.05	0.89	0.87	1.36	1.02	1.03	0.84	1.52	0.94	1.48	1.14
7	0.92	1.08	0.86	0.98	1.34	1.31	1.07	1.01	1.56	1.19	1.46	1.44
8	0.86	1.40	0.83	1.50	1.37	1.48	1.04	1.69	1.65	1.81	1.58	1.33
9	0.81	1.19	0.83	0.79	1.41	1.46	0.97	1.20	1.70	1.22	1.75	1.40
10	0.85	0.94	0.87	0.90	1.31	1.04	0.98	0.89	1.50	0.98	1.54	0.96
11	0.84	0.57	0.91	0.64	1.24	0.90	0.93	0.54	1.37	0.80	1.46	0.79
12	0.95	0.61	0.95	0.98	1.11	1.01	1.01	1.01	1.18	1.01	1.16	0.78
Xav	0.88	1.24	0.88	1.30	1.29	1.47	1.00	0.94	1.48	1.19	1.49	1.03
SD	0.09	0.80	0.04	1.11	0.10	0.74	0.09	0.37	0.17	0.30	0.23	0.31
CV	9.84	64.6	4.76	85.3	7.65	50.4	9.06	39.2	11.5	25.0	15.4	30.1

No.: number of sample from Table 1; TL: total length; WC: width of cephalothorax; LA: length of the abdomen; LC: length of the cephalothorax; Xav: average value; *SD*: standard deviation; CV: coefficient of variation.

Inter-population differences in proportions

All samples were compared with each other in pairs for body proportions; in most cases significant differences were found. For example, Table 6 shows the level of significance of differences between the samples for TL/WC in females. For TL/WC of females differences were observed in 53% of the compared pairs and 50% of the male pairs; for TL/LA − in females was 85%, and 73% in males. For TL/WC 26% of pairwise comparisons of samples revealed significant differences for males and females, and in TL/LA it was significantly different in 64% of comparisons. In 35% of the pairwise comparisons males were significantly different in both proportions, and females were significantly different in 42%. In 14% of the cases both females and males were significantly different in both proportions.

The greatest number of differences with the other samples was observed in samples from Lake Takilskoe in the Crimea (56% comparisons), from Lake Banyoles in Spain (50%) and from Sebkha El Ariana in Tunis (48%).

Clustering was used to identify similarity between different samples separately for males and females as well as for both sexes together, taking into account all linear traits and proportions (Figure 1). Resulting graphs show that there are different grouping pictures for male and females. The distance between the studied lakes varies from less than 50 km to more than 3 000 km. Euclidean distance (Figure 1) did not significantly correlate with geographical distance between sampling sites and differences in salinity.

Table 6 Significance level of inter-sample differences for the proportion of total length to width of cephalothorax of *A. salinus* females

No	0	1	2	3	4	5	6	7	8	9
1	1	-								
2	2	ns	-							
3	3	0.01	0.001	-						
4	4	ns	ns	ns	-					
5	5	ns	ns	0.001	ns	-				
6	6	ns	ns	0.001	ns	ns	-			
7	7	0.001	0.001	ns	0.001	0.001	0.001	-		
8	8	0.01	ns	0.001	ns	0.01	ns	0.005	-	
9	9	0.001	0.001	ns	0.001	0.001	0.001	ns	0.001	-
10	10	0.001	0.05	ns	0.01	0.005	0.05	ns	ns	ns
11	11	0.001	0.01	ns	0.001	0.001	0.001	ns	0.01	ns
12	12	ns	ns	0.001	ns	ns	ns	0.001	ns	0.001

No.: number of sample from Table1.

DISCUSSION

Intra- and inter-population variability

As seen from the above data, the linear dimensions and the level of their variation in *A. salinus* populations were not constant; to a certain extent they depended on temperature, salinity, and density of population. The results lead to the general conclusion that the impacts of factors on linear morphological characteristics and their variability can manifest itself in different ways at intra-population and inter-population levels. For example, it was shown that about 85% of total variability in body length of *A. salinus* can be explained by temperature changes in local populations (Crimean and Sicilian), but only 28% in the total of samples collected in Mediterranean-Black Sea region (Anufriieva & Shadrin, 2014). There is a negative linear correlation between body size and altitudes of *A. salinus* habitats; 22% of total body size variability might be explained by a water body altitude above sea level (Anufriieva & Shadrin, 2014). Similar trend was shown for other diaptomid copepods (Hausch et al, 2013). Temperature, salinity, and population density do not have an influence on body proportions of *A. salinus*, because changes of linear traits correlate each other; these factors may have an influence on intra-population variability of proportions. The presented data show that a level of morphological variability in populations is not constant and reflects on different factors. In copepods, including *A. salinus*, variability can increase when animals are near limit values of factors, such as temperature, salinity or increased population density (Devreker et al, 2007; Jimenez-Melero et al, 2007; Whitehouse & Levis, 1973; Zelikman & Geinrikh, 1959). Those experimental data are consistent with our field data on the impact of the different factors on the morphometrical variability in *A. salinus* populations. Inter-population differences of *A. salinus* cannot be explained by only studied factors, and it is assumed that there are some overlooked factors as well as differences in the genetic architecture of populations.

In this study the authors evaluated total phenotypic variability in the populations. It is known that differences in phenotypes can be caused by genes, environmental factors, or a combination of both. The idea, which originated with Schmalhausen (1941, 1949) and Waddington (1942, 1957), suggested that genetic variation may get canalized under stabilizing selection and released under directional selection or under stress. A decrease in comfort of living conditions to a certain limit leads to destabilization of ontogeny in a population and as a result, to an increase of variability in the population. Upon reaching a higher level of environmental discomfort there is a dramatic increase in the selection pressure against the individuals with a low degree of canalization of ontogeny; a decrease in variability in the populations has been observed. Populations may realize a random search for the better individuals to survive in an extreme environment by increasing intra-population diversity. However, populations need to spend more energy to support their additional diversity. Resources of energy in populations are very limited in extreme environments; populations have not enough energy to support high diversity in a high extreme environment (Anufriieva & Shadrin, 2014). The diversity in the population begins to decrease; the result is that only individuals with the most stable ontogeny are left. Populations switch from one to another survival strategy. When data on female was examined it was seen that highest variability was featured in the unpredictably changing and ephemeral ponds of Tunisia, Sicily, and twice in the lakes of the Crimea, which have the most unstable environment. This suggests that the selection pressure (or intra-population regulation?) also may drive a variability level in populations.

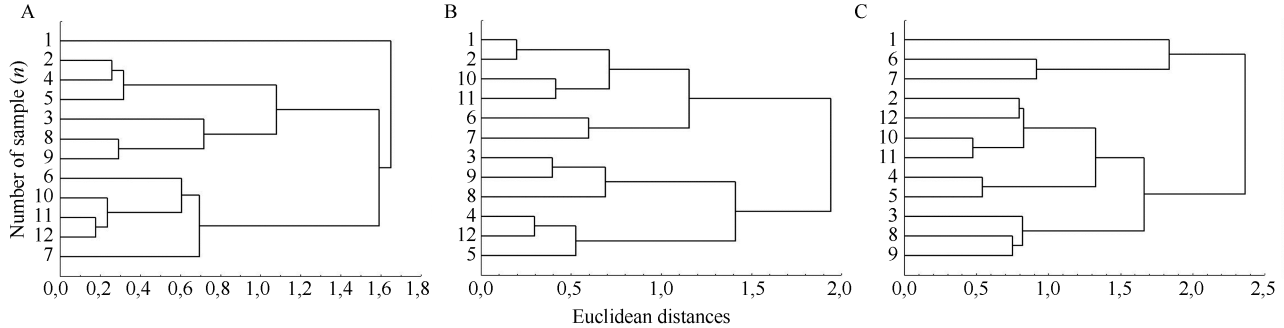

Figure 1 Euclidean distances between the samples of *A. salinus* on the base of measured traits and their proportions (A: males; B: females; C: both males and females)

Sexual dimorphism of *A. salinus* manifests not only in linear dimensions and proportions of the body, but also in variability level and reactions to the fluctuations of environmental conditions. Sexual differences in morphological variability were also observed in other animal species (Elgmork & Halvorsen, 1998; Gaviria & Forró, 2000; Istomin, 2008).

Connections between populations in the region and some sources of their variability

The distance between the Crimean closed lakes does not exceed 50 km, i.e. gene flow between them cannot be limited due to isolation-by-distance. It has been shown that genetic isolating barriers (isolation-by-distance) among *Eudiaptomus graciloides* Lilljeborg, 1888, living in different reservoirs located within 100 km of each other, are non-existent (Zeller et al, 2006). Therefore, in our opinion, all the samples from the Crimean lakes were taken from one local population. However, our data also show that there are significant differences between samples in some parameters, although these differences on average are less than in the inter-population comparisons. This is, in our opinion, valid also for three samples taken from the Sicilian water bodies. The presence of significant differences in the body proportions of males and females in different populations suggests that some local populations are in the initial stages of differentiation. This does not exclude that all the samples could have been taken from a single metapopulation of *A. salinus*. It may be concluded that variations in body proportions were thus related to environmental or genetic factors rather than to geographic distance. It is known that the resting eggs of crustaceans can be carried long distances very successfully by wind, birds, or insects (Caceres & Soluk, 2002; Frisch et al, 2007; Green & Figuerola, 2005; Khomenko & Shadrin, 2009; Van de Meutter et al, 2008). Based on this fact, complete isolation of *A. salinus* local populations may be excluded within the studied area. Episodic transportation of resting eggs between local populations may have occurred; it may be one of the reasons causing fluctuations in individual morphometry and the levels of its variability within populations. This may also explain why Euclidean distance between morphological traits of different populations does not correlate with geographical distance and why there are high morphological differences in samples taken in one single lake at different time. The question of why samples from two close Crimean lakes and one sample from Tunis are in one group cannot be answered by analysing clustering, or why samples taken in Lake Yanyshskoe at different times demonstrate such high Euclidean distance (Figure 1A-C).

The presence of small genetic differences even in adjacent generations was shown for different groups of animals (Altukhov, 2003). Given the ability of the resting eggs to remain dormant in bottom sediments, at least from tens to hundreds of years (Hairston et al, 1995; Marcus et al, 1994), it is easy to imagine how the genetic diversity of the resting egg bank is larger than in the active part of a population. This is shown in particular for *Onychodiaptomus sanguineus* Forbes, 1876 with a long-lived egg bank (Hairston et al, 1996). The wind regime largely determines the output of nauplii from dormant eggs buried in the sediment. Strong wind, which mixes bottom sediments, leads to a resuspension of a thicker layer of sediments, so this can also lead to outbreaks of diversity in populations. Of course, the role of the wind factor depends on the depth of a water body. The wind factor is least pronounced in Lake Banyoles, the deepest lake of those studied here. The wind factor would be highest in ephemeral ponds with a depth of less than 1 m, and a majority of the studied habitats of Crimea are as such.

CONCLUSION

The authors have not been able to quantitatively assess causes of fluctuation in the level of variability in populations of copepods having resting eggs and living in shallow ephemeral ponds. Indicators of phenotypic or genotypic diversity in populations of shallow ephemeral pond copepods cannot be used to assess population sustainability now. It can be concluded that the significant inter-population differences in morphometric traits cannot be explained by only environmental peculiarities or distance between sites. More field and experimental studies are needed to understand the roles of different factors causing variability on different scales. Studied environmental factors had no effect on the body proportions and their female/male ratio; the body proportions and their female/male ratio may be used as species descriptors.

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Stress-relevant social behaviors of middle-class male cynomolgus monkeys (*Macaca fascicularis*)

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ABSTRACT

Stress from dominance ranks in human societies, or that of other social animals, especially nonhuman primates, can have negative influences on health. Individuals holding different social status may be burdened with various stress levels. The middle class experiences a special stress situation within the dominance hierarchy due to its position between the higher and lower classes. Behaviorally, questions about where middle-class stress comes from and how individuals adapt to middle-class stress remain poorly understood in nonhuman primates. In the present study, social interactions, including aggression, avoidance, grooming and mounting behaviors, between beta males, as well as among group members holding higher or lower social status, were analyzed in captive male-only cynomolgus monkey groups. We found that aggressive tension from the higher hierarchy members was the main origin of stress for middle-class individuals. However, behaviors such as attacking lower hierarchy members immediately after being the recipient of aggression, as well as increased avoidance, grooming and mounting toward both higher and lower hierarchy members helped alleviate middle-class stress and were particular adaptations to middle-class social status.

Keywords: Stress; Social behaviors; Beta individual; Male-only; Cynomolgus monkey

INTRODUCTION

People with different socioeconomic status are thought to experience different levels of psychosocial stress, which can lead to various health problems such as physiological and metabolic alternations, disabilities, stress-related diseases and even mortality (Adler et al, 1993, 1994; Brunner, 1997; Manuck et al, 1995; Sapolsky, 2005; Shively et al, 2005). In addition to human studies, Abbott (2003) and colleagues conducted a meta-review on dozens of nonhuman primate species, which showed strong correlations between social rank and stress level, and although the associations varied among species, certain ranks exhibited a corresponding amount of stress. Sapolsky (2005) also reviewed and reported on the close relationship between social status and health conditions in nonhuman primates, and concluded that social status markedly influenced health. Accordingly, investigations on social status and stress levels have drawn increasing attention.

Bupa claims that more than a half of middle-class managers suffer from overwhelming stress problems and experience mental health conditions such as depression (Bupa Research, 2013). Similarly, Edwards et al (2013) found that middle-class barbary macaques experience more social stress than those with relatively higher or lower social status. Thereby, middle-class stress seems to draw increasing attentions from general studies of graded variations between social rank and stress conditions. However, the origins of middle-class stress and how middle-ranked individuals adapt to their particular social status remain poorly understood. [1]

In the present study, we selected beta monkeys in captive, male-only, cynomolgus monkey groups (as middle-ranked individuals), and observed and analyzed four types of social behaviors (aggression, avoidance, grooming and mounting) in the hope of clarifying the origins of middle-class stress and how beta monkeys adapt to their social status from a behavioral point of view.

MATERIAL AND METHODS

Subjects

Data were collected from seven isosexual social colonies of male cynomolgus monkeys (*Macaca fascicularis*), with 10-15

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individuals aged 4-5 years in each group. The monkeys were housed in colonies (3 m×5 m×3 m) at Hainan Jingang Biotech Co., Ltd. (Tian & Ma, 2014), and had free access to water and monkey chow supplemented with fruit and vegetables. The animals were reproductively intact. The members in each group were unchanged for at least two years prior to initial sampling. The treatment of the animals was conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and was approved by the Biological Research Ethics Committee of the Hainan Jingang Biotech Co., Ltd.

Behavioral sampling

The monkeys were familiarized with observers until they were completely adapted to observers remaining in front of the cage. Observers stood or sat at a distance of at least 2.5 m from the cage. Except for taking sampling notes, observers did not move about freely or feed the animals. Actual behavior sampling started after the adaptation period.

Social status was determined via instant scan sampling (Altmann, 1974) during the adaptation period, and was based on the order of eating and the ability to defeat others in each group. More specifically and theoretically, the animal that defeated all other members in its group and ate first when food was provided was ranked as the alpha (top) male; the animal that defeated all but the alpha male was ranked as the beta (second) male; and so forth. In the present study, the top two ranked monkeys in each group were very clear, and their respective statuses were stable during the observation period. However, the ranks of the remaining individuals (excluding the lowest) were unclear and unstable because their observable behavioral differences were difficult to determine. Similar to human middle-ranked managers (Bupa Research, 2013), beta males had to obey the alpha males (boss) as well as maintain their beta status over the other group members. We therefore selected the middle-ranked beta males as the target individuals in this study as they were hierarchically positioned between the alpha and lower-ranked members.

The frequencies of social behaviors, including aggression, avoidance, grooming and mounting, were recorded using focal animal sampling (Altmann, 1974). The frequency of social behaviors between the beta male and other cage mates was recorded. From the action direction point of view, we recorded six sub-categories for each social behavioral type, namely, actions initiated by the alpha male toward the beta male, beta male toward the alpha male, alpha male toward 'others', 'others' toward the alpha male, beta male toward 'others', and 'others' toward the beta male. Each monkey group was recorded for three consecutive days in total, six times a day, 10 min per time between 1700h and 1900h.

Statistical analysis

Student's *t*-tests (paired) were used for comparison of the frequency (over entire observation period) differences among sub-categories in each social behavioral category. The *t*-tests were two-tailed with $P<0.05$ considered to be statistically significant.

RESULTS

Aggressive behavior

No aggressive behavior, across all seven monkey groups and the entire observation period, was initiated by the beta male or 'others' toward the alpha male, nor from 'others' toward the beta male. Aggression mainly occurred between the alpha male and beta male/'others', as well as the beta male and 'others'. As shown in Table 1, the average frequency of the alpha male attacking the beta male or 'others' was 3.14 and 2.57, respectively. The beta males received more than half (55%, 22 out of 40 occurrences) the aggressive actions initiated by the alpha males.

In addition, compared with the alpha male, the beta male attacked the 'others' more frequently (average frequency of 2.57 *v.s.* 7.00, $P=0.0008$, $t=6.16$, $SD=1.9$). The beta male was attacked by the alpha male on 72.7% of occasions (16 out of 22 times per group), after which the beta male immediately initiated aggressive behavior toward the 'others'. By comparing the aggressive behaviors received (A to B, 3.14 times per group on average) and given by the beta male (B to 'others', 7.00 times per group on average), the beta male was more often an aggressor than victim ($P=0.006$, $t=4.12$, $SD=2.47$).

Table 1 Frequencies of aggressive behavior

Group No.	A to B	A to others	B to others	A to all
1	3	3	9	6
2	2	0	3	2
3	3	2	4	5
4	3	1	5	4
5	3	5	8	8
6	4	5	11	9
7	4	2	9	6
Average	3.14	2.57	7	5.71
Sum	22	18	49	40
P		0.0008		
		0.006		

Animal's number from 1-7: the seven monkey groups, respectively; A and B: alpha male and beta male, respectively; *P*: values from student's *t*-tests, and the data pairs for comparison are the two columns indicated by the underscore at both sides of the number (i.e., the paired *t*-test of column "A to others" and "B to others" resulted in $P=0.0008$; similarly, the "A to B" and "B to others" columns showed significant differences with $P=0.006$); Abbreviations and *P* values are the same in Tables 2-4 below.

Avoidance behavior

Avoidance behavior occurred more frequently than the other behaviors, as seen from the total number of each sub-category (97, 68, and 89 times) shown in Table 2. The beta male avoided the alpha male more frequently than the 'others' did (13.86 *v.s.* 9.71 times per group on average, $P=0.018$, $t=3.19$, $SD=3.43$). There was no significant avoidance predilection of 'others' to either the alpha or beta males, although they showed more, in

Table 2 Frequencies of avoidance behavior

Group No.	B to A	Others to A	Others to B
1	13	12	14
2	10	9	12
3	12	7	11
4	12	7	13
5	23	20	11
6	17	6	19
7	10	7	9
Average	13.86	9.71	12.71
Sum	97	68	89
P	0.018		
		0.269	

total number (68 *v.s.* 89 times), avoidance behavior toward the beta males. The alpha males exhibited no avoidance behavior.

Grooming (affinitive behavior)

As shown in Table 3, grooming was dual directional among the alpha male, beta male and 'others'. The beta males groomed the alpha males much more frequently than the reverse (5.43 *v.s.* 1.00 times on average, P=0.003, t=4.55, SD=2.57). The beta males also groomed the alpha males much more frequently than they did the 'others' (5.43 *v.s.* 1.71 time on average, P=0.003, t=4.6, SD=2.14). We did not find any significant differences in the beta male receiving or providing grooming from/to the 'others'. This differed from that observed between the alpha males and 'others', namely, alpha males groomed the 'others' much less frequently than 'others' groomed the alpha males (0.71 *v.s.* 2.29 on average, P=0.017, t=3.27, SD=1.27).

Table 3 Frequencies of grooming behavior

Group No.	A to B	B to A	B to others	Others to B	A to Others	Others to A
1	2	3	1	2	0	2
2	2	6	0	4	0	3
3	1	4	1	1	1	3
4	1	4	0	3	0	1
5	0	5	2	1	2	2
6	0	9	8	0	0	0
7	1	7	0	3	2	5
Average	1	5.43	1.71	2	0.71	2.29
Sum	7	38	12	14	5	16
P	0.003		0.858		0.017	
		0.003				

Mounting behavior

Mounting was the least frequently performed behavior among the four categories of social behaviors. As shown in Table 4, the alpha males mounted the beta males a total of five times, with beta males only mounting an alpha male on one occasion in one group, but mounting the 'others' on seven occasions. No mounting behaviors were observed from the 'others' toward either the alpha or beta males. No significant differences were observed between any two sub-categories in regards to mounting behavior.

Table 4 Frequencies of mounting behavior

Group No.	A to B	B to A	B to others
1	2	0	2
2	1	0	1
3	0	0	1
4	1	1	1
5	0	0	0
6	1	0	1
7	0	0	1
Average	0.71	0.14	1
Sum	5	1	7

DISCUSSION

In the present study, we investigated two aspects relevant to the stress conditions of beta individuals in male-only cynomolgus monkey groups; namely, the origins of stress experienced by beta males and the adaptation of beta males to stress from their own social status. Observations of various social behavioral contacts between the beta male and his cage mates were conducted to clarify these questions from a behavioral point of view.

We suggested that the origin of stress for the beta male was mainly from being attacked by the alpha male. According to our results, the beta male received 55% of aggressive behavior initiated by the alpha male. As a single target, the beta male

received more aggression from the alpha male than the total number of attacks on the other 8-13 individuals. This strongly suggests that the beta male was the main target of aggression from the alpha male. Furthermore, a different research group in our lab found a significant positive correlation between aggressive behavior and hair cortisol (a stress hormone) concentration in adult male rhesus monkeys (unpublished data); namely, the more aggression an individual received, the higher their hair cortisol concentrations, and thus the higher their stress levels. Our results indicated that beta male stress mainly originated from aggressive pressure from the alpha male in each respective group.

Individuals with a certain social status have to adapt to their specific situations, including playing their expected role and dealing with stress. As stated previously, middle-ranked human managers have to obey their higher-ranked bosses, while also leading their lower-ranked subordinates, and thereby hold special status and high levels of stress. Similarly, in the present study, the beta male held a 'middle-class' rank, thus while obeying the alpha male, he also had to maintain his beta status in the group. How do beta males adapt to the stress of middle class? We found 72.7% of aggressions when the beta male was attacked by the alpha male, after which the beta male immediately initiated aggressive behavior toward the 'others'. The beta male also attacked 'others' more frequently than the alpha male did (7.00 *v.s.* 2.57 times per group on average, P=0.0008, t=6.16, SD=1.9). The intense aggressiveness of the beta male (similar rate as that of alpha males in total, 49 *v.s.* 40; and more frequently to 'others' than that of the alpha males, 49 *v.s.* 18, P=0.0008, t=6.16, SD=1.9) was likely performed to maintain his social status as the ability to defeat group members was a determinant of social rank; on the other hand, this aggressive behavior (as suggested from the 72.7% redirection of aggression by the beta male) may be a way to release high tension from being attacked by the alpha male. Previous studies have reported that social behaviors such as grooming, avoidance and mounting can serve as important functions to reduce the stress of opponents, as well as to help decrease the frequency of conflicts (i.e., Bernstein & Ehardt, 1985; Castles & Whiten, 1998; Cords, 1992; Das et al, 1998; Faraut et al, 2015). In particular, affinitive and reconciliation behaviors play a significant role in soothing stress and reducing conflict. Many studies suggest that to maintain a relatively balanced and peaceful social environment, after fighting or threatening one another, most animals show friendliness by touching, hugging or grooming one another (Aureli et al 1989; Cheney & Seyfarth, 1989; De Waal & Roosmalen, 1979; De Waal & Yoshihara, 1983; York & Rowell, 1988). Compared with the 'others', we found that beta males showed more avoidance behavior toward the alpha males (13.86 *v.s.* 9.71 times per group, on average, P=0.018, t=3.19, SD=3.43), and groomed the alpha male more frequently (5.43 *v.s.* 1.71 times, on average, P=0.003, t=4.6, SD=2.14). We considered these beta male avoidance and grooming behaviors as strategies to relieve stress from aggression and reduce potential conflicts with the alpha male. In addition, with no significant differences in frequencies (1.71 *v.s.* 2.00 times per group on average), the grooming behaviors between beta and 'others' could also help with soothing stress of the beta male. Furthermore, although mounting behaviors between the beta and alpha males (six times in total)/'others' (seven times in total) were rare, they may have served to mitigate stress as well.

It is important to note that certain factors that may contribute to stress, such as breeding and survival issues, were controlled in our observation groups, which might also contribute to answering the questions of interest. Specifically, all monkey subjects had free access to food and water, which eliminated the stress for survival. They also had no visual contact with females, which reduced the effects of reproduction and breeding season. Therefore, beta male stress was most likely from social interactions with their male cage mates.

In conclusion, due to its middle rank within the hierarchical social group, the beta males in the captive male-only cynomolgus monkey groups experienced a special stressful situation due to aggressions from the higher-ranked alpha males. To reduce their stress and adapt to their beta status, the beta males were actively involved in social behaviors, i.e., aggression toward 'others' after being attacked, as well as receiving and preforming avoidance, grooming and mounting behavior. Further physiological studies, such as comparison of plasma/hair cortisol levels among individuals holding different social statuses, should be conducted in combination with future behavioral analysis.

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Patterns of reptile and amphibian species richness along elevational gradients in Mt. Kenya

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ABSTRACT

Faunal species richness is traditionally assumed to decrease with increasing elevation and decreasing primary productivity. Species richness is reported to peak at mid-elevation. This survey examines the herpetofaunal diversity and distribution in Mt. Kenya (central Kenya) by testing the hypothesis that changes in species richness with elevation relate to elevation-dependent changes in climate. Sampling along transects from an elevation of approximately 1 700 m in Chogoria forest block (wind-ward side) and approximately 2 600 m in Sirimon block (rain shadow zone) upwards in March 2009. This starts from the forest to montane alpine zones. Sampling of reptiles and amphibians uses pitfall traps associated with drift fences, time-limited searches and visual encounter surveys. The results show that herpetofaunal richness differs among three vegetation zones along the elevation gradient. Chogoria has higher biodiversity than Sirimon. More species occur at low and middle elevations and few exist at high elevations. The trends are consistent with expected optimum water and energy variables. The lower alpine montane zone has high species richness but low diversity due to dominance of some high elevations species. Unambiguous data do not support a mid-domain effect (mid-elevation peak) because the observed trend better fits a model in which climatic variables (rainfall and temperature) control species richness, which indirectly measures productivity. It is important to continue protection of all indigenous forests, especially at low to mid elevations. These areas are vulnerable to human destruction yet are home to some endemic species. Firebreaks can limit the spread of the perennial wildfires, especially on the moorlands.

Keywords: Herpetofauna; Elevation; Rainfall; Temperature; Species diversity

INTRODUCTION

Species of plants and animals do not have random distributions and a central aim of community ecology seeks to understand what drives the patterns (Rahbek, 1997; Sanders et al, 2003). Identifying and understanding the causes of species richness patterns in the tropics is critical as human activities and threats to biodiversity increases (Sanders et al, 2003; Smith et al, 2007). Modern interest has focused on distributions along latitudinal and elevational gradients and the processes that control these patterns (Colwell et al, 2004; Watkins et al, 2006; Sanders et al, 2012). In both patterns, an inverse relationship occurs between species richness, elevation and latitude (Rahbek, 1997; Willig et al, 2003; Carpenter, 2005; Watkin et al, 2006). Studies on elevation gradients have observed several patterns and two main patterns of species richness are common: first a monotonical decrease in richness with increasing elevation and second, a "humped" distribution, with species richness highest near the middle of the gradient (Watkins et al, 2006). Understanding species richness patterns in montane regions is important as most of them are centers of species diversity and endemism in the tropical regions (Smith et al, 2007).

Little is known about the underlying factors that govern the distributional patterns of species although much research has suggested that contemporary climate constrains terrestrial taxonomic richness over broad geographic extents due to its controlling energy dynamics (Hawkins et al, 2003a). Climatic, biological, and historical factors have been suggested to drive variation in species richness along elevational gradients (Rahbek, 1997; Sanders et al, 2003). Climate via the water–energy dynamics hypothesis has influenced primary productivity (Hawkins et al, 2003b), which in turn positively correlates with species richness (Hawkins et al, 2003a; Rahbek, 2005; Malonza & Veith, 2012). [1]

Herein, this study examines the herpetofaunal diversity along elevation gradient in Mt. Kenya and explores the probable causal factors. Mt. Kenya is one of five Kenyan water towers and a United Nations Educational, Scientific and Cultural Or-

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ganization (UNESCO) world heritage site. It is as well a key biodiversity area harbouring endemic and near endemic species. However, it hosts some of the key Kenya endemic herpetofauna namely Mt. Kenya striped Chameleon *Trioceros schubotzi*, Mt. Kenya puddle frog *Phrynobatrachus irangi*, Mt. Kenya Hornless Chameleon *Kinyongia excubitor*, Alpine Meadow Lizard *Adolfus alleni* Mt. Kenya Bush Viper *Atheris desaixi* and Kenya Montane Viper *Montatheris hindii*. Despite its relevance and importance, no comprehensive survey of reptiles and amphibians exists. Most studies involve species descriptions, a few notes on species and general anecdotes. Andrén (1976) provided a list of reptiles with brief comments from the lower alpine zone (Ontulili, northwestern Mt. Kenya) and Reilly (1982) noted the ecological characteristics of *Trioceros schubotzi*, an alpine zone species. No detailed analysis considers elevation, ecology, natural history or distribution of the herpetofauna of Mt. Kenya. To rectify this dearth of analyses, this manuscript reports on a short survey done in Chogoria (windward side) and Sirimon (leeward side) blocks of forest that start at approximately 1 700 m and 2 600 m a.s.l., respectively.

This study evaluates the influence of elevation on the distribution of herpetofaunal assemblages. The hypotheses that amphibian and reptilian composition does not change with elevation and that patterns of species richness relate to climatic factors as a function of changes in elevation were tested.

MATERIALS AND METHODS

Study area

Located in central Kenya, the administration of Mt. Kenya is shared by greater Meru (Meru and Tharaka-Nithi), Embu, Kirinyaga and Nyeri counties. The lower slopes are covered by mixed indigenous forest that depending on the windward or leeward side gives way to bamboo then montane grasslands and heather (alpine) zones. On the windward side, forest starts at elevations of about 1 700 m a.s.l., while on the extreme leeward side at about 2 600 m a.s.l.. Herpetofaunal sampling was carried out between March 4th and March 24th 2009 at elevations for about 1 700 m to 3 200 m in Chogoria and from 2 600 m to 3 800 m in Sirimon.

Sampling sites

On each transect, four sampling points were selected for both Chogoria and Sirimon based on the length of each transect from the forest edge to the alpine zone. Chogoria was longer than Sirimon. The former was sampled every 6 km and the latter every 4 km. For Chogoria, sampling started at the Chogoria Forest Station and for Sirimon around Sirimon Gate. These sites fell within different vegetation/habitat zones that associated with elevation (Table 1). Vegetation zonation occurred clearly with increasing elevation along Chogoria but Sirimon had a mixture of *Erica* and bamboo occurring almost from the base of the forest zone.

Patterns of species richness

Reptiles and amphibians were sampled using three methods. First, time-limited searches (TLS) were conducted as described by Karns (1986), Heyer et al (1994) and Sutherland (1996). All possible amphibian and reptilian microhabitats, such as wetlands, under leaves debris, on trees, decomposing tree stumps and logs, including digging, were searched intensively for one person/hour. Second, visual (VES) and acoustic encounter surveys (AES) were used only for gathering qualitative and semi-qualitative data mainly for presence or absence of species (Rödel & Ernst, 2004; Veith et al, 2004). Finally, drift fences and pitfall traps were set up in X-shaped arrays a modification of Corn (1994) with segments of 5 m in length. The pitfall traps consisted of 10 L plastic buckets located flush with the ground and every trap had five buckets. Traps were set for three days (trap nights) and checked once every morning before 0730h. Equal sampling effort was applied within mixed indigenous forests, bamboo and montane grassland zones. Amphibian specimens were humanely euthanized with MS222 and reptiles with pentobarbital solution and then preserved in 10% formalin and in the lab stored in 70% ethanol solution. Selected tissue samples for later molecular analysis were taken and stored in absolute ethanol. Voucher and DNA materials collected are deposited in the Herpetology Section reference collection at the National Museums of Kenya (NMK), Nairobi.

Table 1 Eight sampling sites selected on both Chogoria and Sirimon transect routes

Sampling site	Coordinates	Vegetation zone
Chogoria 6 km	S00°14′06.7″, E037°32′32.28″, 2 030 m	Mixed forest (scattered *Podocarpus* and *Ocotea*)
Chogoria 12 km	S00°12′27.4″, E037°29′57.7″, 2 402 m	Mixed forest (Scattered *Podocarpus*)
Chogoria 18 km	S00°10′30.6″, E037°27′32.6″, 2 741 m	Bamboo(with *Podocarpus* on hillsides)
Chogoria 24 km	S00°08′45.3″, E037°25′10.2″, 3 188 m	Montane grassland: *Erica/Protea*; *Juniperus/Hagenia* on valleys
Sirimon 4 km	N00°00.860′, E037°14.802′, 2 636 m	Mixed forest (*Juniperus* dominated)
Sirimon 8 km	S00°00.936′, E037°16.257′, 2 906 m	Mixed forest (*Podocarpus* and bamboo dominated)
Sirimon 12 km	S00°02.589′, E037°17.122′, 3 270 m	Montane grassland (*Erica* dominated)
Sirimon 16 km	S00°02.589′, E037°18′06.5″, 3 778 m	Montane grassland

Statistical analysis

Species richness and diversity for every collecting site was estimated from the TLS data using EstimateS 8.2 (Colwell, 2009). Species diversity was estimated using the Shannon Index (*H*'). Chao 1, ACE, and Jackknife 1 species richness estimators were used and compared to the observed species (*Sobs*). Species accumulation curves of observed species were generated using EstimateS based on 1 000 randomizations. In the curves, the species richness was plotted as a function of the accumulated number of samples (number of time-limited

searches). One way ANOVA was used to test the differences in mean species diversity among the different vegetation types in the two transects. Data were analyzed with STATISTICA 6.0 (StatSoft, 2001) with a significance level of 5%.

RESULTS

Species richness and diversity

Trapping was unsuccessful; only one specimen was caught during the sampling period. TLS and VES were the most successful methods (Table 2) of assessing the species diversity. Species diversity indices in the Chogoria transect for forest, bamboo and montane zones were 1.71, 0.92 and 0.92, respectively, and 0.69, 0.56 and 0.92 for Sirimon, respectively. Species diversity among the two transect vegetation zones differed highly significantly (ANOVA: $F_{1,4}$=47.02, n=6, P=0.0023). Though diversity was highest in the forest zone in Chogoria, the alpine zone hosted the most species. In comparison, Sirimon had very poor species richness and diversity (Table 2). Whereas the entire Sirimon transect had five species, species were confined to habitat edges.

Observed species richness and number of individuals per transect differed among vegetation types. In the alpine/monatne zone *Trachylepis varia* and *Adolfus alleni* were the most abundant species in Chogoria and *Adolfus jacksoni* on the forest zone. In Sirimon, species abundance was generally very low in the entire transect. In its alpine/montane zone *Adolfus alleni* and *Trioceros schubotzi* were the only species encountered, and then only occasionally.

The first order Jackknife estimator of species richness was higher than the real number of species observed (Sobs) in most of the counts. In the lower alpine/montane and indigenous zones of Chogoria, the species accumulation curve did not reach an asymptote (Figure 1), indicating that more species could be recorded with additional sampling. However, the curves plateaued for Sirimon transect sites

Table 2 Numbers of species recorded in different habitat types using the three methods along the two transects

Transect	Mixed indigenous forest			Bamboo dominated			Montane/alpine zone		
	TLS	Trap	VES	TLS	Trap	VES	TLS	Trap	VES
Chogoria									
Reptiles	5	0	4	3	0	0	8	1	4
Amphibians	1	0	3	0	0	0	0	0	2
Sirimon									
Reptiles	2	0	1	2	0	0	2	0	2
Amphibians	0	0	0	0	0	0	0	0	0

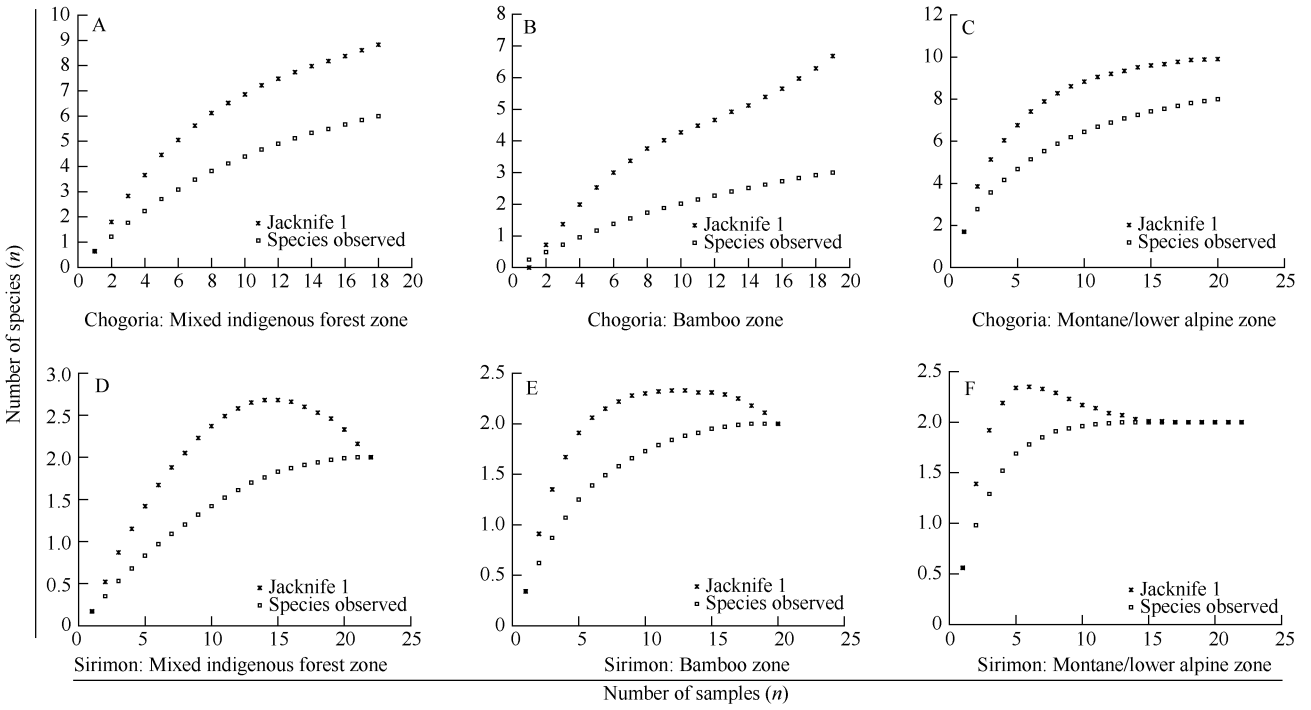

Figure 1 Species accumulation curves of the two transect sites with more than one observed species showing species richness in different vegetation zones

indicating that it was unlikely that additional sampling would discover more species.

Elevational species turnover

There is no support for the hypothesis that amphibian and reptilian composition does not change with elevation. Ecological separations of some species occurred clearly as elevation changed (Figure 2). The chameleon *Trioceros schubotzi* was found mainly above 3 000 m while its relative, *Trioceros hoehnelii*, occupied in areas from around 3 100 m and below. *Trioceros jacksoni* also occurred up to an elevation of about 3 000 m. Some lizards normally known to occur above 3 000 m, such as *Trachylepis irregularis* and *Adolfus alleni*, were recorded below this elevation. For example, *A. alleni* occurred in a grassland (natural seasonal grassed dam or forest glade) patch at 2 400 m. This was within the Chogoria forest zone inhabited by *A. jacksoni*. There was no clear species richness pattern because species richness peaked twice (Figure 3).

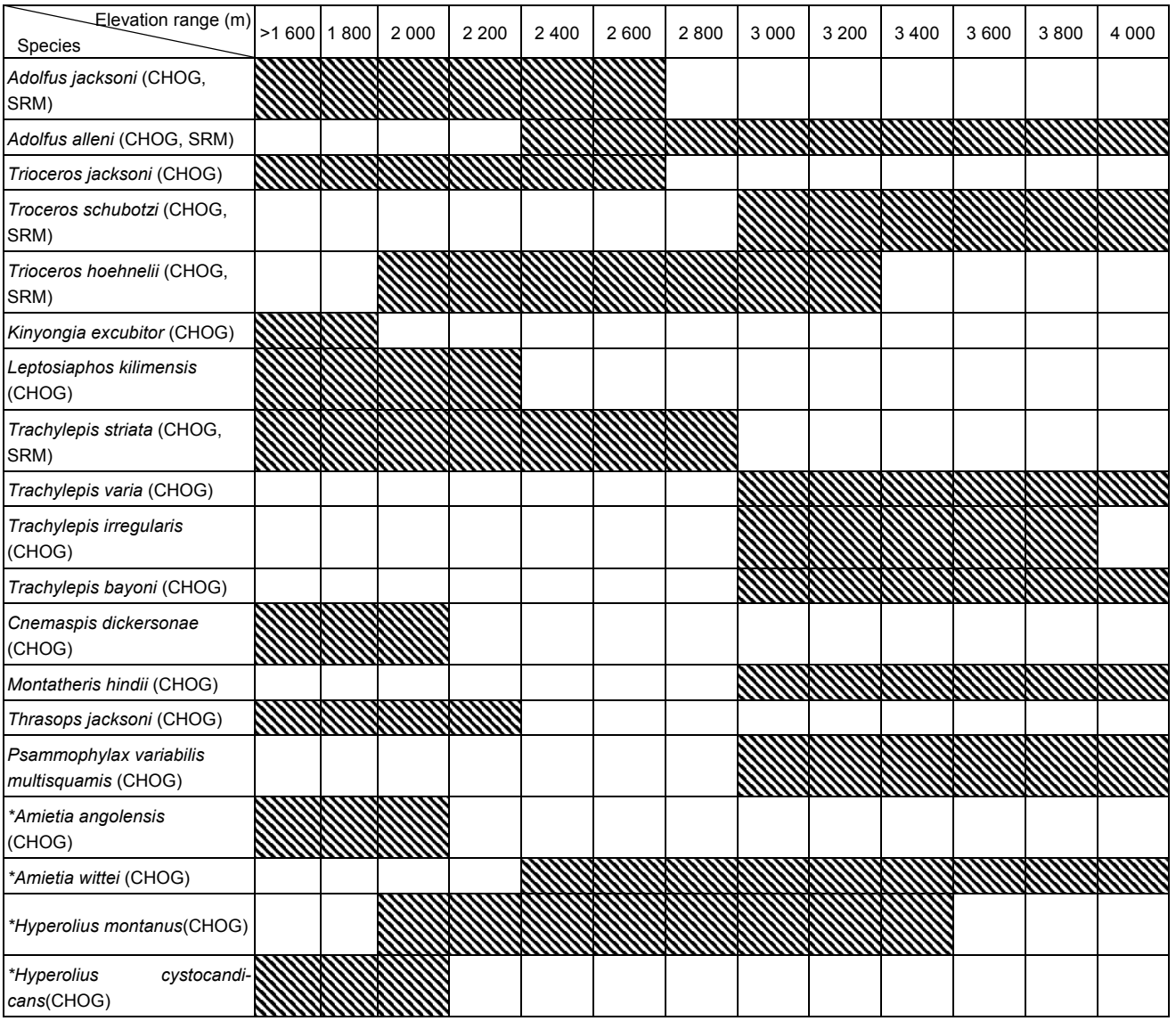

Figure 2 Elevational ranges (m) of reptiles and amphibians on Mt. Kenya

Transect where a species was recorded is given in parentheses (CHOG=Chogoria, SRM=Sirimon); Amphibians are denoted with an asterisk.

DISCUSSION

The results show that the abundance and richness of species differ significantly among the three vegetation types (forest, bamboo, montane). Montane (lower alpine, 3 000-4 000 m) and the lower forest zone (1 700-2 000 m) have the greatest number of species. Time-limited searching shows that the bamboo zone is very depauperate in both species richness and

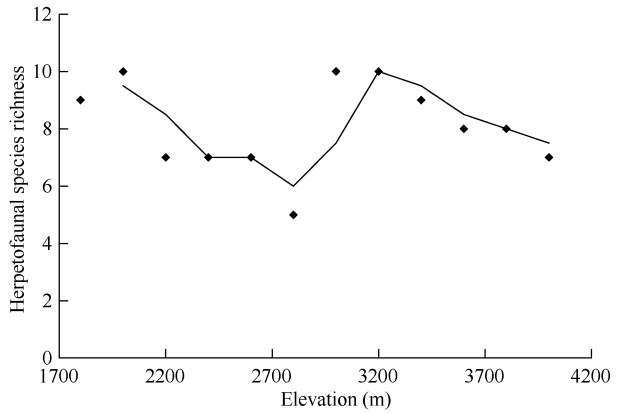

Figure 3 Elevational pattern of species richness along the Chogoria transect

species abundance. This is a common feature of pure stands of vegetation such as exotic plantations (Evans, 1982). In general, more species occur in Chogoria, which is on the windward side and receives more rainfall, than Sirimon, which is on the leeward side and dry.

The results demonstrate mixed patterns of species turnover with more species at the mixed forest zone around 1 700-2 200 m and at mid-elevation around 3 000 m as the mountain rise up to around 5 200 m. The results does not support the hypothesis that amphibian and reptilian composition does not change with elevation. Species richness increases with increasing elevation up to a certain level. Although there is no data for amphibians during the wet season in this study, reptile species richness peaks between 3 000 m and 4 000 m (lower alpine zone) and exceeds that of the forest zone (1 700-3 000 m). The species of reptiles in the lower alpine zone are those reported by Andrén (1976) from the lower alpine zone of Aberdare and Mt. Kenya National Parks.

The higher number of species in Chogoria compared to Sirimon likely owes to high rainfall and intermediate temperatures. Rainfall and temperature have been shown to serve as indirect measures of primary productivity for birds (van Rensburg et al, 2002), ants (Sanders et al, 2003) and amphibians (Malonza & Veith, 2012). The pattern of more species at low and mid elevations in this study agrees with patterns reported for a wide range of taxonomic groups, such as small mammals (Heaney, 2001) and tree frogs (Smith et al, 2007). High species richness at lower elevations (Chogoria) associates with high energy and productivity (e.g., Hawkins et al, 2003a; Willig et al, 2003). However, past studies have repeatedly shown that many taxonomic groups including birds, amphibians, invertebrates, mammals and plants exhibit mid-elevational peaks, the so-called mid-domain effect (MDE) in species-richness (e.g., Rhabek, 1997; Sanders, 2002; Sanders et al, 2003; McCain, 2004; Smith et al, 2007). However, in the Mt. Kenya ecosystem this, this does not occur due to the concurrent influences of latitude and elevation.

The total number of species recorded in this study may not be exhaustive and more studies are highly recommended covering different seasons. Mt. Kenya is a diverse ecosystem that is underexplored and new species may still exist. The endemic Mt. Kenya Puddle frog *Phrynobatrachus irangi* was not recorded during this period despite being expected especially in Chogoria forest block.

The results of this study show that elevation, temperature and rainfall are highly interdependent variables. In high elevation areas, rainfall is high while temperatures and species richness are low. Therefore, the occurrence of few species at high elevations may be due to physiological constraints (Navas, 2006). Climate (rainfall and temperature) influences primary productivity that in turn affects species diversity (Hawkins et al, 2003b; Sanders et al, 2003).

Future studies on the drivers of variation in species richness along environmental gradients should consider the multiple mechanisms that contribute to the pattern. This study indicates that elevation, temperature and rainfall influence the regional species richness of reptiles and amphibians on Mt. Kenya.

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Morphometric studies of genus *Placocheilus* (Teleostei: Cypriniformes) from Red River, China

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ABSTRACT

It is practically difficult to differentiate *Placocheilus robustus* and *Placocheilus caudofasciatus* from Red River drainage of China. Without stated reasons, *P. robustus* has been assumed as the synonyms of *P. caudofasciatus*. The present study aims to decipher the morphological differences between two species so as to provide reliable clues for their classification by multivariate morphometry. A total of 72 specimens of two species in genus *Placocheilus* were examined. Besides morphological character comparisons, 10 anatomic landmarks were utilized and 23 frame structures and 15 general characters measured. The scatter plot results of principal component analysis showed that all specimens were clustered together and could not be defined as two distinct species. No significant morphological differences existed in four diagnostic characters between *P. robustus* and *P. caudofasciatus*. Thus the results of the present study fail to support *P. robustus* as a valid and independent species.

Keywords: *Placocheilus caudofasciatus*; *Placocheilus robustus*; Mophomemetrics; Principal component analysis; Evidence for taxonomy

INTRODUCTION

The genus *Placocheilus* was established by Wu (1977) on the basis of the description of type species *Discognathus caudofasciatus* Pellegrin et Chevey. *Placocheilus* was originally described as of order Cypriniformes, subfamily Barbininae and later re-classified into subfamily Labeoninae. The genus *Placocheilus* is distributed in Dulong River, Nujiang River and Yuanjiang River-Honghe River (Red River) drainage, Yunnan Province, China; Nam Na Basin in Lai Chau, Vietnam and Nam Ma Basin in Laos (Chen et al, 2012; Chu & Cui, 1989; Cui & Li, 1984; Kottelat, 2001; Wu, 1977; Zhang et al, 2002). The species of genus *Placocheilus* is rheophilic and its lower lip has been modified into a mental adhesive disc (Chu & Cui, 1989). So far, the genus contains four species: *P. caudofasciatus* Pellegrin & Chevey, *P. cryponemus* Cui & Li, *P. robustus* Zhang et al and *P. dulongensis* Chen et al.

For quite some time, the *Placocheilus* from Yuanjiang River-Honghe River drainage was deemed as *P. caudofasciatus*. Zhang et al (2002) re-evaluated the structures of scaleless midventral belly region, ratio of depth/length of caudal peduncle, and length/width of mental adhesive disc. Herein the *Placocheilus* from Yuanjiang River-Honghe River drainage was re-classified into two independent species: *P. caudofasciatus* from Lixian River with its branches in Yunnan, China as well Tuojiang River (Heishui River or Black River, lower Lixian River Basin), Vietnam and Nam Ma Basin in Laos, all belong to the tributaries of Honghe drainage; and a new species, *P. robustus*, from Yuanjiang River and its tributaries of Red River Basin. However, due to the difficulties of discriminating *P. robustus* and *P. caudofasciatus*, the classification has been usually based on collecting sites. As stated in *Checklist of Fishes of Yunnan*, *P. robustus* was considered to be the synonym of *P. caudofasciatus* without explanations (Chen, 2013). The taxonomic status of these two species should be further clarified. [1]

The conventional morphological measurements have limitations in comprehensiveness and accuracy. That is to say, the measuring distance extends along both horizontal and vertical coordinates and it is rather restricted to head and caudal peduncle areas. As a result, it is impossible to cover the entire body surface (Xie et al, 2003). The multivariate morphometry overcomes the above shortcomings (Bookstein et al, 1985) so that it has been successfully applied for population measurements. In other words, determining the validity of existing species or conjecture unknown species by evaluating the morphological differences among congeners (Cai et al, 2001; Xie et al, 2003; Yang et al, 2003). Through multivariate

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morphometry, Li et al (2008) reported that one single fish species *Pseudecheneis sulcata* from different drainages actually belonged to several different species. The findings of Yang et al (2011) and Yang et al (2013) accorded with the previous results of *Discogobio yunnanensis* and *Garra orientalis,* and no intraspecies difference was detected. And Min et al (2009) revealed no morphological differentiations among different populations of *Sinocyclocheilus grahami* from the same drainage and confirmed the validity of the species.

In the present study, by adopting multivariate morphometry and principal component analysis, through measuring external morphological characters, including longitudinal, lateral and oblique distances, the major morphological differentiations and morphometric differences between *P. robustus* and *P. caudofasciatus* were compared. These findings provide evidence for clarifying the taxonomic status of these two species.

MATERIALS AND METHODS

Materials

The specimens (*n*=72) of *P. caudofasciatus* and *P. robustus* were deposited in Museum of Animal Section of Southwest Forestry University (SWFU) and Museum of Fish Section of Kunming Institute of Zoology (KIZ) (Table 1, Figure 1). The specimens Zhang et al (2002) used for describing *P. robustus* as a new species are currently conserved at KIZ. And 53 specimens at SWFU collected from Phona Tho at Lai Chau, Noire [Song Da] River at Lai Chau, Black River, Vietnam, were utilized for observing scale coverage only.

Study methods

Conventional measuring methods and multivariate morphometric studies were combined for specimen measurements. Multiple variable statistics (principal component analysis) was used for statistical analysis.

Morphological character measurements

A total of 10 anatomical coordinates were selected (Figure 2). With the left side of body taken as the bench mark, 23 frame characters and 15 general characters were measured. The measurements of countable and general characters were performed per Kottelat (2001); disc width and length per Zhang et al (2002). The scale coverage was observed under binocular microscope (Nikon, SMZ645). The length of scaleless region (distance from the origin line of pectoral fins to scaleless midventral belly region) was determined and its percentage in total length from the origin of pectoral fin to ventral fin calculated. Lineal distances between anatomical coordinates were obtained by an electronic digital caliper (accuracy=0.1 mm).

Statistical analysis

Logarithmic (log10) transformation of all morphological character data were processed with Microsoft Excel 2003 for eliminating the variations caused by size differences among the specimens (Xie et al, 2003; Li et al, 2008). Principal component analysis was conducted by SPSS 17.0 for Windows. Standard

Table 1 List of examined specimens

Species	Specimen origin	Collection site	Collection date	Numbers of specimens (*n*)
P. robustus	KIZ	Lvshuihe hydrostation (upstream), Pingbian	2012.04	2
		Honghe	1960.05	12
		Yuanjiang	2012.04	1
	SWUF	Biaheqiao, Pingbian	1997.12	1
		Yuanyang	1999.02	1
		Huayudong, Hekou	2014.07	3
		Biaheqiao, Pingbian	2013.07	3
		Huayudong, Hekou	2013.07	5
		Yuanjiang Town, Yuanjiang	2006.05	1
P. caudofasciatus	KIZ	Nama River (upstream), Vietnam	2008.11	20
		New food market, Yuanyang, Lvchun	2009.06	3
	SWUF	Bashahe River, Daheishan, Lvchun	2009.11	5
		Bashahe River, Daheishan, Lvchun	2005.06	8
		Jinshuihe Town	2003.01	2
		Daheishan Village, Lvchun	2014.07	2
		Bashahe River, Daheishan, Lvchun	2013.11	3
Total				72

KIZ: Museum of Fish Section of Zoology Kunming Institute of Zoology, Chinese Academy of Sciences; SWFU: Museum of Animal Section of Southwest Forestry of University Southwest Forestry University

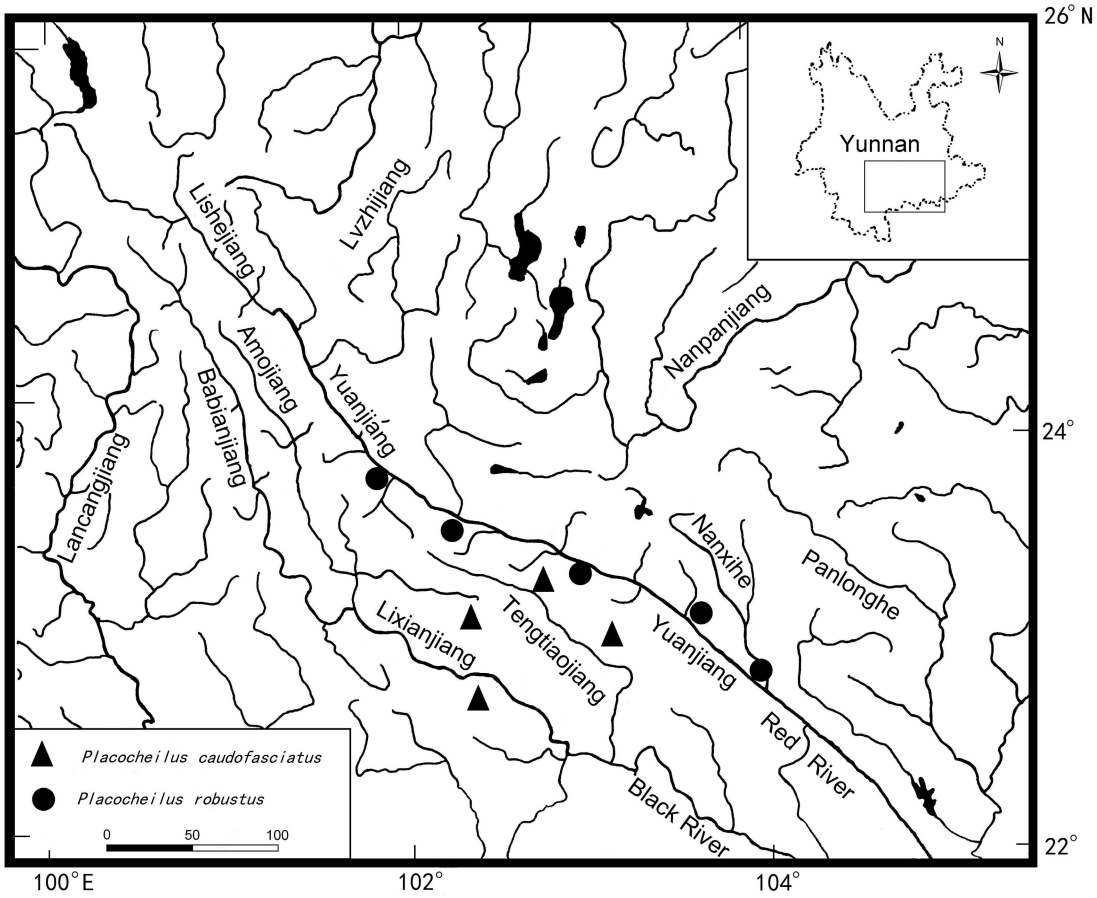

Figure 1 Distribution maps of *Placocheilus caudofasciatus* and *Placocheilus robustus*

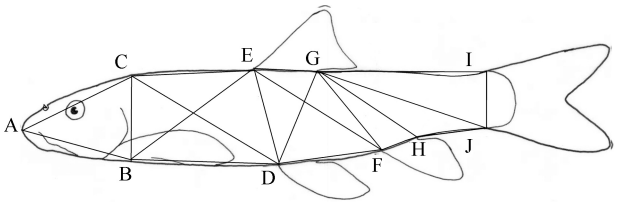

Figure 2 Morphometric frame characters of *Placocheilus caudofasciatus*

A: Snout tip; B: Pectoral fin origin; C: Supraoocipital end; D: Ventral fin origin; E: Dorsal fin origin: ; F: Anal fin origin; G: Dorsal fin base end; H: Anal fin base end; I: Caudal fin dorsal origin; J: Caudal fin ventral origin

data transformation was completed by the default settings for factorial analysis. Covariance matrix and Varimax were applied for factorial analysis. And scatter plots were constructed on the basis of the scores of principal components.

RESULTS

Comparison of morphological characters

Besides scale coverage of midventral belly region, caudal peduncle and length/width of mental adhesive disc were listed (Zhang et al, 2002). In the present study, the rays of fins, scales in lateral line and scales in peduncle were also compared between *P. caudofasciatus* and *P. robustus*. Yet no significant differences existed in none of the relevant characters (Table 2, Table 3).

Principal component analysis

The specimens were divided into two groups according to their collection sites: group from Yuanjiang River Basin and its tributaries (*P. robustus*) and group from Lixian River Basin (*P. caudofasciatus*). The principal component analysis of 23 frame characters and 15 general characters showed that the variances of PC1, PC2 and PC3 were 48.093%, 29.866% and 12.297% respectively with an accumulative variance of 90.257% (Table 4). Scatter plots regarding PC1 vs PC2 and PC2 vs PC3 were constructed (Figure 3). The scatter plots indicated that these two *Placocheilus* species were non-distinguishable.

DISCUSSION

Pellegrin (1936) described *Discognathus caudofasciatus* on the basis of one holotype of MNHN 1935-0327. And it was collected from Noire (Song Da) River at Lai Chau, Black River, Vietnam.

Table 2 Morphological character comparisons between *Placocheilus caudofasciatus* and *Placocheilus robustus*

Species	*P. caudofasciatus*		*P. robustus*	
	Present study	Zhang et al (2002)	Present study	Zhang et al (2002)
Number of specimens (*n*)	43	22	29	20
Standard length (mm)	32.3-81.7	48.0-76.5	31.7-116.8	48.6-117.2
Dorsal fin (D)	3, 8	3, 8	3, 8	3, 8
Anal fin (A)	3, 5	3, 5	3, 5	3, 5
Caudal fin (C)	i+8-7+i	–	i+8-7+i	–
Ventral fin (V)	1, 8	1, 7	1, 8	1, 8
Scales in lateral line	39-42	40-41	39-41	39-42
Scales in peduncle	12	12	12	12
Scale coverage of midventral belly region	–	1／4	–	3／4
	Range (mean±*SD*) (%)	Range (%)	Range (mean±*SD*) (%)	Range (%)
Disc length/disc width	74.0-92.6 (83.5±4.1)	44.5-49.2	70.1-91.7 (83.5±6.2)	37.0-43.1
Disc width/head length	41.2-63.2 (53.6±4.4)	54.8-66.2	44.3-65.0 (55.1±5.0)	58.9-65.2
Disc length/head length	33.0-53.4 (44.7±4.0)	44.5-49.2	35.1-52.9 (45.9±4.5)	37.0-43.1
Caudal peduncle depth/length	50.0-97.0 (72.1±10.5)	63.7-71.3	52.4-78.3 (67.7±8.9)	72.4-82.5

Table 3 Midventral region scale coverage of *Placocheilus caudofasciatus v.s. Placocheilus robustus*

	P. caudofasciatus		*P. robustus*
	Lixiang River & its tributaries (upper Black River)	Song Da River at Lai Chau, Vietnam	Yuanjiang River & its tributaries
Number of specimens (*n*)	43	53	29
Stand length (mm)	32.3-81.7	53.0-106.8	31.7-116.8
Length of scaleless region /P-V distance (%)	0.00-0.72 (0.28±0.20)	0.00-0.85 (0.40±0.27)	0.29-0.94 (0.62±0.20)

Table 4 Principal component analyses of *Placocheilus caudofasciatus v.s. Placocheilus robustus*

Characters	Results			Characters	Results		
	PC1	PC2	PC3		PC1	PC2	PC3
Ventral fin origin-dorsal fin origin (DE)	0.8467	0.4004	0.2344	Caudal peduncle depth	0.6900	0.4428	0.4204
Body depth	0.8414	0.3916	0.2489	Snout length	0.6820	0.5803	0.3692
Pectoral fin origin-supraoccipital end (BC)	0.8379	0.4490	0.0085	Caudal fin ventral origin-caudal fin dorsal origin (JI)	0.6816	0.4283	0.4192
Head depth	0.8303	0.4332	0.2781	Caudal fin dorsal origin-anal fin base end (IH)	0.6735	0.5576	0.4214
Ventral fin origin-dorsal fin base end (DG)	0.8301	0.4373	0.2199	Caudal fin ventral origin-dorsal fin base end (JG)	0.6587	0.2965	0.3419
Dorsal fin origin-anal fin origin (EF)	0.7990	0.5026	0.2338	Head length	0.6557	0.6102	0.3066
Dorsal fin base end-anal fin base end (GH)	0.7846	0.5097	0.3173	Pectoral fin origin-dorsal fin origin (BE)	0.6107	0.4793	0.3990
Anal fin origin-dorsal fin base end (FG)	0.7800	0.4992	0.3123	Caudal fin ventral origin-anal fin base end (JH)	0.5887	0.5219	0.5079
Supraoocipital end-dorsal fin origin (CE)	0.7767	0.3866	0.3632	Eye diameter	0.3401	0.8249	0.0984
Interorbital distance	0.7716	0.4604	0.3684	Anal fin length	0.3237	0.6925	0.4077
Body depth at anus	0.7690	0.4437	0.3247	Snout tip-supraoocipital end (AC)	0.6396	0.6788	0.2408
Snout tip-dorsal fin origin (AE)	0.7409	0.5603	0.3513	Ventral fin length	0.6416	0.6545	0.3436
Head width	0.7386	0.5648	0.3272	Pectoral fin length	0.6455	0.6513	0.3577
Snout tip-anal fin origin (AF)	0.7245	0.5879	0.3068	Snout tip-pectoral fin origin (AB)	0.5953	0.6488	0.2843
Snout tip-ventral fin origin (AD)	0.7245	0.5906	0.3095	Anal fin origin-anal fin base end (FH)	0.6172	0.6454	0.1985
Standard length	0.7236	0.5780	0.3450	Head length of posterior eye	0.5796	0.6364	0.3901
Dorsal fin base end-caudal fin dorsal origin (GI)	0.7140	0.5509	0.3669	Caudal peduncle length	0.1770	0.1601	0.8959
Ventral fin origin-anal fin origin (DF)	0.7083	0.5715	0.3349	Total	18.2756	11.3491	4.6730
Dorsal fin origin-dorsal fin base end (EG)	0.6937	0.6411	0.1702	Variance (%)	48.0936	29.8662	12.2974
Supraoocipital end-ventral fin origin (CD)	0.6911	0.5966	0.2302	Cumulative variance (%)	48.0936	77.9598	90.2572
Pectoral fin origin-ventral fin origin (BD)	0.6903	0.5880	0.2950				

Alphabet codes are the same as in Figure 2

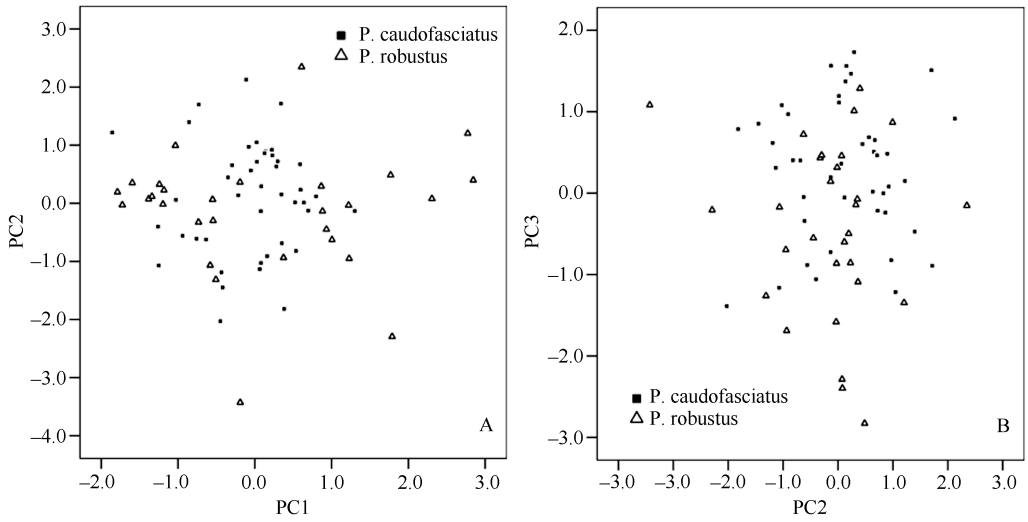

Figure 3 Scatter plots of principal component analysis

The specimens currently preserved at SWFU were also collected from Noire (Song Da) River at Lai Chau, Vietnam. Thus the above specimens are de facto topotypes.

As reported by Zhang et al (2002), comparing with *P. caudofasciatus*, *P. robustus* was characterized by stouter caudal peduncle, smaller mental adhesive disc, medium-sized scaleless midventral belly region extends slightly beyond halfway from pectoral fin to ventral fin origin, whereas scaleless midventral region in *P. caudofasciatus* is limited to basal area of pectoral fin. Significant variations of scale coverage existed in specimens from Phona Tho at Lai Chau, Noire [Song Da] River at Lai Chau, Black River, Vietnam, in fact, these scale coverage variations include all the midventral belly region scale coverage status in *P. caudofasciatus* and *P. robustus* specimens from Vietnam and China. Here the character data were different from those in Zhang et al (2002). Other than inevitable measurement variations from different experiments, we assume that the major reason for these differences was the body lengths of specimens fell into a wide range. Therefore, among various-sized individuals, significant variations in some body parts were observed due to allometry. Based upon the data here and Zhang et al (2002), most diagnostic character measurements from Zhang et al (2002) were lined up end-to-end between two species. Therefore some inter-species differences were implicated. However there were significant overlaps with the data here (Table 3). It is concluded that the diagnostic characters of Zhang et al (2002) for distinguishing *P. caudofasciatus* and *P. robustus* were not firmly supported.

Long-term geographic isolation blocks both genetic and unit communications among different populations. Thereafter, differentiations in morphology, ecology, physiology and biochemistry, or even reproductive isolation would occur as outcomes. When reproductive isolation happens, two populations are two independent species. However, in taxonomic studies, populations from different collection sites, but with similar or close physical forms are usually classified on the basis of their morphological characters. In the present study, the results of both external measurements and morphometry showed that no significant morphological differences existed between *P. caudofasciatus* and *P. robustus*, indicating that both were actually of one species and the latter was the synonym of the former. And it was in accordance with the results of Chen (2013). These conclusions are also supported by the results of muscular and skeletal anatomy (Li et al, 2016). The phylogenetic tree showed that *P. caudofasciatus* and *P. robustus* are gathered together. The genetic distance based on *Cyt b* showed that the distance among the effective specices of *Garra* and *Placocheilus* were 5% or higher; whereas, that between *P. caudofasciatus* and *P. robustus* was only 4.2%, suggesting that *P. robustus* was the synonym of *P. caudofasciatus* (Wang, 2012). Even though the morphological and molecular results were mutually supportive, the genetic distance between *P. caudofasciatus* and *P. robustus* (4.2%) was still higher than the standard criteria (3%) for discriminating species in DNA barcoding studies. Thus for clarifying the species effectiveness, further molecular phylogenetic studies are warranted.

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