

SPECIES-SPECIFICITY IN COMMUNICATION CALLS OF TREE SHREWS (*TUPAIA*: SCANDENTIA)

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Tree shrews are small mammals living in the tropical forest of Southeast Asia. The habitus of species within the genus *Tupaia* is often quite similar, so that it is difficult to differentiate the species based on their morphology. We applied comparative bioacoustics, a tool successfully used to discriminate cryptic species of nocturnal mammals, to investigate whether species in the diurnal genus *Tupaia* can be recognized noninvasively on the basis of a conspicuous loud call, the chatter. We studied to what extent the chatter call of 2 tree shrew species, *Tupaia glis* and *T. belangeri*, differed in acoustic structure. We also acoustically analyzed the chatter call of *T. chinensis*, a subspecies or closely related parapatric species of *T. belangeri*. Analyzed acoustic features allowed assigning chatter calls with a probability of more than 73% to the species that produced them. Bioacoustical differences are in line with subtle morphological differences, supporting species status for all 3 studied tree shrew species and corroborating immunodiffusion and genetic data that differentiate *T. glis* and *T. belangeri*. Loud calls may offer a reliable noninvasive tool for species diagnosis and discrimination in cryptic species of this diurnal mammalian group.

Key words: evolution, species diagnosis, species-specificity, taxonomy, *Tupaia*, vocalization

Species- or subspecies-specific differences in vocalizations used for intraspecific communication have been identified as an important taxonomic supplement to traditional morphological and more recent molecular systematic data sets for species diagnosis and discrimination (e.g., nocturnal primates [Ambrose 2003; Anderson et al. 2000; Braune et al. 2005; Olivieri et al. 2007; Zimmermann et al. 2000], diurnal primates [Hodun et al. 1981; Macedonia and Taylor 1985; Oates et al. 2000; Snowdon et al. 1986], mole rats [Nevo et al. 1987], fur seals [Page et al. 2002], song sparrows [Patten et al. 2004], and túngara frogs [Pröhl et al. 2006]). Furthermore, comparative bioacoustical analyses according to the methods of phylogenetic systematics helped to assess phylogenetic affinities among different species of a genus (orangutans [Davila Ross and Geissmann 2007], sportive lemurs [Méndez-Cardenas et al. 2008], felids [Peters and Tonkin-Leyhausen 1998], and bushbabies [Zimmermann 1990]).

Tree shrews belong to the order Scandentia and are grouped within the Euarchonta combining Scandentia, Primates, and Dermoptera (Janecka et al. 2007; Murphy et al. 2001;

Nishihara et al. 2002). These diurnal, small-bodied mammals inhabit tropical forest areas of southern and southeastern Asia. Because of the similar habitus of the species within the genus and the difficulty to differentiate species based on their morphology, tree shrews are considered a cryptic species complex (e.g., Mandahl 1976). In the last 90 years, the number of species included in the genus *Tupaia* has decreased because of taxonomic revision from 32 (Lyon 1913) to 11 (e.g., Corbet and Hill 1992; Martin 2001). Since the 1st species of tree shrew was described in 1820, 120 species or subspecies have been proposed within the Scandentia (Olson et al. 2005).

Tupaia glis and *T. belangeri* are distributed in the Indo-malayan region and seem to be separated geographically by the Isthmus of Kra in southern Thailand (Corbet and Hill 1992; Endo et al. 2000a; Lyon 1913). However, there is also evidence that both species occur sympatrically between the Isthmus of Kra and the Hai Yai District, south of the Isthmus of Kra (Endo et al. 2000b; Helgen 2005; Hirai et al. 2002). *Tupaia chinensis* is distributed in southern China, Laos, northern Thailand, and Vietnam as well as in eastern Burma (Lyon 1913) and occurs allopatrically to *T. glis* and *T. belangeri*. *T. chinensis* differs from both of the latter species being about 30% smaller (Toder et al. 1992), having a smaller skull as well as a shorter rostrum (Lyon 1913). Examination of morphologic, immunodiffusion, and genetic data suggests a clear separation of *T. glis* from *T. chinensis* and *T. belangeri*, whereas the status of *T. chinensis* is still unanswered. Often *T. chinensis* is treated as a subspecies

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(Dene et al. 1980; Lyon 1913) or closely related parapatric species of *T. belangeri* (Helgen 2005).

Tree shrews show a high auditory sensitivity (Heffner et al. 1969; Zimmermann 1993), are vocal, and use various vocalizations during social interactions (Kaufmann 1965; Martin 1968). The vocal repertoire was described in detail for *T. belangeri* (Binz and Zimmermann 1989). It consists of 8 acoustically distinct call types, which were associated with specific contact, aggression, defense, and alarm situations. Moreover, Schehka et al. (2007) showed for *T. belangeri* that the arousal state of females is expressed acoustically during defensive threat displays toward males. No comparable information is available yet for *T. glis* or *T. chinensis*. Based on listening to tree shrews in Borneo, Emmons (2000) postulated that each species can be identified by its distinctive loud call, which is uttered during alarm situations as well as in the context of disturbance. Toder et al. (1992) also mentioned differences in vocalizations of *T. glis*, *T. belangeri*, and *T. chinensis*. However, to date no comparative quantitative analysis has been carried out to test these assumptions.

We explored to what extent comparative bioacoustics can help in diagnosing cryptic species in this diurnal, nonprimate mammalian group and may provide insight into taxonomic questions. We tested the hypothesis of Emmons (2000) that the loud call in tree shrews, uttered when disturbed, can be used as a vocal fingerprint for noninvasive diagnosis and discrimination of species. We induced the loud call in individuals of 3 tree shrew species, *T. glis*, *T. belangeri*, and *T. chinensis*. We then quantified the acoustic structure of this context-homologous call for each species by multiparametric sound analysis and compared it between species by multiparametric statistics. We postulated that differences in acoustic structure are more distinct between different species (*T. glis* and *T. belangeri*), than between subspecies or closely related parapatric species (*T. belangeri* and *T. chinensis*).

MATERIALS AND METHODS

Tupaia glis and *T. chinensis* were kept in the animal facility of the Department of Animal Physiology, University of Bayreuth, Bayreuth, Germany, and *T. belangeri* in the animal facility of the Institute for Zoology, University of Veterinary Medicine, Hannover, Germany. Animals were maintained under standardized conditions as described by von Holst (1969) and Hertenstein et al. (1987). All studied individuals were born in captivity. Founders of *T. glis* came from Kuala Lumpur (Malaysia), *T. chinensis* from Kuming (Republic of China), and *T. belangeri* from the Bangkok area and Bang Saphan (Thailand). Founders of tree shrews used in this study were genetically characterized by Toder et al. (1992).

Animals were housed singly in wire mesh cages from 0.25 m³ to 1.8 m³, provided with at least 2 nest boxes and a variety of branches and several wooden resting shelves. Up to 8 animals of 1 species were maintained together in 1 room, in which they had acoustic and olfactory contact with each other, but no tactile contact. Under these conditions individuals of all 3 species uttered a specific loud call, named chatter (Binz and

Zimmermann 1989), as soon as the experimenter, an individual not known to the tree shrews, entered the animal room. Over a period of 10 min all calls of 1 focal animal were recorded. Four to 10 calls per individual, uttered during silence of the other animals present and with best acoustic quality, were selected for further acoustic analyses. Calls of *T. glis* and *T. chinensis* were recorded in Bayreuth either with an Uher report monitor 4200 tape recorder (Assmann, Bad Homburg, Germany) and a Sennheiser microphone (type MKH 816 [20–40 kHz]; Sennheiser Electronic, Wedemark, Germany) or with a Nagra IV-SJ tape recorder (Nagra Kudelski, Cheseaux, Switzerland) and Bruel and Kjaer B&K microphone (type 4133 [20–40 kHz]; Bruel and Kjaer, Svendborg, Denmark). Tape recordings of chatter calls with best acoustic quality were digitized with BatSound Pro software (version 3.31; A/D-card: sampling rate 44,100 Hz, 16 bits, mono; Petterson Elektronik AB, Uppsala, Sweden). Chatter calls of *T. belangeri* were recorded in Hannover with a Sennheiser microphone (type ME 64 [20–40 kHz]; Sennheiser Electronic, Wedemark, Germany), amplified by a M-Audio DMP3 preamplifier (Avid Technology, Öhringen, Germany), digitized at a sampling frequency of 100 kHz using the software NIDisk (Engineering Design, Belmont, Massachusetts; A/D converter: DAQCard-6062E; National Instruments, Austin, Texas) and stored as uncompressed audio files on a laptop (Toshiba Satellite A10-s100; Toshiba, Irvine, California). The experiments are licensed by the Bezirksregierung Hannover, Germany (reference number 509.6-42502-03/660) and comply with the guidelines of the American Society of Mammalogists (Gannon et al. 2007).

We analyzed the acoustic structure of chatter calls from 6 adult individuals of *T. glis* ($n = 60$ calls), 7 of *T. chinensis* ($n = 66$ calls), and 6 of *T. belangeri* ($n = 24$ calls) by oscillograms and power spectra using BatSound Pro 3.31 (Petterson Elektronik AB). In all 3 species a chatter call consisted of a variable number of short, frequency-modulated harmonic units called syllables. We determined the middle point of each analyzed chatter call by dividing the duration (time between onset of the 1st syllable and offset of the last syllable) by 2 and chose 2 syllables next to this point for further detailed acoustic analyses. We analyzed a total of 292 syllables by characterizing 3 acoustic parameters: peak frequency (kHz), syllable duration (ms), and intersyllable interval (ms). The 2 temporal parameters were measured by oscillograms, and peak frequency by power spectra over the first 10 ms of a syllable (Fig. 1). We selected this time interval at call onset to exclude potential echo effects.

Statistical analyses were performed with SPSS 12.01 for Windows (2003) (SPSS Inc., Chicago, Illinois) and Statistica 6.1 for Windows (StatSoft, Tulsa, Oklahoma). Using univariate statistics, we calculated the median for each measured acoustic parameter per subject. Using these medians, we applied the nonparametric Kruskal–Wallis test for each parameter to test whether there were any significant differences among the 3 species. To analyze the extent to which pairs of species differ acoustically from each other, we applied nonparametric 2-tailed Mann–Whitney *U*-tests and adjusted afterward the *P*-value according to the Bonferroni correction. To analyze whether the 3 tree shrew species can be discriminated by acoustic

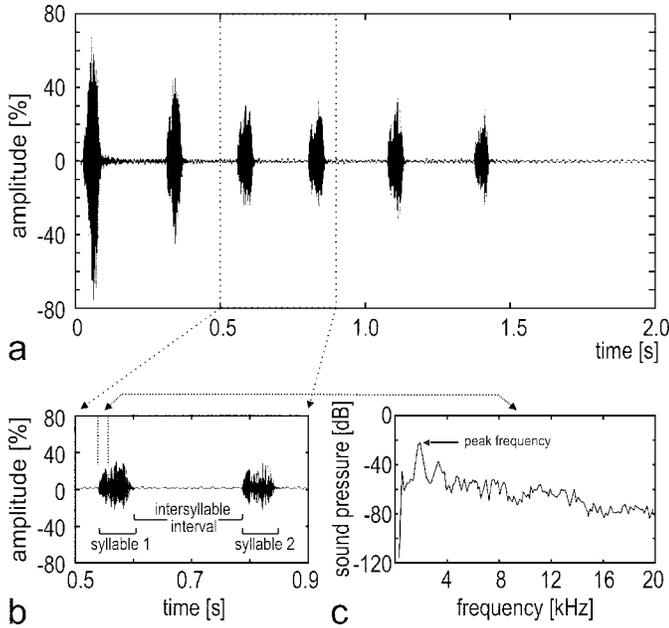


FIG. 1.—Acoustic measurement by a, b) oscillogram and c) power spectrum of a chatter call of *Tupaia belangeri*. Peak frequency was measured from a power spectrum over the first 10 ms of a syllable.

characteristics of the chatter call, we performed a permutation-based discriminant function analysis for a nested design as described in Mundry and Sommer (2007). To balance the effect of individuals, we used 4 randomly selected chatter calls per subject. We repeated the random selection 100 times and performed a discriminant function analysis with each of the resulting data sets. We then calculated the average numbers

of correctly classified selected calls and cross-classified calls and used them as the overall results. We then performed a permutation-based discriminant function analysis with 1,000 permutations. Within each randomized data set, we again randomly selected 4 chatter calls per subject to be included in the calculation of the discriminant functions. The permutation-based discriminant function analyses were done using a script written by R. Mundry (MPI for Evolutionary Anthropology, Leipzig, Germany). This script runs in R (R Development Core Team 2007) and requires the library MASS (Venables and Ripley 2002) to be loaded. For all tests, $P < 0.05$ was chosen as the level of significance. Because our data were generally not normally distributed, results are reported as medians and interquartile ranges (IQRs).

RESULTS

Chatter calls of all 3 species were uttered as series composed of multiple syllables. Their measured peak frequency corresponded to the fundamental frequency in all 3 species. However, there were remarkable species-specific differences in acoustic structure (Fig. 2).

Acoustic characteristics of chatter calls in the 3 tree shrew species.—A distinct feature of the chatter call in *T. glis* is that syllables are generally fused with each other at the beginning of the call. A syllable has a median peak frequency of 0.84 kHz (IQR 0.14 kHz) and a median duration of 45.13 ms (IQR 8.08 ms). The median duration of the intersyllable interval is 31.90 ms (IQR 10.21 ms).

A characteristic feature of the chatter call in *T. belangeri* is that the syllable interval is always longer than the syllable

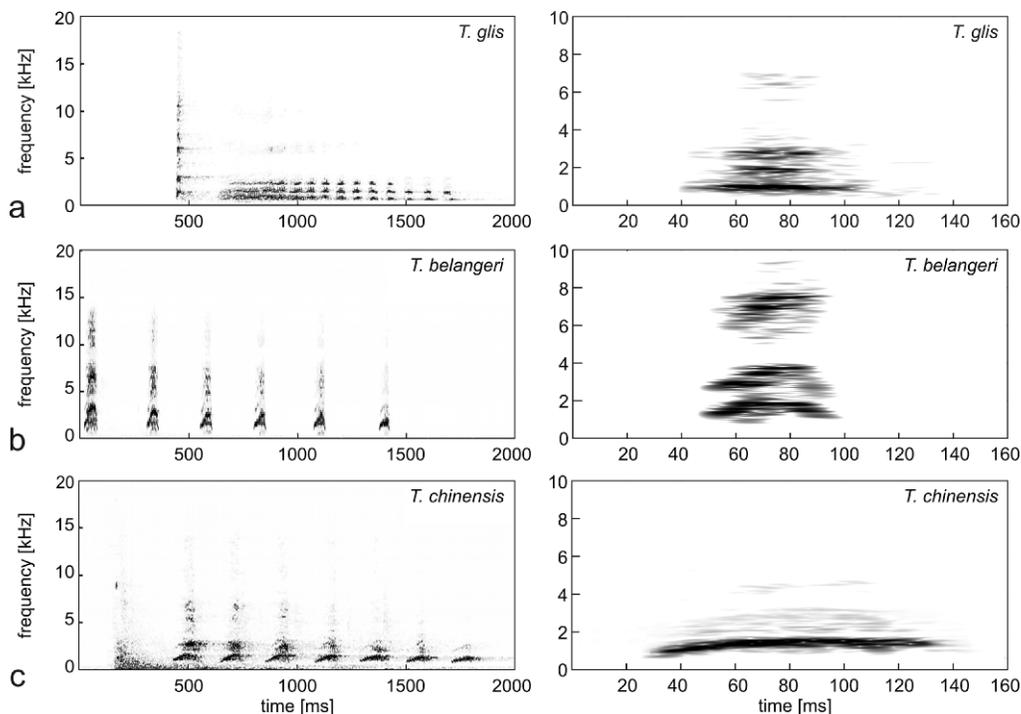


FIG. 2.—Sonograms of a representative chatter call (left side) and enlarged sonograms of a syllable (right side) of a) *Tupaia glis*, b) *T. belangeri*, and c) *T. chinensis*.

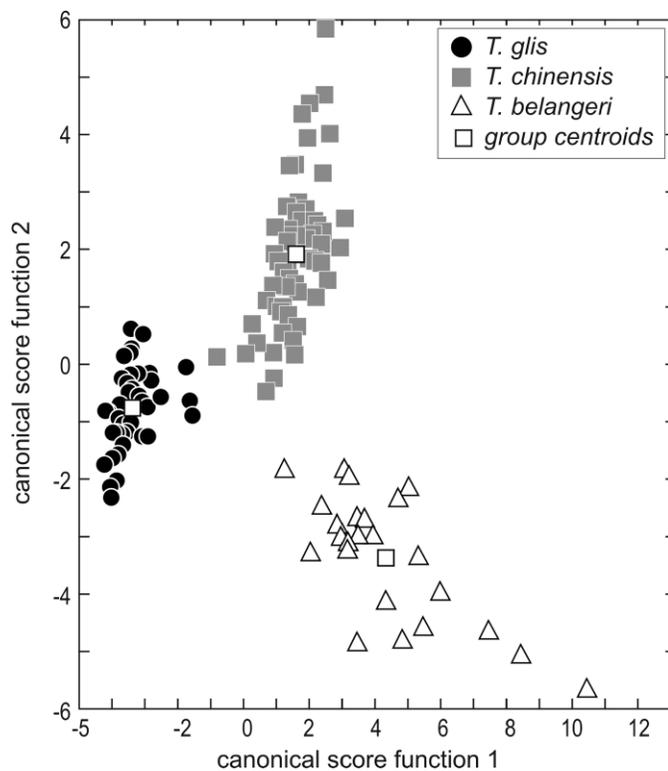


FIG. 3.—Scatterplot of the canonical variates based on the discriminant analysis of the acoustic parameters of the chatter calls of 3 species of tree shrews (*Tupaia glis*, *T. belangeri*, and *T. chinensis*). Each point represents the median value of an analyzed chatter call; different symbols represent the respective species. Squares indicate the group centroids.

duration. Therefore, the median interval with 225.25 ms (IQR 73.38 ms) is more than 4 times longer than the median duration with 51.70 ms (IQR 7.75 ms). The median peak frequency lies at 1.26 kHz (IQR 0.23 kHz).

The chatter call of *T. chinensis* shows a similar acoustic design as that of *T. belangeri*; however, it differs in rhythm. Thus, syllable duration is the longest of all 3 species with a median of 100.75 ms (IQR 20.00 ms). This duration is nearly as long as the calculated median intersyllable interval of 105.00 ms (IQR 20.88 ms). The median peak frequency is 1.16 kHz (IQR 0.15 kHz).

Comparison of chatter calls between the 3 species.—A significant effect of species on the acoustics of the chatter call was revealed for all measured acoustic parameters (Kruskal–Wallis test, duration: $H = 13.87$, $P = 0.001$; interval length: $H = 16.01$, $P < 0.001$; peak frequency: $H = 12.79$, $P = 0.002$).

Between *T. glis* and *T. belangeri*, significant differences were found in intersyllable interval (Mann–Whitney U -test with followed Bonferroni correction, $Z = -2.88$, $P = 0.007$) and peak frequency ($Z = -2.88$, $P = 0.007$), but not in duration ($Z = -1.76$, $P = 0.28$). Between *T. chinensis* and *T. belangeri*, significant differences were revealed in duration ($Z = 3$, $P = 0.004$) and intersyllable interval ($Z = -3$, $P = 0.004$), but not in peak frequency ($Z = -1.50$, $P = 0.413$).

Furthermore, significant differences between *T. glis* and *T. chinensis* appeared in all measured acoustic variables (duration: $Z = -3.0$, $P = 0.004$; intersyllable interval: $Z = -3.0$, $P = 0.004$; peak frequency: $Z = -3.0$, $P = 0.004$). In all statistical analyses, the temporal parameter intersyllable interval differed significantly among the 3 species. Consequently, this parameter seems to be the most important acoustic parameter for discriminating the different tree shrew species.

The discriminant function analysis yielded an average rate of correctly classified selected calls of 75.72% and an average rate of correctly cross-classified calls of 73.41%. Fig. 3 visualizes the conspicuous separation of analyzed chatter calls according to the 3 species. The permutation-based discriminant function analysis supported our revealed clear assignment of calls to the respective species and showed a highly significant ability to discriminate among the acoustic characteristics of chatter calls of the 3 tree shrew species (originally included chatter calls: $P = 0.001$; cross-classified chatter calls: $P = 0.001$). This result showed that the measured acoustic parameters provided reliable information for identifying and discriminating the 3 species.

DISCUSSION

Our results revealed that cryptic species of tree shrews can be recognized reliably not only by listening, but also on the basis of the acoustic structure of a context-homologous loud call, the chatter. Differences were found in frequency- or temporal-related acoustic features, or both, among all 3 species and allowed assigning calls with a probability of >73% to the species that produced them.

The chatter call of *T. glis* and *T. belangeri* differed significantly in intersyllable interval and peak frequency, acoustic features that are related to the temporal as well as frequency domain. The extent of acoustic variation supports cytogenetic and immunodiffusion findings and reflects species-specificity. Differences in the structure of the chatter call between *T. belangeri* and *T. chinensis* were only found in temporal-related acoustic features, intersyllable interval, and syllable duration. This result corroborated our hypothesis that the variation in acoustic structure was more distinct between different species than between subspecies or closely related parapatric species.

The conspicuous differences in the acoustic contour of the chatter call among all 3 species are on a level on which species status is suggested for members in other mammalian groups, such as nocturnal and diurnal primates, birds, or frogs (e.g., Ambrose 2003; Anderson et al. 2000; Braune et al. 2005; Hodun et al. 1981; Macedonia and Taylor 1985; Nevo et al. 1987; Oates et al. 2000; Olivieri et al. 2007; Page et al. 2002; Patten et al. 2004; Pröhl et al. 2006; Snowdon et al. 1986; Zimmermann et al. 1988, 2000). Because in mammals dialectal acoustic differences are subtle variations of the same acoustic contour, not easily detectable by listening (e.g., see Hafen et al. [1998] for this situation in nocturnal lemurs), this phenomenon does not seem to explain our results. The chatter call of 2 colonies of *T. belangeri* (German Primate Center in Göttingen

[J. Kirchof, pers. comm.], and Institute for Zoology in Stuttgart-Hohenheim [Binz and Zimmermann 1989]) corresponds in acoustic contour to the chatter call described for this species in the present study. Thus, acoustic differences detected between species were not likely related to colony-specificity, which has been reported in mammals as subtle changes of the same general frequency contour (Zimmermann and Hafen 2001). Our bioacoustical results strongly suggest that all 3 species differed on species-specific levels. Our findings support not only cytogenetic and immunodiffusion differences found between *T. belangeri* and *T. glis*, but also morphological differences between *T. belangeri* and *T. chinensis*.

All 3 species of tree shrews probably live in different habitats with differing sound-producing communities and differing habitat acoustics. Therefore, the acoustic adaptation hypothesis (Hansen 1979; Morton 1977; Rothstein and Fleischer 1987) may help explain the evolution of differences in chatter calls. However, to what extent this hypothesis may explain the acoustic differences in the chatter call between the 3 species of tree shrews is not yet known and has to be explored by further studies including the habitat acoustics and socioecology of the respective species in sympatry and allopatry.

Differences in acoustic structure may be caused by vocal learning, for example, as revealed for songbirds (Catchpole and Slater 1995). However, there are theoretical reasons and empirical evidence arguing against vocal learning in our case and favoring the assumption that these differences are inherited. Mammals are not known for having extensive abilities for vocal production learning (Egnor and Hauser 2004; Janik and Slater 2000). Furthermore, the "absentee" maternal care system in tree shrews (Martin 1968), in which the mother returns to her infants every 2nd day to suckle them for about 5 min until they leave their nest at 4 weeks, does not predispose infants for vocal production learning. Indeed, infant tree shrews of at least 1 species already exhibit chatter calls with an almost adultlike structure (*T. belangeri*—Benson et al. 1992). Thus, vocal production learning in tree shrews seems to be fairly implausible for explaining the revealed species-specific acoustic differences. Instead, these differences may be caused by genetic divergence between the species.

Genetic divergence may lead to changes in the vocal production and most likely perception system. Divergences in the size and mass of laryngeal structures (often related to body size), in the rate at which vocal cords open and close as well as in the control of muscles for breathing and vocal production (Fitch and Hauser 2002; Lieberman and Blumstein 1988; MacLarnon and Hewitt 2004) may explain acoustic differences according to the source filter theory of speech production. They may partly account for species-specific differences in the median peak frequency (equivalent in our measurements to fundamental frequency) and the rhythm of syllables of the chatter call in tree shrews. Regarding the effect of body size on fundamental frequency, we found that *T. glis* with the largest body size of all 3 studied species of *Tupaia* (Lyon 1913; Toder et al. 1992) has indeed the lowest median peak frequency. However, *T. belangeri* with a medium body size has a higher median peak frequency than *T. chinensis* with the smallest

body size. Thus, fundamental frequency cannot fully be predicted by differences in body size. The same also is true for the measured temporal characteristics. There is no relation between body-size differences of the species and species-specific divergences in syllable duration or intersyllable interval. It is likely that the revealed species-specific acoustic differences of the chatter call of tree shrews are linked to specific differences in the vocalization-related neural network, as found for squirrel monkeys (Hage and Jürgens 2006).

The process of allopatric speciation with the evolution of specific communication calls may be initiated by geographic events that induce the separation of the gene pool of a species (e.g., Campbell and Reece 2006). Specific species assemblages of plants (Ridder-Numan 1998), insects (Corbet 1941), amphibians and reptiles (Inger and Voris 2001), and mammals, including tree shrews (Corbet and Hill 1992), were found at either side of the Isthmus of Kra separating Sundaic and Inochinese biotas. It was suggested that this distribution pattern of species is explained by fluctuations of sea level. According to Woodruff (2003), Neogene marine transgressions flooded the Thai–Malay Peninsula around the Isthmus of Kra in 2 areas. During the early–middle Miocene (24–13 million years ago [mya]) and again during the early Pliocene (5.5–4.5 mya) sea levels were about 100 m above the present-day level. This phylogeographic scenario might have favored allopatric speciation in tree shrews. Paralleled by specific acoustic adaptation to the environment, it may have accounted for the emergence of species-specificity in the loud call chatter of *T. glis* and *T. belangeri*. A similar scenario may explain the acoustic distinctiveness of the chatter call in *T. chinensis*. In addition to adaptation, genetic drift and founder effects may be responsible for changes in call structure, as suggested for other mammalian radiations (Blumstein 1999; Daniel and Blumstein 1998; Nikolsky 1981). Moreover, further selection factors such as predator pressure (Marler 1955), social complexity (Blumstein and Armitage 1997), or abundance (Fotheringham et al. 1997; Sorjonen 1986; Tubaro and Segura 1994) may have played a role in the evolution of species-specificity in chatter calls of tree shrews.

All in all, our results confirmed that cryptic species of tree shrews show specific and conspicuous acoustic differences in a context-homologous loud call, the chatter. Thus, acoustic fingerprinting may be used as a noninvasive tool for species diagnosis and discrimination in the order Scandentia. Moreover, our results suggest that comparative bioacoustics provides an insight into taxonomic questions of cryptic diurnal mammalian groups. Acoustic fingerprinting offers an easy and inexpensive way to determine species in the field and in the laboratory. It provides a practical means for monitoring the biodiversity of this secretive mammalian group in nature. Furthermore, vocal fingerprinting can be used for species diagnosis in laboratories, in which tree shrews are used as models for biomedical research, as well as in zoos.

ZUSAMMENFASSUNG

Spitzhörnchen sind kleine, in den tropischen Regenwäldern Südostasiens beheimatete Säugetiere. Die verschiedenen Arten der Gattung *Tupaia* ähneln sich äußerlich sehr stark, weshalb eine Unterscheidung allein aufgrund ihres äußeren Habitus sich häufig als sehr schwierig erweist. Mittels der Methode der vergleichenden Bioakustik, einem nichtinvasivem Verfahren, das schon erfolgreich zur Diskriminierung kryptischer Arten nachaktiver Säugetiere angewendet wurde, haben wir in dieser Studie ermittelt, ob verschiedene Arten innerhalb der tagaktiven Gattung *Tupaia* anhand ihres spezifischen Loud Calls, dem sogenannten Chatter-Laut, erkannt werden können. Wir haben untersucht, inwieweit sich der Chatter-Laut zweier Spitzhörnchenarten, *Tupaia glis* und *T. belangeri*, hinsichtlich seiner akustischen Struktur unterscheidet. Zudem haben wir den Chatter-Laut von *T. chinensis*, einer unter- oder nah verwandten parapatrischen Art von *T. belangeri*, akustisch analysiert. Anhand der analysierten akustischen Parameter konnte eine 73%ige Zuordnung der Chatter-Laute zur entsprechenden Art erfolgen. Die bioakustisch ermittelten Unterschiede stehen im Einklang mit geringen morphologischen Divergenzen und stützen damit die Annahme des Artstatus für alle 3 untersuchten Spitzhörnchenarten. Des Weiteren untermauern die akustischen Ergebnisse Daten zur Immunodiffusion und Genetik, auf denen die Unterscheidung der 2 Spitzhörnchenarten *T. glis* und *T. belangeri* beruht. Die Studie zeigt zudem, dass Loud Calls ein verlässliches nichtinvasives Werkzeug zur Erkennung und Diskriminierung kryptischer Arten tagaktiver Säugetiergruppen darstellen.

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