Kasetsart J. (Nat. Sci.) 44 : 590 - 603 (2010)

Review Article

A Multi-Approach Survey as the most Reliable Tool to Accurately Assess Biodiversity: an Example of Thai Murine Rodents

Yannick Chaval[1]*, Gauthier Dobigny[1], Johan Michaux[1], Marie Pagès[1], Céline Corbisier[1], Jean-François Cosson[1] and Vincent Herbreteau[2]

ABSTRACT

Wildlife surveys rely on an accurate taxonomic framework. Identification tools used to reach this goal are not equivalent and may depend on several objectives and constraints, including sampling conservation difficulties, the invasiveness of the sampling techniques, sampling capacity, the relevance of the results, materials needed, the cost and the user time required in the field and laboratory. This article presents and discusses the advantages and limits of each identification tool used in the Ceropath (Community ecology of rodents and their pathogens in South East Asia) program to reach a fast and relevant identification of the rodents sampled. It is concluded that there needs to be a combination of the results from different methods, including the most recent ones, to achieve an improvement in taxonomic identification.

Keywords: morphology, morphometry, cytotaxonomy, molecular phylogeny, barcoding

INTRODUCTION

Species-specific identification remains one of the most critical steps in biology. This is particularly true for applied programs, such as epidemiological studies. Indeed, each species may display a specific immunity or to be the host of a specific pathogen. It is also the case for agronomic and ecological studies, since species may have specific crop incidence, population dynamics, behavior and/or ecology. Consequently, all field surveys must rely on a rigorous systematic framework. South East Asian ecosystems are exceptionally rich and shelter a number of important endemic species (Myers *et al*., 2000). Unfortunately, this biodiversity is currently threatened by rapid and extensive habitat destruction. Yet, it remains poorly documented (Baillie *et al*., 2004; Srikosamatara *et al*., 2004) and needs urgent investigation. In Thailand, the Order Rodentia represents a substantial part of mammalian diversity, including 26% of the known species. Within the subfamily Murinae (Old World rats and mice), at least 35 species have been inventoried on the basis of morphological criteria (Marshall, 1977a). Most of these species are

1 Centre de Biologie et de Gestion et des Populations (CBGP), Campus International de Baillarguet, CS 30016, 34988 Montferrier sur lez, France.

2 Maison de la télédétection, 500 rue Jean-FranSois Breton, 34093 Montpellier Cedex 5

* Corresponding author, e-mail: chaval@supagro.inra.fr

Received date : 07/02/09 Accepted date : 01/04/10

considered as vectors for many human pathogens (Wangroongsarb *et al*., 2002; Coleman *et al*., 2003) and are the focus of several epidemiologic studies. Morphology, morphometry and karyology (Marshall, 1977b, 1986; Corbet and Hill, 1992; Musser and Brothers, 1994; Aplin *et al*., 2003a; Aplin *et al*., 2003b) have long served as references to identify Thai rodents. However, recent technical advances, especially involving DNA-based approaches, have allowed the development of new powerful tools for species assignment. In an attempt to apply such molecular approaches, clear discrepancies with species assignment based on morphological characters were evidenced (Pagàs *et al*., *In Press*), thus highlighting the requirement of a deep taxonomic revision of Thai rodents. Therefore, such work has been initiated through a Thai-French collaborative project that aimed at investigating Murid rodent communities, as well as their associated parasites and pathogens (ANR projects "Hantavirus Roboviroses", 2006-2009, and "Ceropath", 2009-2012). The current study reviews some of the main tools that are now available to field workers for a valuable assessment of species-specific status in rodents, with a special emphasis on murid rodents in Thailand. Although alternative methods may also be useful (for example, eco-ethology features), this study focuses on the approaches that are currently used in the authors' projects, that is, morphology, morphometry, cytotaxonomy and DNA-based methods. Finally, the relevance of each approach is discussed in regard to scientific and/or management purposes, techniques accessibility and funding aspects.

Review of rodent species assignment tools

Morphology and morphometry

One of the major challenges in systematics is to distinguish phenotypic and genotypic variations within one single species from those that are associated with species-specific differences. Morphology and morphometry-based identification largely relies on metric and non-metric traits that result from genetic expression, but are also greatly influenced by animal status (for example, male versus female, juvenile versus adult) and environmental conditions. As such, they may be very sensitive to local adaptations, as well as to age and sex. As a consequence, such criteria usually require the rigorous establishment (taking into account at least the age, sex and geographic origin of the animals) of identification keys, as well as users with a good level of experience. This method remains the most widely employed technique to identify rodents, as it is historically among the oldest, cheapest and most accessible for quick identification. Moreover, it is the most practical method when working in the field.

The first step in the identification of a rodent usually relies on the general morphology and often refers to the absolute and/or relative proportions of the head + body versus the tail, the hind foot and the ear shape. In the particular case of Thai rodents, the tail morphology has also proven to be informative. Indeed, sometimes the difference between species is based on scale shape or coloration pattern. For example, in Thailand, *Rattus* species present a dark tail, while *Niviventer fulvescens* (Gray, 1847) has a characteristic tail that is bi-colored over its full length. *Maxomys surifer* (Miller, 1900) also has a bi-colored tail, but it ends with a conspicuous white tip. At the species level, the head + body:tail ratio allows the rapid differentiation between *Rattus losea* (tail shorter than head + body) and *Rattus tanezumi* (tail nearly equal to the head + body), even though the tails of both species display a similar morphology.

In addition, some genera present distinctive colors for the dorsal and ventral furs. *Leopoldamys sabanus*, *Maxomys surifer* and *Niviventer fulvescens* have similar coloration, with a red-brown back and a white-cream belly, both parts being sharply demarcated. On the contrary, in Thailand, species from the *Rattus* genus present a dark-brown fur above and creamy underbody

(note that *Rattus rattus* despite this coloration is commonly named the black rat). The texture of the fur sometimes also reveals some diagnostic features. The variations in hair length, thickness, form and/or density may provide characteristics peculiar to each species. Four different furs can be distinguished from the outside toward the inside: guard hairs (longer and slightly thicker than the others), contour hairs (that contribute to the major coloration), spine hairs (a part of the contour hairs and are very rough to touch) and under-fur hairs (with a woolly texture, covering the skin). The most striking example is probably the rough texture of the fur of the representatives of the genus *Maxomys*, which are commonly called the spiny rats.

The hairs covering the hind foot may also display various patterns, ranging from pure white (for example *Niviventer* sp., *Rattus norvegicus*) to dark (for example *Bandicota indica*). This fur is usually a mix of dark and clear hairs (for example *Rattus tanezumi*), but some species present also a clearly delimited dark strip on the hind foot (for example *Berylmys bowersi*, *Leopoldamys* spp.).

The color of incisors may sometimes serve as an identification criterion. For instance, the genus *Berylmys* is characterized in Thailand by white teeth, while the genera *Bandicota* and *Maxomys* possess yellow-orange teeth. This particular characteristic has even driven the Thai naming of the two species from the genus *Berylmys* (*B. bowersi* and *B. berdmorei*) which are locally called white-teeth rats (หนูฟันขาว = Nu Fan Kao).

The second step towards a more precise identification relies on morphometrics, which implies taking some measurements, usually starting with the head and body length, the tail length and the hind foot length without claws. The latter are classical linear parameters used in rodent systematics. Their comparative analysis may be powerful for species-specific diagnosis, although this may also have limitations. From there, osteological (usually cranial) preparations open the gate to more linear measurements (on skulls and teeth) that help greatly when further discrimination is required (Musser and Brothers, 1994). They can be used for diagnostic purposes through either independent comparisons of each measurement (the so-called univariate approach) or through more complex calculations that take into account several measurements within the same analysis, thus increasing the diagnostic potential. The latter quantitative approach usually relies on multivariate statistics, such as principal components analysis, canonical analysis and multivariate analysis of variance, amongst others.

Importantly, these criteria, whether qualitative (morphological) or quantitative (univariate and multivariate morphometrics), reflect phenotypic variations, and so all have the drawback of being dependant on age and sex, as well as potential local adaptations. This is the reason why, like any other method, a morphologically-based approach must take into account the intra-specific variability and users must be able to distinguish it from the interspecific variance. Another important constraint of morphometric tools is that they rely on statistical analyses, thus requiring large series of references and well documented records.

Finally, identification based on morphology and morphometry can be misleading due to biological reasons. Indeed, many cases of sibling (that is morphologically identical though reproductively isolated) species have been documented in several instances, especially in rodents (see part 3). In such cases, morphology and morphometry are not helpful, due to the intrinsic lack of interspecific variance. Then, it is necessary to rely on alternative methods based on different characters, such as genetic factors. This is the main reason why a multi-approach appears to be reasonable and preferred in most instances. As a conclusion, the adequacy of differentiating between different features (morphology,

karyotypes, DNA sequencing and ecology, amongst others) is probably the most reliable argument to accurately identify biological species, and to assess biodiversity (Ducroz *et al*., 1997; Volobouev *et al*., 2002; Denys *et al*., 2003; Volobouev *et al*., 2007).

Cytotaxonomy

Cytotaxonomy is the application of cytogenetic techniques to α-systematics. It relies on the assumption, now well documented, especially in mammals, that sufficiently divergent karyotypes (the chromosomal complement of all eukaryotic cells) ensure reproductive isolation (King, 1993). As such, the comparison of karyotypes may be a valuable way to quickly demonstrate most probable inter-sterility, thus demonstrating that two specimens belong to two different biological species. As a consequence, like any other method described in this paper, cytotaxonomy may point out differences between specimens that may be species-specific. Contrary to alternative methods, these differences may be identified as a real cause of inter-sterility, thus, making them unambiguously species-specific differences. However, like any other approach, there are limits that can be illustrated once again through Thai rodent examples. The karyotypes of some Thai murids were described by several authors (Yong, 1968; Yong, 1969; Yosida *et al*., 1971; Markvong *et al*., 1972; Gropp *et al*., 1973; Markvong *et al*., 1973; Yosida, 1973; Marshall, 1977a). However, no recent work is available, and banding data remain scarce and incomplete. For this reason this study revisited some of the karyotypic features on the basis of a reasonably large sample. To do so, 76 specimens belonging to 17 taxa were karyotyped either from bone marrow in the field, or from fibroblast cell lines that were settled in the laboratory (CBGP, Montpellier, France).

This study, which is based on conventional banding, as well as some molecular cytogenetic techniques, is completed (Badenhorst, 2009) and provide results for 17 rodents (including 15 murine) species, namely *Mus cookii* (Ryley, 1914), *Mus cervicolor* (Hodgson, 1845), *Mus caroli* (Bonhote, 1902), *Rattus tanezumi* (Temmink, 1844), *Rattus exulans* (Peale, 1848), *Rattus losea* (Swinhoe, 1871), *Leopoldamys edwardsi* (Thomas, 1882), *Maxomys surifer*, *Niviventer fulvescens*, *Berylmys berdmorei* (Anderson, 1879), *Berylmys bowersi* (Blyth, 1851), *Bandicota indica* (Bechtein, 1800), *Bandicota savilei* (Thomas, 1916), *Chiropodomys gliroides* (Blyth, 1856) and *Hapalomys delacouri* (Thomas, 1927), as well as the squirrel *Menetes berdmorei* (Blyth, 1849) and the Rhizomyid *Cannomys badius* (Hodgson, 1841). In essence and relevant to the present study, the focus was on some of the interspecific karyotypic differences that had previously been described as diagnostic (see references above). It could be possible to show that many of the latter characters seem to be polymorphic but shared, rather than really species-specific, thus rendering their diagnostic value obsolete. On the contrary, no reliable and unambiguous karyotypic differences could be identified between congeneric species, whereas each genus displays a proper karyotype.

In conclusion, cytotaxonomy appears to be largely irrelevant for species-specific identification of most of the Thai rodents, despite the fact that it has been proven to be so useful, notably for African rodent systematics (Duplantier *et al*., 1990; Granjon *et al*., 1997; Dobigny *et al*., 2002a; Dobigny *et al*., 2002b; Dobigny *et al*., 2003; Granjon and Dobigny, 2003, references above). This appears to be especially true for *Rattus, Bandicota* and *Mus* species, which are pivotal to many epidemiological studies in South East Asia. Resolution is satisfactory only at the generic level, which is of poor value, since morphology can be applied more quickly and cheaply in this case.

Molecular phylogeny for the assessment of species-specific clades

The discriminative value of DNA markers at the specific level has never been tested in Thai rodents. To do so, the strict association of a DNA marker with a well defined species first requires an unambiguous delimitation of that particular species, which is something that may not be easy when intraspecific polymorphism is important and when species are morphologically similar (see earlier sibling species discussion).

This is the case for the *Rattus sensu lato* group in South East Asia that encompasses the genera *Rattus, Bandicota, Leopoldamys, Berylmys* and *Niviventer*. The situation is even more complex within the sub-group of *Rattus sensu stricto* for which seven species have been described in Thailand (*Rattus tanezumi, R. exulans, R. losea, R. argentiventer, R. rattus, R. norvegicus* and *R. andanamensis*). The taxonomy of these two groups

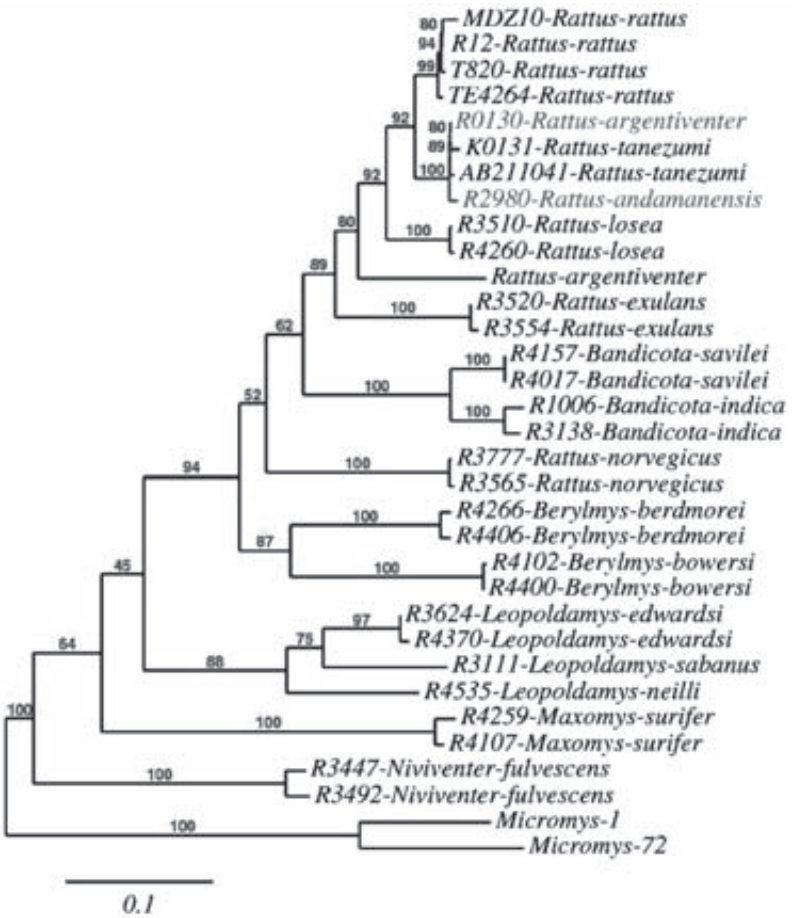

Figure 1 Agarose gel showing the different lengths of DNA amplification obtained by polymerase chain reaction among species of the genus *Bandicota* and the genus *Rattus*. On the left, bands of the ladder separated by 200 bp allow determination of the size of the amplicons. The two bands of the sample R1001 allow identification of *Bandicota savilei*, while the single band of R1006 is characteristic of *Bandicota indica*, and no specific band of R4003 represents *Rattus* sp.. Otherwise, the sequence of the cytochrome *b* of the R4003 individual identifies it as *R.tanezumi*.

is very confused and corresponds to an overabundance of synonymous designations. As a striking example, at least 80 names were proposed for what correspond in fact to the sole *R. tanezumi* and *R. rattus* species (Musser and Carleton, 2005). In order to further investigate specific diversity in the *Rattus sensu lato* lineage, a molecular phylogenetic study was developed based on the analysis of the complete cytochrome *b* gene (see Figure 2). Although the phylogenetic signal provided by this mitochondrial gene was insufficient to draw a robust phylogeny of the group (that is, some bootstrap values of important branches did not exceed 42%), it was, however, highly relevant for species assignment, since clear and robust (bootstrap values ranging between 88 and 100%) species-specific clades were retrieved (Figure.2). For instance, two specimens misidentified as *Rattus argentiventer* and *Rattus andamanensis* (highlighted in green in Figure 2)

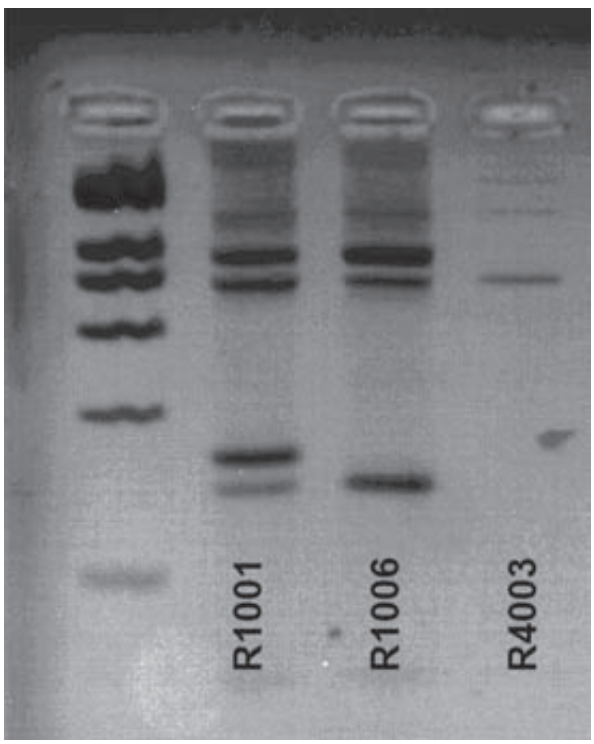

Figure 2 Tree of the *Rattus "sensu lato"* group based on the analysis of the complete cytochrome *b* gene. All the samples come from Thailand, except *Rattus rattus* ones (used here for species assignment). AB211041 corresponds to a Japanese *Rattus tanezumi* sequence extracted from GenBank data base. Phylogenetic analysis was performed with *Micromys*, used as an outgroup. The appropriate model of evolution for the cytochrome *b* gene was determined using Modeltest (Posada and Crandall, 1998): GTR+I+G. ML analysis was performed with PHYML version 2.4.4 (Guindon and Gascuel, 2003). The transition:transversion ratio, the proportion of invariable sites, as well as the gamma distribution parameter were estimated and the starting tree was determined by BioNJ analysis of the datasets (default settings). Using optimization options, 500 bootstrap replicates were performed. The tree was edited using the online interface http://www.phylogeny.fr/ recently developed by Dereeper *et al.* (2008).

according to their unusual measurements were proven to be in fact atypical individuals of *Rattus tanezumi*.

However, a phylogeny based on a single gene may sometimes be misleading, due to various genetic and/or historic factors. In order to more confidently define species-specific clades, the next step will be to analyze two other markers, with the first being nuclear (the first intron of the IRBP) and the second being mitochondrial (the cytochrome *c* oxydase 1, CO1) genes (Pagàs *et al*., 2010). IRBP sequences will provide a nuclear picture of the phylogeny, which is something that is pivotal to discriminate between gene versus species trees. Concomitantly, the analysis of CO1 sequences will open the gate to a barcoding approach (see paragraph 2d, and (Robins *et al*., 2007)). The ultimate aim of this ongoing work is to test and potentially reconsider some of the morphological criteria that were proposed to be species-specific. While waiting for such an investigation within the *Rattus sensu lato* group, some molecular tests (see below) are already available for clear species identification, in particular, for the *Bandicota* species.

Molecular test (species-specific primers)

In the context of such a confused systematic framework, and considering the interest of the genus *Bandicota* as a vector for several human diseases, such as hantavirosis, leptospirosis, typhus and babesiosis, among others (Wangroongsarb *et al*., 2002; Herbreteau *et al*., 2005; Ahmed *et al*., 2006; Thaipadungpanit *et al*., 2007), it was considered urgent to develop a DNA-based method using species-specific primers (Michaux *et al*., 2001; Galan *et al*., 2005; Lecompte *et al*., 2005) to easily, quickly and unambiguously assign *Bandicota* specimens to a single species. This technique relies on the definition of PCR primer sets that amplify only one single species within the genus (see references above for examples in other rodent groups). This tool is now available (Chaval *et al*., InPrep) and is suitable for investigations on large series. It will also be very useful for field surveys, since it requires simple and easily accessible DNA-preserved samples (such as dry material or preserved in ethanol). However, the complete treatment needs access to a molecular biology laboratory.

In addition to the screening of animals directly caught in the field, the specific primer approach could be used for collecting specimens that are conserved in alcohol, or as dry and/or osteological material, thus making it a pivotal tool to fulfill the gap between genetics and morphology. In particular, it may open the gate to the proper use of nomenclature by the inclusion of typical material.

In the near future, the aim is to extend such a promising identification tool to every murine species found in Thailand.

Barcoding

DNA barcoding, formerly a way of identifying DNA within foodstuffs, is now considered an answer to the lack of specialists in the systematics area. The purpose of barcoding is to find a unique piece of DNA (a part of cytochrome *c* oxidase subunit 1) for every described species and to tag it like a commercial barcode. Since the proposal of a standardized method to identify species (Hebert *et al*., 2003a; Hebert *et al*., 2003b), barcoding interests and caveats remain hotly debated (Ebach and Holdrege, 2005; Gregory, 2005; Moritz and Cicero, 2005; Schindel and Miller, 2005). Today, it appears that this mitochondrial gene cannot be considered as a universal systematic tool due to its poor resolution capacity for inferring phylogenetic relationships within many parts of the tree of life. Moreover, as briefly quoted above, the use of a single mitochondrial gene is not always sufficient to posit species frontiers. This is why CO1-based barcoding is not considered as the panacea. Nevertheless, the CO1 gene has been

shown to have the capacity to provide resolution for many taxa, including most of the mammals. It is clear now that it constitutes a useful marker that may optimize the time-consuming work of species identification. There is little doubt that the international scientific community will benefit from contributing to and sharing the dedicated database (the Barcode of Life Data (BOLD), Available: http://www.barcodinglife.org/views/login.php) with sequences from samples that are accurately identified and referenced. The BOLD tool can offer a reliable expert system that can be applied to systematic assessment based on the comparison of K2P (Kimura 2 Parameter) distances between an unknown sequence submitted by the user and a reference sequence collection. Interestingly, the BOLD network aims to gather all available molecular, morphometric and spatial information and ensure that these data are all clearly linked to a voucher "specimen". In the current study, the skull was selected as the representative voucher, since most rodent taxonomy has been based on cranial and mandibular features. Of main interest for such standardization of knowledge gathering and structuring is: 1) settling diagnostic species-specific characters that, ideally, can be applied by non-specialists; and 2) pointing out those parts of the tree of life that are still in need of systematic investigation. It also allows taxonomists to have quick access to an important amount of (if not all) data that have been collected on one particular taxon through a wide range of sometimes independent studies. Importantly, such an enlarged sampling, in both the number of individuals and geographic localities (Robins *et al.*, 2007; Borisenko *et al.*, 2008), would be highly regarded, since it would cover most of the intra-specific diversity.

Advantages and drawbacks of the different species identification tools

The disciplines quoted above have a wide range of purposes in the field of comparative biology. For instance, phylogenetic topologies based on morphological and/or molecular characters can be used to address various questions, such as life traits shifts, relative rates of evolution and co-evolution processes, among others. As another example, cytogenetics opens the gate to investigations about genome structure and evolution. This paper, however, did not focus on these parallel axes of research, but got rather interested in contribution of morphology, DNA-based methods and cytogenetics in taxon identification.

Mammalogists can now benefit from a panel of various tools to assess the systematic status of rodents. These tools emanated from morphology, morphometry, cytogenetics and molecular biology. Recently, DNA-based techniques have been increasingly advocated for systematic purposes. Among them, the barcoding and primer-specific methods constitute new molecular identification tools, which aim at assigning a specimen to a predefined genetic entity. They do not have the ability to define the limits of these entities, which are rather defined by phylogenetics. However, these approaches are not equally accessible to different users, and, unfortunately, are too rarely used jointly through a true multi-disciplinary strategy. In addition, it must be conceded that the choice of one particular tool is sometimes driven by purely pragmatic reasons, such as financial aspects or technique availability.

In order to provide a practical and complete view of each method, their advantages and drawbacks are presented, with special emphasis on their reasonable use in the field and/or in the laboratory using the particular example of Thai murid rodents. Tables 1 and 2 compile the respective strengths and caveats of the various approaches for field (Table 1) and laboratory (Table 2) surveys, with an attempt to classify several "practical"/logistic criteria from "very poorly" (—) to "highly" accessible (+++).

Table 1 Advantages and drawbacks of major techniques used in the field for species identification ("practical"/logistic criteria are ranked from "very poorly" (—) to "highly" accessible (+++).

		Field sampling	Comments
External morphology/morphometry	Cost[a]	+++	- The only method allowing species identification in the field.
	Time	+++	
	Conservation[b]	+++	- Identification with determination keys.
	Equipment Needed[c]	+++	- Cheap and popular tool in the field for rodent identification.
	Invasiveness[d]	++	- Body can be easily conserved in alcohol or formol (take care: formol is contraindicated if user wants the possibility to sample for DNA on the contraindicated if user wants have the possibility to sample for DNA on the body).
	Sampling Capacity[e]	+++	
	Result in the field	for some genus	-Requires a systematic update to be fully relevant. - Not reliable for all genera if used alone.
Sampling for Karyotype	Cost	+	- A robust and well documented tool for South- East Asia.
	Time	-	
	Conservation	+++	- Easy but rather time-consuming in the field.
	Equipment Needed	+	- Relevance depends on rodent lineages: highly relevant for the African murids for instance, but unfortunately poorly discriminatory for Thai rodents, including the *Rattus s.l.* complex.
	Invasiveness Sampling	- - -	
	Sampling capacity	-	
	Result in the field	No	
Sampling for Culture cell "for karyotyping"	Cost	+++	- Used to maintain living material in reference laboratories, thus allowing for subsequent cytogenetic and genomic studies, as well as molecular identification of parasites. - May also be used to obtain DNA or RNA. RNA.
	Time	++	
	Conservation	- - -	
	Equipment Needed	+++	
	Invasiveness Sampling Capacity	++ +++	
	Result in the field	No	
Sampling for Molecular test (primer specific)	Cost	+++	- A quick, reliable and cheap tool for species identification. - Adapted for biomedical sampling (large sampling). - Requires a previous work in laboratory to develop specific primers for each species and an access to a platform of molecular biology (PCR). - Available for *Bandicota* genus.
	Time	+++	
	Conservation	+++	
	Equipment Needed	+++	
	Invasiveness Sampling Capacity	++ +++	
	Result in the field	No	
Sampling for barcoding	Cost	+++	- Quick and reliable tool for species identification. - Relevant for biomedical sampling, biological inventory or survey, food control, etc. - Requires a previous work to establish the phylogeny of species in focus. - Need access to a platform of molecular biology. - More expensive than molecular tests (PCR + sequence).
	Time	+++	
	Conservation	+++	
	Equipment Needed	+++	
	Invasiveness	++	
	Sampling Capacity	+++	
	Result in the field	Not yet	

Table 2 Advantages and drawbacks of major techniques once back in the laboratory for species identification ("practical"/logistic criteria are ranked from "very poorly" (—) to "highly" accessible (+++).

		Laboratory work	Comments
Voucher/cranial morphology/morphometry	Cost	++	- Cheap with few technical materials for a basic diagnosise.
	Time	- - -	- Requires an important work for skull preparation.
	Conservation	- (skin/fur) +++ (bones)	- Systematic studies using this method
	Equipment needed	+++	necessitate expensive technical material to record shapes or three-dimensional measurements.
	Sampling capacity	- - -	- Finding diagnostice criteria requires the study of numerous individuals in various localities scattered over the distribution of the focus.
Karyotype	Cost	+	- Consumables and chemicals are quite cheap but associated cost is increased by
	Time	+	the requirement of a microscope.
	Equipment needed	+	- Important tool for mammalian taxonomy, especially for sibling species complex, such as those found in rodents and bats. - -
	Sampling capacity	+	- Indeed, iImportant karyotypic differences mean reproductive isolation, hence non conspecificity.
Culture cell "for karyotyping"	Cost	- - -	- Cell culture requires highly equipped labs and specialized consumables.
	Time	- -	- May be very expensive, except for labs that routinely deal with living cells.
	Conservation	+++	- Growing cells also requires an important
	Equipment needed	- - -	working time.
	Sampling capacity	- - -	- Allows the conservation and use of the biological samples indefinitely through cryopreservation.
Molecular test (primer specific)	Cost	+	- Relatively expensive to develop because of the need forof numerous DNA sequences for a
	Time	++	good evaluation of the molecular variation
	Equipment needed	-	within and between taxa.
	Sampling capacity	+++	- Requires low sample sizes within localities, but high number of distant localities. - Relatively cheap (DNA extraction and PCR without sequencing) once developed.
barcoding	Cost	-	- Adapted to treat large samples.
	Time	+	- Relatively expensive because of DNA
	Equipment needed	- - -	extraction and sequencing.
	Sampling capacity	++	- BecomGetting affordable for biological survey, if considering the decreasing cost for sequencing.

In Tables 1 and 2, several aspects were considered, such as cost, invasiveness, time consumption, equipment requirements and sampling conditions.

(a) The "cost" criterion refers to the total cost of materials, consumables and human time used for processing. Note that this cost only covers the work of identification by itself, and does not include the cost of methodological development, which is usually mandatory at the early stages of any systematic study.

(b) The "conservation" criterion refers to the stability of the sample in time, once conserved under proper conditions (in appropriate buffer, frozen, stuffing, etc.).

(c) Although the "equipment" criterion could have been considered as a part of the total cost and the sampling capacity, it was considered independently here. Indeed, bulky, expensive and/or delicate equipment can constitute on its own an important handicap for field or laboratory works.

(d) The "invasiveness" criterion is the ability to use a particular tool with the animal being kept alive (e.g. threatened species, population dynamics studies).

(e) The "sampling capacity" criterion evaluates the volume of samples that can be reasonably processed by one user. It represents a trade off between the time consumption, the cost and the equipment that is needed.

The integrative approach towards Thai rodent taxonomy is an ongoing study that has not been completed yet, so that the results presented here should be considered as preliminary. However, the current paper aims at summarizing several of the diagnostic tools that are available to workers in both the field and the laboratory. As a conclusion, the importance of a multidisciplinary approach should be emphasized, when applicable, as it should be preferred for the assessment of specific diversity. For instance, it was very helpful in the context of other tropical rodents, such as *Mastomys* spp., which is a Murid genus that has had important detrimental impacts on Sahelian and Sudanian agriculture (seed depletion and crop field damage). In addition, they were identified as major reservoirs for human pathogens, such as *Yersinia pestis* and Lassa Fever virus, the agents responsible for plague and potentially lethal hemorrhagic fevers, respectively. The knowledge of specific diversity within this epidemiologically pivotal group has been largely improved through the use of different but complementary approaches. Until 1990, two *Mastomys* species were known in Senegal (*Mastomys erythroleucus* and *M. huberti*) that were described only on morphological and morphometric grounds. However, a karyologic study (Duplantier *et al.*, 1990) clearly demonstrated the presence of a third sibling species *M. natalensis*. Finally, species-specific primers and restriction sites generating species-specific profiles were recently identified and made available (Lecompte *et al.*, 2005). Altogether, this set of identification tools of the different *Mastomys* species was a substantial step forward, since they now allow the unambiguous identification of a large series of *Mastomys* individuals, and, for instance, were critical for understanding the species-specific role of *M. natalensis* vs. *M. erythroleucus* in the Lassa fever virus transmission (Lecompte *et al.*, 2006).

CONCLUSION

As a conclusion, it is important to keep in mind that the view of the classification of living organisms should not be fixed, but should rather be considered as representing a snapshot of biodiversity through the filter of given knowledge at a given time. Taking into account technical advances, the viewpoint necessarily needs to be regularly questioned, in order to be updated. Hopefully, such tools will be a great help in many applied biological fields, such as human health and agronomic or environmental problems in South East Asia.

LITERATURE CITED

Ahmed, N., S.M. Devi, L. Valverde Mde, P. Vijayachari, R.S. Machang'u, W.A. Ellis and R.A. Hartskeerl. 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic leptospira species. **Annals of Clinical Microbiology and Antimicrobials** 5: 28.

Aplin, K., P. Brown, J. Jacob, C. Krebs and G. Singleton. 2003a. Field methods for rodent studies in Asia and the Indo-Pacific, **ACIAR Monograph**, Canberra.

Aplin, K.P., A. Frost, N. Phu Tuan, L.P. Lan and N. Manh Hung. 2003b. Identification of rodents of the genus Bandicota in Vietnam and Cambodia, pp. 499-506. *In* R.G. Singleton, L.A. Hinds, C.J. Krebs and D.M. Spratt (eds.). **Rats, Mice and People: Rodent Biology and Management**. ACIAR Monograph No. 96 Canberra.

Badenhorst, D., V. Herbreteau, Y. Chaval, M. Pages, T.J. Robinson, W. Rerkamnuaychoke, S. Morand, J.-P. Hugot and G. Dobigny. 2009. New karyotypic data for Asian rodents (Rodentia, Muridae) with the first report of B-chromosomes in the genus Mus. **Journal of Zoology** 279: 44-56.

Baillie, J.E.M., C. Hilton-Taylor and S.N. Stuart. 2004. A global species assessment. The IUCN Species Survival Commission.

Borisenko, A.V., B.K. Lim, N.V. Ivanova, R.H. Hanner and P.D.N. Hebert. 2008. DNA barcoding in surveys of small mammal communities: a field study in Suriname. **Molecular Ecology Resources** 8: 471-479.

Chaval, Y., V. Herbreteau, M. Galan, L. Anne, S. Morand, S.Y, J.-P. Hugot and J.F. Cosson. InPrep. A polymerase chain reaction test enlightens the respective role of two close bandicoot rat species in leptospirosis transmission in Thailand.

Coleman, R.E., T. Monkanna, K.J. Linthicum, D.A. Strickman, S.P. Frances, P. Tanskul, T. M.J. Kollars, I. Inlao, P. Watcharapichat, N. Khlaimanee, D. Phulsuksombati, N. Sangjun and K. Lerdthusnee. 2003. Occurrence of *Orientia tsutsugamushi* in small mammals from Thailand. **American Journal of Tropical Medicine and Hygiene** 69: 519-524.

Corbet, G.B. and J.E. Hill. 1992. **Mammals of the Indomalayan Region.** A systematic review. Oxford University Press, Oxford. 488 pp.

Denys, C., E. Lecompte, L. Granjon, M. Baylac, P. Cordeiro, R. Cornette, G. Dobigny, E. Fichet-Calvet, J.-P. Hugot, C. Meslage, V. Millien-Parra, P. Petrillo, V. Volobouev and M. Welz. 2003. Integrative systematics: the importance of combining techniques for increasing knowledge of african Murinae, pp. 531-535. *In* G.R. Singleton, L.A. Hinds, C.J. Krebs and D.M. Spratt (eds.). **Rats, Mice and People: Rodent Biology and Management**. ACIAR Monograph No. 96 Canberra. 564 pp.

Dereeper, A., V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.M. Claverie and O. Gascuel. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. **Nucleic Acids Res** 36:W465-469.

Dobigny, G., A. Nomao and J.C. Gautun. 2002a. A cytotaxonomic survey of rodents from Niger: implications for systematics, biodiversity and biogeography. **Mammalia** 66: 495-523.

Dobigny, G., V. Volobouev and V. Aniskin. 2002b. Explosive chromosome evolution and speciation in the gerbil genus Taterillus (Rodentia, Gerbillinae): a case of two new cryptic species. **Cytogenetic and Genome Research** 96: 117-124.

Dobigny, G., L. Granjon, V. Aniskin, K. Bâ and V. Volobouev. 2003. A new sibling species of Taterillus (Rodentia, Gerbillinae) from West Africa. **Mammalian Biology** 68: 299-316.

Ducroz, J.-F., L. Granjon, P. Chevret, J.-M. Duplantier, M. Lombard and V. Volobouev. 1997. Characterization of two distinct species of Arvicanthis (Rodentia: Muridae) in West Africa: cytogenetic, molecular and reproductive evidence. **Journal of Zoologie of London** 241: 709-723.

Duplantier, J.M., J. Britton-Davidian and L. Granjon. 1990. Chromosomal characterization of three of the genus *Mastomys* in Senegal. **Zeitschrift für Zoologie Systematic Evolution** 28: 289-298.

Ebach, M.C. and C. Holdrege. 2005. DNA barcoding is no substitute for taxonomy. **Nature** 434: 697-697.

Galan, M., C. Baltzinger, A.J.M. Hewison and J.-F. Cosson. 2005. Distinguishing red and roe deer using DNA extracted from hair samples and the polymerase chain reaction (PCR) method. **Wildlife Society Bulletin** 33: 89-95.

Granjon, L., J.M. Duplantier, J. Catalan and J. Britton-Davidian. 1997. Systematics of the genus *Mastomys* (Thomas 1915) (Rodentia, Muridae): A review. **Belgium Journal of Zoologie** 127: 7-18.

Granjon, L. and G. Dobigny. 2003. Chromosomally characterised murid rodents from the edges

of lake Tchad: Evidence for an African biogeographical crossroads, or a centre of endemism ? **Mammal Review** 33: 77-91.

Gregory, T.R. 2005. DNA barcoding does not compete with taxonomy. **Nature** 434: 1067-1067.

Gropp, A., J.T. Marshall and A. Markvong. 1973. Chromosomal findings in the spiny mice of Thailand (genus *Mus*) and occurence of a complex intraspecific variation in *M. shortridgei*. **Zeitschrift für Säugetierkunde** 38: 159-168.

Guindon, S. and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. **Systematic Biology** 52: 696-704.

Hebert, D.N.P., A. Cywinska, S.L. Ball and J. DeWaard. 2003a. Biological identifications through DNA barcodes. **Proceedings of the Royal Society of London B: Biological Sciences** 270: 313-321.

Hebert, D.N.P., S. Ratnasingham and J.R. DeWaard. 2003b. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. **Proceedings of the Royal Society of London B: Biological Sciences** 270: S96-S99.

Herbreteau, V., J.-P. Gonzales, A. Haja, P. Kittayapong and J.-P. Hugot. 2005. Mapping the potential distribution of *Bandicota indica*, vector of zoonoses in Thailand, by use of remote sensing and geographic information systems (a case of Nakhon Pathom province). **The Natural History Journal of Chulalongkorn University** 5: 61-67.

King, M. 1995. Species evolution. **The Role of Chromosome Change**. Cambridge University Press. 358 pp.

Lecompte, E., C. Brouat, J.-M. Duplantier, G. Maxime, L. Granjon, L. Anne, K. Mouline and J.-F. Cosson. 2005. Molecular identification of four cryptic species of Mastomys (Rodentia, Murinae). **Biochem. Syst. and Ecol.** 33: 681-689.

Lecompte, E., E. Fichet-Calvet, S. Daffis, K. Koulémou, O. Sylla, F. Kourouma, A. Doré, B. Soropogui, V. Aniskin, B. Allali, S. Kouassi Kan, A. Lalis, L. Koivogui, S. Günther, C. Denys and J. ter Meulen. 2006. *Mastomys natalensis* and lassa fever, West Africa. **Emerging Infectious Diseases** 12: 1971-1974.

Markvong, A., J.T. Marshall and A. Gropp. 1972. Karyotype analysis of certain murid rodents of Thailand. **Mammalia Chromosome News** 13: 16-20.

Markvong, A., J.T. Marshall and A. Gropp. 1973. Chromosomes of rats and mice of Thailand. **Natural History Bulletin of the Siam Society** 25: 23-32.

Marshall, J.T. 1977a. Family Muridae: rats and mice, pp. 396-487. *In* L. Boonsong and J.A. McNelly. **Mammals of Thailand.** Association for wildlife conservation, Bangkok.

Marshall, J.T. 1977b. A synopsis of Asian species of *Mus* (Rodentia, Muridae). **Bulletin of the American Museum of Natural History** 158: 175-220.

Marshall, J.T. 1986. Systematics of the genus *Mus*. **Current Topics in Microbiology and Immunology** 127: 12-18.

Michaux, J.R., S. Kinet, M.-G. Filippucci, R. Libois, A. Besnard and F. Catzeflis. 2001. Molecular identification of three sympatric species of wood mice (*Apodemus sylvaticus, A. Flavicollis, A. Alpicola*) in Western Europe (Muridae: Rodentia). **Molecular Ecology Notes** 1: 260-263.

Moritz, C. and C. Cicero. 2005. Promises and pitfalls on DNA barcoding. **Genes & Genetic Systems** 80: 443-443.

Musser, G.G. and E.M. Brothers. 1994. Identification of bandicoot rats from Thailand (Bandicota, Muridae, Rodentia). **American Museum Novitates** 3110: 56

Musser, G.G. and M.D. Carleton. 2005. Family

Muridae, pp. 894-1531. *In* R.D. Wilson DE (ed.). **Mammal species of the world: A taxonomic and geographic reference**. The John Hopkins University Press, Baltimore.

Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca and J. Kent. 2000. Biodiversity hotspots for conservation priorities. **Nature** 403: 853-858.

Pagàs, M., G. Corbet, A. Orth, V. Volobouev, J. Michaux and F. Catzeflis. Submitted. Morphological, chromosomal and genic differences between *Rattus rattus* and *Rattus satarae*, a sibling, sympatric species of South India. **Journal of Mammalogy.**

Pagàs, M., Y. Chaval, V. herbreteau , S. Waengsothorn, J.F. Cosson, J.P. Hugot, S. Morand and J. Michaux. 2010. Revisiting the taxonomy of the Rattini tribe: a phylogeny-based delimitation of species boundaries. **BMC Evolutionary Biology.** 10: 184.

Posada, D. and K.A. Crandall. 1998. Modeltest: Testing the model of DNA substitution. **Bioinformatics** 14: 817-818.

Robins, J.H., M. Hingston, E. Matisoo-Smith and H.A. Ross. 2007. Identifying *Rattus* species using mitochondrial DNA. **Molecular Ecology Notes** 7: 717-729.

Schindel, D.E. and S.E. Miller. 2005. DNA barcoding a useful tool for taxonomists. **Nature** 435: 17-17.

Srikosamatara, S., W.Y. Brockelman, A. Pattanavibool, J. Milne and P. Round. 2004. **Thailand Environment Monitor 2004, Biodiversity**, World Bank, Bangkok.

Thaipadungpanit, J., V. Wuthiekanun, W. Chierakul, L.D. Smythe, W. Petkanchanapong, R. Limpaiboon, A. Apiwatanaporn, A.T. Slack, Y. Suputtamongkol, N.J. White, E.J. Feil, N.P. Day and S.J. Peacock. 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. **PLoS Neglected Tropical Disease** 1: e56.

Volobouev, V.T., V.M. Aniskin, E. Lecompte and J. F. Ducroz. 2002. Patterns of karyotype evolution in complexes of sibling species within three genera of African murid rodents inferred from the comparison of cytogenetic and molecular data. **Cytogenetic and Genome Research** 96: 261-275.

Volobouev, V., V. M. Aniskin, B. Sicard, G. Dobigny and L. Granjon. 2007. Systematics and phylogeny of west African gerbils of the genus *Gerbilliscus* (Muridae: Gerbillinae) inferred from comparative G- and C-banding chromosomal analyses. **Cytogenetic and Genome Research** 116: 269-281.

Wangroongsarb, P., W. Petkanchanapong, S. Yasaeng, A. Imvithaya and P. Naigowit. 2002. Survey of leptospirosis among rodents in epidemic areas of Thailand. **The Journal of Tropical Medecine and Parasitologie** 25: 55-58.

Yong, H.-S. 1968. Karyotype of four malayan rats. (Muridae, genus *Rattus* fischer). **Cytologia (Tokyo)** 33: 174-180.

Yong, H.-S. 1969. Karyotypes of Malayan rats (Rodentia, muridae, genus *Rattus* fischer). **Chromosoma** 27: 245-267.

Yosida, T.H. 1973. Evolution of karyotypes and differentiation in 13 *Rattus* species. **Chromosoma** 40: 285-297.

Yosida, T.H., H. Kato, K. Tsuchiya and K. Moriwaki. 1971. Karyotypes and serum transferrin patterns of hybrids between asian and oceanian black rats, *Rattus rattus*. **Chromosoma** 34: 40-50.