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An integrative approach to species discovery in odonates: from character-based DNA barcoding to ecology

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Abstract

Modern taxonomy requires an analytical approach incorporating all lines of evidence into decision-making. Such an approach can enhance both species identification and species discovery. The character-based DNA barcode method provides a molecular data set that can be incorporated into classical taxonomic data such that the discovery of new species can be made in an analytical framework that includes multiple sources of data. We here illustrate such a corroborative framework in a dragonfly model system that permits the discovery of two new, but visually cryptic species. In the African dragonfly genus *Trithemis* three distinct genetic clusters can be detected which could not be identified by using classical taxonomic characters. In order to test the hypothesis of two new species, DNA-barcodes from different sequence markers (ND1 and COI) were combined with morphological, ecological and biogeographic data sets. Phylogenetic analyses and incorporation of all data sets into a scheme called taxonomic circle highly supports the hypothesis of two new species. Our case study suggests an analytical approach to modern taxonomy that integrates data sets from different disciplines, thereby increasing the ease and reliability of both species discovery and species assignment.

Keywords: character-based barcoding, conservation genetics, integrative approach, new (cryptic) species, Odonata, taxonomic circle

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Introduction

It is widely accepted that a species identification system based on DNA sequences offers a rapid, reliable and consistent method, which is especially important for crisis disciplines like conservation biology and biodiversity research (Goldstein & DeSalle 2000; Janzen 2004; Wheeler *et al.* 2004; DeSalle *et al.* 2005; Vogler & Monaghan 2007; Waugh 2007). The recent introduction of DNA barcoding as a fast identification method for assessing biodiversity of known species, has created excitement about a new, powerful tool for taxonomy (e.g. Hebert & Gregory 2005; Vences *et al.* 2005; Clare *et al.* 2007; Pfenninger *et al.* 2007). However, problems arise when new species are discovered, in other words, when specimens come from the major part of biodiversity that has not been described yet (DeSalle 2006; Rubinoff 2006). DNA barcoding studies have mainly been focussing on distance-based methods to identify and delimitate species (e.g. Hebert *et al.* 2003). This however can prove difficult for various reasons. For example, substitution rates of mtDNA vary between different groups of species resulting in a broad overlap of intra- and interspecific distances (Will & Rubinoff 2004; Hickerson *et al.* 2006).

A recently applied new technique, the character-based DNA barcode method, characterizes species through a unique combination of diagnostic characters rather than genetic distances (DeSalle *et al.* 2005; Rach *et al.* 2008). This way species boundaries can be defined by a diagnostic set of characters which can be increased

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to any level of resolution by applying multiple genes (Rach *et al.* 2008). Another advantage of character-based barcoding is the fact that DNA characters can be combined with characters from other disciplines, e.g. ecology, morphology, geography and behaviour. Therefore a comprehensive database can be established to test new species hypotheses based on an analytical rather than descriptive approach.

An analytical discovery process is especially important when traditional taxonomy fails to identify a species that is genetically distinct, as in the case of 'cryptic species' (e.g. Witt *et al.* 2006; Bickford *et al.* 2007). The taxonomic circle introduced by DeSalle *et al.* (2005) describes a way in which different data sets can interact to discover new species. In this scheme, a genetically, morphologically or geographically discovered entity can only be raised to species status when at least two disciplines support the species discovery hypothesis. The advantage of this corroborative approach is the reliability of at least two different data sets of qualitatively different characters. Although this scheme displays the evolutionary process in a highly oversimplified way, it demonstrates that species discovery could be based on biological and evolutionary species concepts.

In this case study on odonates (dragonflies and damselflies) we apply the scheme of a taxonomic circle to prove the discovery of the first two 'cryptic' species within the order. Odonates are highly mobile organisms. Their complex life cycle, consisting of an aquatic larval stage and terrestrial adults, coupled with species-specific habitat requirements make them excellent indicators for assessing biodiversity and wetland health (Corbet 1999; Stoks *et al.* 2005; Hadrys *et al.* 2006; Groeneveld *et al.* 2007). Their complex reproductive system and behaviour, e.g. with the 'lock and key' arrangement of the genitalia and the typical copulation wheel, is unique in the animal kingdom and has made them model organisms for a variety of evolutionary studies (Waage 1979; Hadrys *et al.* 1993, 2005; Turgeon *et al.* 2005; Córdoba-Aguilar 2008). Despite a vast literature on the ecology, behaviour, and taxonomy of odonates, the application of modern genetic tools into conservation studies has been slow. The specificities that make odonates particularly valuable for biodiversity assessment on the one hand also make them technically difficult to study on the other hand, particularly with respect to the identification of larvae (Stewart & Samways 1998; Sahlen & Ekestubbe 2001; Hornung & Rice 2003; Flenner & Sahlen 2008; Clausnitzer *et al.* 2009; Simaika & Samways 2009).

Despite a variety of phylogenetic and population genetic studies and an estimated high number of still undescribed species, to date, species discovery has been based solely on classical taxonomic descriptions and no cryptic odonate species have been discovered (Misof *et al.* 2000; Weekers *et al.* 2001; Stoks *et al.* 2005; Hadrys *et al.* 2006; Hasegawa & Kasuya 2006). We originally started a population genetic study in the libellulid dragonfly *Trithemis stictica*, but surprisingly discovered three genetically distinct clusters based on genetic evidence from ND1 (NADH dehydrogenase 1) and COI (cytochrome c oxidase subunit I) sequences. In this study we report the discovery process of the first two cryptic species in odonates. The hypotheses of one or two new *Trithemis* species are tested by using the taxonomic circle. In addition to the genetic data, we analyse the morphology, ecology and biogeography of three genetic clusters and integrate all data sets into one total evidence character-based barcode matrix.

Materials and methods

Field studies and geography

The genus *Trithemis* (Libellulidae) is widely distributed in Africa and Asia, and includes 40 described species (Pinhey 1970) that are found in a variety of habitats. Species range from habitat generalists that are dispersed throughout Africa and Asia, to regionally restricted specialists. *Trithemis stictica* (Burmeister 1839) is a generalist and a common species in Sub-Saharan Africa. It inhabits swamps, pools or streams in open and forested areas and depends on permanent water bodies with an abundance of vegetation (Pinhey 1970). In Namibia, one of the most arid countries in the world such habitats are rare. From 133 monitored localities, *T. stictica* was found in only two regionally restricted areas, the Naukluft Mountain region in western-central Namibia and the Caprivi Stripe that includes the Okavango and Kwando rivers in the north-eastern corner. Between 2000 and 2006, 108 samples of *T. stictica* were collected from 14 localities in Namibia, Botswana (Okavango Delta), Zambia (Zambezi River), South Africa (Western Cape, Royal Natal Park), Tanzania (East Usambara Mountains), Kenya (Kiboko River, Nairobi National Park) and Ethiopia (Ambo) to broadly cover its geographical distribution (see Table 1 and Fig. 1). Habitat characteristics were mapped for each location. For comparative phylogenetic analyses, five closely to distantly related *Trithemis* species were also sampled and included into the study.

DNA extraction and sequencing

Total genomic DNA was isolated from leg tissue using a modified phenol-chloroform extraction (Hadrys *et al.* 1992). For initial population genetic analyses, the mitochondrial marker ND1 was used. A 610 bp fragment

Table 1 Population sites (country, locality, abbreviation) and number (*n*) of analysed individuals of *T. stictica* (clades 1, 2 and 3) as well as five other *Trithemis* species

Species	Country	Locality	Abbreviation	*n*
T. stictica	Namibia	Naukluft	Nauk	8
	Namibia	Zebra River	Zebra	9
	Namibia	Popa Falls	Popa	32
	Namibia	Andara	And	3
	Namibia	Rundu	Rund	4
	Namibia	Kwando River	Kwan	7
	Botswana	Okavango Delta	Bot	11
	Zambia	Zambezi River	Zam	17
	Kenya	Kiboko River	KR Ken	5
	Kenya	Nairobi NP	NNP Ken	1
	Tanzania	East Usambara Mt.	Tanz	5
	South Africa	Western Cape	WC SA	2
	South Africa	Royal Natal Park	RN SA	3
	Ethiopia	Ambo	Eth	1
T. annulata	Namibia	Rehoboth		2
	Namibia	Popa Falls		3
T. furva	Ethiopia	Nekemte		3
	South Africa	Wakkerstrom		2
T. grouti	Liberia	Gola Forest		2
	Liberia	Lorma Nat. Forest		3
T. nuptialis	Congo	Lingomo		1
	Congo	Lukomete		1
T. kirbyi	Namibia	Tsaobis		3
	Namibia	Waterberg		2

was amplified using the primer pair P 850 fw and P 851 rev (Abraham *et al.* 2001). The amplification product included $tRNA^{Leu}$ and a 3′ partial fragment of the 16S rDNA fragment and the ND1 gene region. The PCR thermal regime was performed as described in Rach *et al.* (2008). A second marker, the suggested universal barcode region COI, was used on a subset of individuals covering the previously identified genetic clades (five individuals in each clade). Here, a 630 bp fragment was amplified using universal primers (Hebert *et al.* 2003). PCR conditions were as follows: 3 min initial denaturation at 95 ° C, followed by 35 cycles of 95 °C for 30 sec, 50 °C for 40 sec and 72 °C for 40 sec, and 2 min extension at 72 °C. PCR was carried out in a total volume of 25 μL, containing 1× amplification buffer (Invitrogen), 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 7.5 pmol each primer, and 0.75 U Taq DNA polymerase (Invitrogen).

Cycle sequencing of purified PCR-products was done using the ET Terminator Mix from Amersham Bioscience and sequenced on an automated sequencer (MegaBACE 1000; Amersham Bioscience). Sequences were assembled and edited using Seqman II (version 5.03; DNAStar, Inc.). Consensus sequences were aligned by means of MUSCLE 3.6 (Edgar 2004). Sequences of each haplotype of all species were deposited into GenBank under accession nos FJ358436–FJ358482.

Genetic distance and phylogenetic analysis

The number of haplotypes and variable nucleotide positions were calculated using Quickalign (Müller & Müller 2003). Pairwise genetic distances for ND1 and COI were calculated using Kimura-2-Parameter distance model implemented in PAUP version 4.0b10 (Swofford 2002). For estimation of gene flow between populations, F_{st}-values were computed in ARLEQUIN version 3.1 (Excoffier *et al.* 2005) and tested for significance by permuting haplotypes between samples (10 000 replicates).

For phylogenetic analyses, two different tree building methods, Bayesian (BA) and Maximum Parsimony (MP) were compared. Using the Akaike Information Criterion

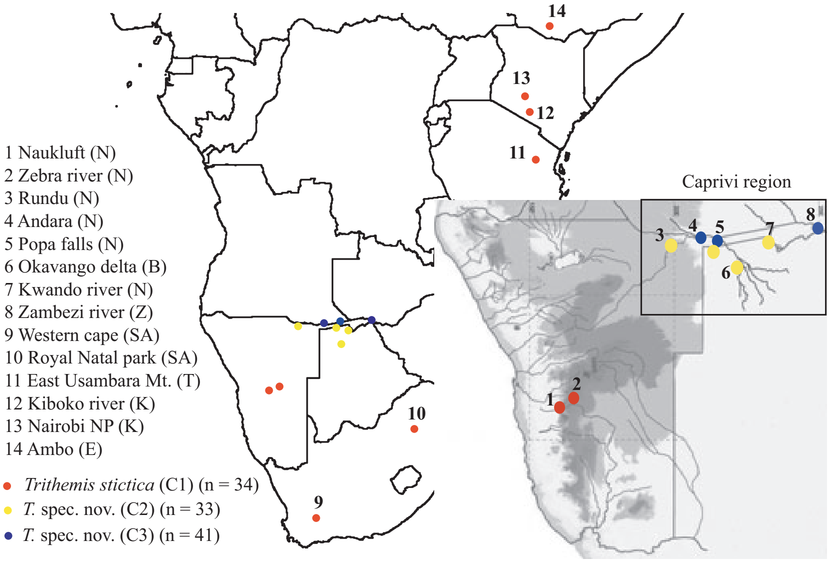

Fig. 1 Map of the analysed sample sites of *T. stictica* (C1 = clade 1), *T.* spec. nov. (C2 = clade 2) and *T.* spec. nov. (C3 = clade 3). The true *T. stictica* is distributed across Southern Africa with five different countries included in this study (N: Namibia; SA: South Africa; T: Tanzania; K: Kenya; E: Ethiopia), whilst *T.* spec. nov. (C2) and (C3) are restricted to the Caprivi region, at the borders between Namibia (N), Botswana (B) and Zambia (Z).

in Modeltest 3.7 (Posada & Crandall 1998), the TrN+I model for ND1 and the GTR model for COI were selected for BA which were performed with MrBAYES version 3.1.2 (Huelsenbeck & Ronquist 2001). The most appropriate parameters for amongst site variation, base frequencies and discrete gamma distribution were employed and Markov-Chain Monte-Carlo posterior probabilities determined. The Markov-Chain Monte-Carlo search was performed with four chains for 1 500 000 generations and trees were sampled every 750th generation. MP analyses were performed as implemented in PAUP version 4.0b10 (Swofford 2002). A heuristic search for each marker was employed using TBR branch swapping and random addition of taxa for 100 replicates. Bootstrap values were calculated based on 1000 replicates (Felsenstein 1985).

Character-based barcode analysis

The identification of diagnostic characters within ND1 and COI sequences was performed in two steps. First, for pairwise comparisons of *T. grouti, T. nuptialis* and the three genetic *T. stictica* clades, the numbers of nucleotide substitutions distinguishing all individuals of one species or clade from the others were listed for each species pair. Nucleotide substitutions occurring only in single individuals of a species were ignored and only pure diagnostic characters mentioned (see Rach *et al.* 2008). These pure diagnostic characters consisted of all the nucleotide positions of individuals from one species that differed from those in all individuals of a second species.

In the second step, a search for species specific combinations of character states from both markers (ND1 and COI) was performed with all species by employing the CAOS algorithm (Sarkar *et al.* 2002; Rach *et al.* 2008). The most variable sites that distinguish all the species were chosen and the character states at these nucleotide positions were listed. This way, unique combinations of character states (character-based DNA barcodes) were identified. For a more detailed description of character-based DNA barcoding using CAOS see Rach *et al.* (2008).

Morphological analyses

A total of 43 (previously genetically analysed) male specimens from Namibia (Zebra River, Kwando, Andara and Popa Falls), Botswana, Zambia, Kenya, Tanzania and South Africa were examined using a stereoscopic microscope, a stage micrometer and a scanning electron microscope (SEM) (Damm & Hadrys 2009). We included the morphological data on wing venation and colouration patterns of the specimens as well as eleven other variables commonly used in dragonfly identification (e.g. the length of the abdomen, wing length and the shape of secondary genitalia) from a former study (Damm & Hadrys 2009). We calculated the mean and standard deviation for all length variables (see Table 4A). All measurements were first tested for Normality (Shapiro–Wilk test) using SAS. The significance of morphological differences between the genetic entities was determined by the Wilcoxon test.

To identify a potential reproductive barrier, the inner part of the secondary copulatory apparatus (SCA) was examined via SEM (ETEC-AUTOSCAN). The distal segments of the SCA (including the penis) of five selected individuals of each locality were dissected. The specimens, previously preserved in 80% ethanol, were vacuum dried, sputter coated with gold and examined in the vacuum chamber of the SEM.

Results

Genetic distance patterns

The alignment of the ND1 marker contained sequences of all 108 individuals of *T. stictica*. The fragment of 496 bp harboured 62 variable and 60 parsimony informative sites. One deletion occurred at position 126 in the region of the tRNA^{Leu} in 73 sequences (all from the Caprivi region, which includes Popa Falls, Andara, Kwando, Rundu, Zambia and Botswana). In total, 26 haplotypes were identified with no haplotype shared by all localities. Genetic distances ranged from 0% to 9.0% (Table 2) with high values amongst three groups of individuals resulting in three haplotype clades with no intermediate haplotypes (see Fig. S1, Supporting information). The alignment of the COI marker contained 630 bp, including 67 variable and 59 parsimony informative sites. Nine different haplotypes were found and genetic distances ranged from 0 to 8.3% (Table 2). The individuals grouped together in the same three distinct clades as in ND1.

All but one locality could be assigned to one of the three clades. The first genetic clade consisted of localities separated by long distances: South Africa, Ethiopia, Tanzania, Kenya and two sites in central Namibia, the Naukluft and Zebra River region (red dots in Fig. 1). The second clade contained regionally restricted individuals from the Caprivi region, the localities Okavango Delta in Botswana, Kwando River, Rundu and a part of the Popa Falls individuals in Namibia (yellow in Fig. 1). The remaining Popa Falls individuals belonged to the third clade together with individuals of the sites Zambezi River (Zambia) and Andara, again all from the Caprivi region (blue in Fig. 1). Genetic distances between the clades were high

Table 2 Sequence divergence (in %) based on the Kimura-2-parameter of ND1 (above) and COI (below) of the three clades (C1 = clade 1, C2 = clade 2, C3 = clade 3) of *T. stictica* and four *Trithemis* species

	C1 (*T. stictica*)	C2 (*T.* spec. nov)	C3 (*T.* spec. nov)	*T. grouti*	*T. nuptialis*	*T. annulata*	*T. furva*
C1 (*T. stictica*)							
C2 (*T.* spec. nov)	9.0						
C3 (*T.* spec. nov)	8.5	5.0					
T. grouti	6.8	8.1	8.1				
T. nuptialis	2.2	7.6	8.7	7.0			
T. annulata	10.6	6.5	7.3	10.0	9.4		
T. furva	9.1	8.0	8.3	10.2	8.3	8.3	
C1 (*T. stictica*)							
C2 (*T.* spec. nov)	7.9						
C3 (*T.* spec. nov)	8.3	5.7					
T. grouti	3.3	8.9	8.9				
T. nuptialis	3.3	9.5	9.3	1.0			
T. annulata	9.1	10.6	11.4	8.1	8.5		
T. furva	9.7	10.1	10.4	10.1	10.6	9.3	

between the first and the second clade (9.0% in ND1 and 7.9% in COI), and between the first and third clade (8.5% in ND1 and 8.3% in COI). The regionally restricted clades 2 and 3 with individuals of the Caprivi region were separated by 5.0% in ND1 and 5.7% in COI (see Table 2). In contrast, the genetic distance within clades was low, ranging from 0 to 1%. At one site in the Caprivi region, Popa Falls, individuals of clades 2 and 3 occur sympatrically. Interspecific genetic distances between the five known *Trithemis* species included in this study ranged from 2.2 to 10.6% in ND1 and 1.0 to 11.4% in COI (Table 2). Here e.g. the genetic distance between clade 1 and the known species *T. nuptialis* (2.2% in ND1, 3.3% in COI) and *T. grouti* (6.8% in ND1, 3.3% in COI) was lower than that between clades 2 and 3.

Comparisons of F_{st}-values revealed high genetic substructuring between the populations, independent of geography. Grouping individuals according to their genetic clade, the F_{st}-values between these groups ranged from 0.906 to 0.960 in ND1 and from 0.921 to 0.984 in COI. These high levels of F_{st}-values suggested an absence of gene flow amongst the populations in the Caprivi region (clades 2 and 3), as well as between the Caprivi region and clade 1 (Namibia Naukluft, Kenya, Tanzania, South Africa and Ethiopia).

Phylogenetic analyses

Maximum Parsimony (MP) and Bayesian analyses (BA) revealed the same topology for both markers (ND1 and COI), and was consistent with results of the distance analyses, which grouped individuals into three clusters (see Fig. S1, Supporting information).

In order to position the genetic clades of *T. stictica* in a phylogenetic tree, MP and BA were performed of the three clades together with two closely and three more distantly related *Trithemis* species. The resulting trees showed a clear separation of clade 1 (red) from clades 2 and 3 (yellow and blue, Fig. 2). Clade 1 grouped together with *T. grouti* and *T. nuptialis,* which is congruent with the classical taxonomic position of *T. stictica* (Pinhey 1970). Based on this tree topology, clade 1 was identified as the originally described *T. stictica*. A sister species relationship between the putative new species (clades 2 and 3) was highly supported (PP = 1.00; 100% bootstrap).

Character-based DNA barcodes

Table 3A lists the barcodes, i.e. species-specific nucleotide positions (pure diagnostic barcode characters), for the three *Trithemis* clades and two closely related species. The three clades were distinguishable by unambiguous barcodes. Clades 1 and 2 were distinguished by 26 variable nucleotide positions (VNPs) in ND1 and 43 in COI, clades 1 and 3 by 27 positions in ND1 and 43 in COI, and clades 2 and 3 by 13 positions in ND1 and 28 in COI (see Table S1, in Supporting Information). In contrast, *T. nuptialis* differed from clade 1 in only four positions in ND1 and 19 in COI. A comparison of clade 1 and *T. grouti* revealed 21 different position in ND1, and 20 in COI. Interestingly, the VNPs between clades 2 and 3 were nearly the same as between *T. grouti* and *T. nuptialis*, with around 30 variable positions in ND1 and 50 in COI.

To establish character-based barcodes for all *Trithemis* species studied, 13 nucleotide positions of ND1 and 15 of COI were chosen. These particular nucleotide positions revealed the highest numbers of diagnostic characters (Table 3B). Using only these chosen positions, all species could be distinguished by at least four diagnostic characters in both markers.

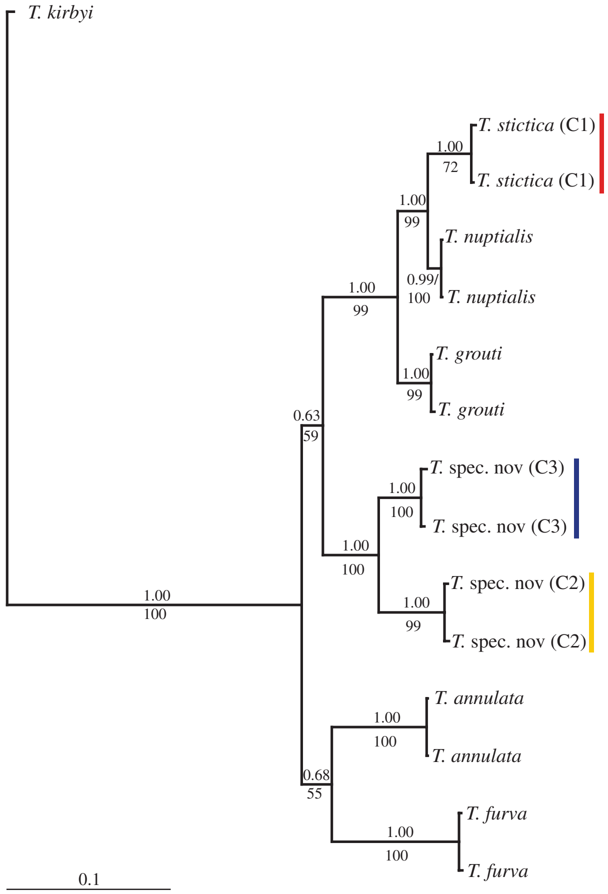

Fig. 2 Bayesian tree of selected *Trithemis* species based on a concatenated matrix of COI and ND1. Posterior probabilities and bootstrap values are included. For the different species at least two individuals were incorporated as well as the two most common haplotypes of each newly found clade. *T. stictica* groups together with *T. nuptialis* and *T. grouti*, whilst *T.* spec. nov. (C2) and *T.* spec. nov. (C3) form two separate sister taxa.

Morphological analyses

Originally, all individuals collected in the field for population genetic studies were identified as *T. stictica*. After re-examination of selected 43 male specimens, slightly different colouration patterns of the abdomen and the thorax were found. These differences were not consistent with the genetic clades. However, two phenotypic traits were identified that unambiguously separated individuals from different genetic groups: eye colour and the colouration of the base of the wings (Table 4). All individuals of the two clades from the Caprivi region had bicoloured eyes and a yellow wing base, whereas specimens from clade 1 had a single eye colour and a clear wing base (Damm & Hadrys 2009).

More ambiguous, but still significant differences were obtained from more detailed measurements of different morphological traits (details see Table 4). Most importantly, SEM analyses of the secondary genitalia revealed differences in penis morphology (Fig. 3). The shape of the two cornuti (indicated by arrows in Fig. 3), located at the distal penis segment, differed between two groups of individuals. The cornuti of all individuals from clade 1 (Kenya, Tanzania, South Africa, Ethiopia, Zebra River and Naukluft) were curved and pointed at the end as described for the holotype of the true *T. stictica* (Pinhey 1970) whereas the cornuti of clades 2 and 3 consistently differed from the former (see Fig. 3). The only difference found so far between clades 2 and 3 was body size, with the abdomen and segment four significantly shorter in clade 3 compared to clade 1 or 2. Thus, whilst the true *T. stictica* could be identified and delimitated morphologically from the other two clades by eye and wing colouration, and penis structure, the differences between the putative new species (clades 2 and 3) were, except for slight size differences, cryptic (Table 4).

Ecological pattern

Mapping the habitats of the sampled sites onto the phylogenetic trees revealed that the three genetic clades differed in habitat preference. Habitats of *T. stictica* (clade 1) were well-vegetated ponds, streams and rivers, some of which were shady (Naukluft populations). Individuals of clade 2 were exclusively found at open habitats with standing or slow flowing waters of the Okavango River, the smaller Kwando River and the Okavango Delta (see Fig. 1), where the surrounding bank vegetation was dominated by grassland and reed. Most of the gallery forest along the Okavango was deforested. Clade 3 was discovered at two sites within the Nature Reserve Popa Falls (Okavango River, including Andara) and at the Zambezi River near Victoria Falls (Zambia). These sites had a mostly in tact gallery forest along the fast-flowing river, characterized by rapids and bordered by high trees providing shade. Interestingly, at one site in the Caprivi region, Popa Falls, a highly heterogeneous landscape, clades 2 and 3 occurred sympatrically. The flight season of the three species was between August and May and the two clades at Popa Falls were caught during the same season at the same time.

Discussion

DNA sequence data now offers a powerful tool for taxonomic studies by greatly expanding the number of characters that can be used to distinguish species. The inclusion of such data, along with the more traditional morphological variables, promises to rectify the problem of subjectivity in current species descriptions (Cracraft 1992; Dayrat 2005; Rubinoff 2006; Vogler 2006; Cardoso *et al.* 2009).

Table 3 (A) Total number of pure diagnostic characters discriminating all individuals from a specific clade or species from each other in a pairwise comparison listed for *T. stictica* (C1 = clade 1), the two putative new species *T.* spec. nov (C2 = clade 2), *T.* spec. nov. (C3 = clade 3) and two closely related sister species based on ND1 (422bp) and COI (630bp) sequences. (B) Character-based DNA barcodes for seven *Trithemis* species, including *T. stictica* and T. spec. nov. (C2 and 3) for ND1 and COI. Shown are diagnostic character states at 13 selected nucleotide positions for ND1 and 16 for COI which are different in at least four positions per species combination

(A) Pairwise comparison	ND1	COI
T. stictica (C1)/*T.* spec. nov. (C2)	26	43
T. stictica (C1)/*T.* spec. nov. (C3)	27	43
T. stictica (C1)/*T. nuptialis*	4	19
T. stictica (C1)/*T. grouti*	21	20
T. spec. nov. (C2)/*T.* spec. nov. (C3)	13	28
T. spec. nov. (C2)/*T. nuptialis*	32	51
T. spec. nov. (C2)/*T. grouti*	30	49
T. spec. nov. (C3)/*T. nuptialis*	28	52
T. spec. nov. (C3)/*T. grouti*	30	50

	Nucleotide positions												
(B) ND1 Species	101	132	135	152	185	191	245	287	290	326	342	355	419
T. stictica (C1)	G	A	G	A	T	A	C	A	T	A	T	C	T
T. spec. nov. (C2)	C	G	A	A	G	T	T	A	C	G	T	C	T
T. spec. nov. (C3)	T	G	A	A	G	T	T	G	T	A	C	C	T
T. grouti	A	A	T	A	T	A	T	G	T	A	T	C	C
T. nuptialis	G	A	G	G	G	A	T	A	T	A	T	T	T
T. annulata	T	G	A	A	C	T	T	A	T	A	T	T	T
T. furva	T	T	A	A	C	C	G	T	T	T	C	T	G

	Nucleotide positions														
COI Species	45	144	162	180	279	288	294	297	330	333	360	393	396	454	459
T. stictica (C1)	C	C	A	C	T	A	A	T	T	G	T	A	A	A	T
T. spec. nov. (C2)	C	G	A	A	A	A	T	T	T	T	C	A	A	C	T
T. spec. nov. (C3)	A	G	A	A	G	G	C	T	T	G	T	G	A	C	T
T. grouti	A	G	G	C	T	A	A	T	T	G	T	A	A	T	C
T. nuptialis	C	G	G	C	T	A	A	C	C	G	T	A	A	A	T
T. annulata	A	T	T	C	A	A	A	T	T	A	T	A	C	A	T
T. furva	A	A	A	T	T	A	A	A	T	T	A	T	T	T	T

Our application of the taxonomic circle (DeSalle *et al.* 2005) to a case study in dragonflies suggests that this simple scheme is able to provide a framework for the discovery of new species. Our analyses of 108 *T. stictica* individuals combined genetic data with morphology, ecology and geography and led to the discovery of two new species that have phenotypically been *cum grano salis* 'cryptic'.

Application of the taxonomic circle

The genetic data provided the most obvious evidence for the existence of two new *Trithemis* species. None of the other disciplines alone would have led to the discovery of the new species. This highlights the importance of DNA analyses for the discovery of new species, particularly of so-called 'cryptic species'. On the other hand, DNA approaches alone can hardly fulfil a species concept in a satisfying way. The taxonomic circle suggested by DeSalle *et al.* (2005) captures, in a simplified way, the components of such a modern taxonomic system: hypothesis testing, corroboration, reciprocal illumination and revision. In this scheme more than one of the five components of the circle (DNA, morphology, reproduction, ecology and geography) has to support the hypothesis of a new species. This approach is based on the assumption that with increasing support from independent data sets, the likelihood for false identifications decreases. In our case study, initially a DNA-based hypothesis was postulated and tested against classical taxonomic components (see Fig. 4). After testing the multiple DNA-based profiles

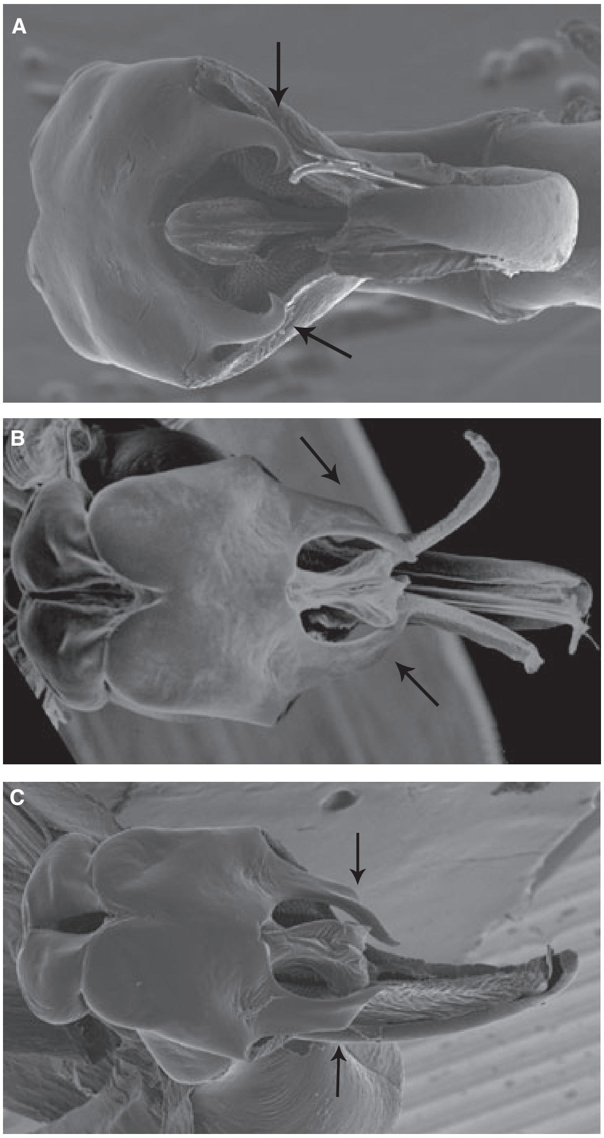

Fig. 3 SEM photographs of the distal segments of the penis of *Trithemis* males including the paired hook-shaped extensions of the hood (also called 'cornuti'): (a) *T. stictica* (b) Clade 3 and (c) Clade 2. Different shapes of the cornuti are marked with arrows.

of the new species against morphology, ecology and geography, we could leave the taxonomic circle, confirm our hypothesis, and also bridge gaps to both the biological and evolutionary species concept.

The initial molecular study started with one species (*T. stictica*) that revealed three genetic clusters. Therefore we analysed two different hypotheses with the help of the taxonomic circle (as displayed in Fig. 4). In the first hypothesis we asked if the two clades (2 and 3) from the Caprivi region could be differentiated from *T. stictica*. Fixed differences in morphology (eye and wing colouration, cornuti shape), geography and ecology supported the hypothesis of two separate entities. In the second hypothesis we tested if clades 2 and 3 can be raised to species status. A separate species status was supported by DNA (e.g. genetic isolation), morphology (fixed size differences) and ecology (niche separation). Thus, with three components supporting the hypothesis we concluded that there are two separated species. Genetic isolation, supported here by mitochondrial data, was consistent with nuclear data in a phylogenic analysis of the genus *Trithemis* (Damm *et al.* 2010). In summary, the significant genetic isolation of the two lineages, the ecological niche shift, the fixed size differences and the most likely reproductive isolation provide substantial corroborative evidence to support the hypothesis of two new sympatric *Trithemis* species in the Caprivi region (Fig. 4b).

In our case study, the taxonomic circle proved to be a valuable tool for the discovery of new species in one of the hardest of all possible cases, in sympatric and 'cryptic species'. Whereas the components of the circle used in our study are likely also applicable for many animal groups, they may not be appropriate for other groups, such as microbial species, for which geographical and morphological information is often lacking (DeSalle *et al.* 2005). In such problematic cases, other components such as additional gene regions or more ecological information could be incorporated to support or refute a species hypothesis. In addition, the quality of hypothesis testing relies on additional aspects such as sample size, the chosen genetic marker and the geographical range of the sampling regime. Morphological as well as genetic variation also occurs intraspecifically, and often varies with geography. The optimal procedure would be to cover the whole distributional range of a hypothetical species. This will not always be possible in most cases, but highlights the importance of the integration of different disciplines for decision making. Often DNA data will suggest a separation, which then leads to more intensive and specific investigation at different organismal levels. Consequently the taxonomic circle presents a practical framework which requires more than one line of evidence to support a species hypothesis. It provides sufficient strictness for species discovery by serving as a bridge between traditional morphological and modern molecular approaches. We assume that the *Trithemis* case study is just one example out of many yet undiscovered examples for the presence of valid species that are easily overlooked at the organismal level.

Advantages of character-based DNA barcoding

In this case study, traditional DNA barcoding methods would also have led to the discovery of two new

Table 4 (A) Means and standard deviations of eleven morphological length parameters (in mm) of males of *T. stictica* (clade 1), clade 2 and clade 3. The statistical significance of the Wilcoxon test for pairwise comparisons of clades is given in the lower half of the table (modified after Damm & Hadrys 2009). Hw: Hind wing; Pt (Hw): Pterostigma; Bs Hw: width of Hw base; Abd: abdomen; App: appendices; S4: segment 4; A: length of outer secondary genitalia; B: length of genital lobe; C: length of the anterior lamina; D: length of the hook of the hamule; E: width of the hamule. (B) Morphological difference of the outer appearance of the three clades (modified after Damm & Hadrys 2009)

(A)	Hw	Pt (Hw)	Bs Hw	Abd	App	S4	A	B	C	D	E
T. stictica (n = 20)	27.95 ± 0.931	3.49 ± 0.153	1.64 ± 0.123	23.10 ± 0.840	1.53 ± 0.126	3.83 ± 0.16	1.41 ± 0.08	0.76 ± 0.04	0.69 ± 0.03	0.51 ± 0.04	0.39 ± 0.03
Clade 2 (n = 11)	26.48 ± 0.576	3.35 ± 0.112	1.43 ± 0.083	23.28 ± 0.526	1.50 ± 0.000	3.90 ± 0.11	1.30 ± 0.03	0.74 ± 0.02	0.67 ± 0.03	0.48 ± 0.02	0.40 ± 0.04
Clade 3 (n = 12)	25.73 ± 1.100	3.39 ± 0.145	1.45 ± 0.081	21.60 ± 0.752	1.46 ± 0.080	3.40 ± 0.10	1.21 ± 0.06	0.75 ± 0.07	0.66 ± 0.04	0.42 ± 0.05	0.38 ± 0.02
P-values											
T. stictica/clade 2	**0.01**	0.15	**0.01**	0.76	0.14	0.69	0.15	0.35	0.21	0.16	0.91
T. stictica/clade 3	**0.00**	0.11	**0.00**	**0.00**	**0.02**	**0.00**	**0.00**	0.33	0.10	0.08	0.87
Clade 2/clade 3	0.21	0.68	0.75	**0.01**	0.36	**0.01**	0.09	0.94	0.76	0.06	1.0

(B)	Eyes	Wing base	'Cornuti' of penis
T. stictica	Unicoloured	Clear	As described by Pinhey (1970)
Clade 2	Bicoloured	Amber	Broader in the middle
Clade 3	Bicoloured	Amber	Broader in the middle

Trithemis species. Sequence divergences between the relevant groups are in concordance with those of taxonomically well described *Trithemis* species, and the 3% cut-off value and the 10× rule were fulfilled (Hebert *et al.* 2003, 2004). In many cases, however, distance methods relying on DNA data alone are ill suited for species delineation. The main reason is that substitution rates of mtDNA vary largely between different groups of species resulting in a broad overlap of intra- and interspecific distances (Will & Rubinoff 2004; Hickerson *et al.* 2006). In dragonflies a universal genetic distance cut-off value may not be valid, since there are several examples in which intrapopulation variation exceeds divergences between species (Cordero Rivera *et al.* 2004; Svensson *et al.* 2006). Thus it seems understandable that 'DNA barcoding' in general was criticized to fail in new species discovery (Hickerson *et al.* 2006).

The introduction of character-based DNA barcoding (Sarkar *et al.* 2002; Rach *et al.* 2008) seems to be a promising complement that avoids the problem of subjective distance thresholds. In the *Trithemis* study, character-based DNA barcoding distinguished all three clades easily through the presence of diagnostic characters or specific combinations of character states. The established character-based DNA barcodes for all *Trithemis* species (using 13 character states of the ND1 and 15 of the COI sequences) represent unique and unambiguous combinations of character states for each species. In some cases, the use of a single barcode marker may not suffice. For example in a former study, the species pair *Aeshna grandis* and *Aeshna cyanea* differed only at a single position in ND1 (Rach *et al.* 2008). Here the application and combination of a second barcode marker, e.g. COI, would be helpful. Another example is the genus *Calopteryx*, where several species exhibited low genetic distances and few diagnostic character states (Rach *et al.* 2008), although the three sister species (*Calopteryx virgo*, *C. splendens* and *C. haemorrhoidalis*) were clearly distinguishable by morphology (Misof *et al.* 2000; Dumont *et al.* 2005). Such examples highlight the overall advantage of character-based barcoding, particularly the possibility to expand the DNA based barcodes with characters from other disciplines.

A character-based database can also contribute more directly to conservation biology, since, in conservation management, information about genetics, ecology and geography is equally important. As our study demonstrated, the two new clades of the described *Trithemis* species complex were hidden for a long time, because habitat preferences of *T. stictica* (Pinhey 1970) seemed to perfectly match the habitats of the Caprivi region with its rivers Okavango, Kwando and Zambezi. Our genetic data fuelled the discovery of the new species and

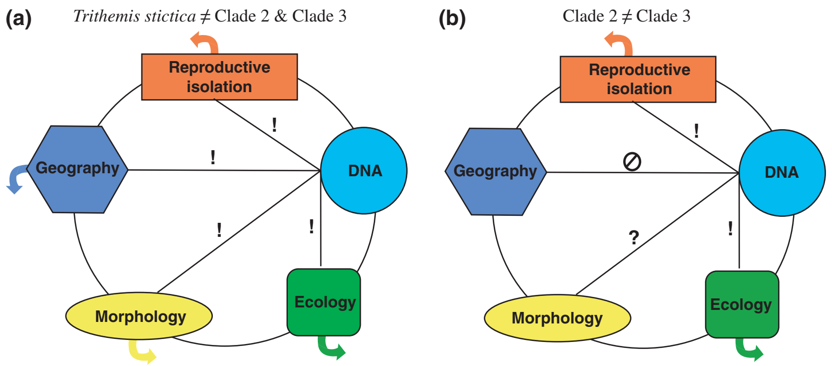

Fig. 4 Taxonomic circles demonstrating an integrative species discovery approach. In this scheme a new taxon could be delineated if at least two disciplines corroborate and verify the hypothesis of a new taxon, which is indicated by an exclamation mark at the interior traversal line. In both circles, the species discovery hypothesis is based on DNA-evidence. (a) The first hypothesis tests the distinctiveness of *T. stictica* and the two new clades. Here all components of the circle corroborate the hypothesis of new species. (b) Based on multiple DNA evidence, the hypothesis tests if the two clades, *T.* spec. nov. (C2) and (C3) are separate species. Here ecology and reproductive isolation corroborate the hypothesis of two new species in the genus *Trithemis*, whilst morphological characters differ only weakly.

resolved differences in habitat choice. We can now map ecological characters to each of the three species.

While the character-based DNA barcode consists of fixed characters for each species, the most critical parameters in establishing a barcode are sample size and the number of CAs (characteristic attributes). The confidence level of a CA fixed in a species increases with sample size. Although there is no absolute certainty for a given CA to be fixed, the reliability of a barcode increases with each additional independent CA added (Rach *et al.* 2008). In endangered or rare species with small population sizes, e.g. in rainforest species like *Chlorocypha centripunctata* (Dijkstra *et al.* 2010), high sample sizes are not easy to obtain. Nevertheless, a DNA barcode of a single individual is still useful and provides important information for this species within a group of interest. Incorporating characters from other disciplines will then increase the reliability of species identification. Criticism of this integrative approach may arise because the establishment of such a database might not be fast enough for conservation concerns. However, DNA based identification will allow an initial decision and non-DNA data can later on complement the database. Thus, DNA based information can be associated with biological information to incorporate also the evolutionary and taxonomically background (Vogler & Monaghan 2007).

Independent of the form, a reliable and fast method for species identification is needed for any kind of conservation management and biodiversity programme. We suggest the integration of distinct DNA characters and traditional information such as morphology, ecology and geography in a comprehensive character based barcode database which would allow fast species identification and discovery.

Cryptic speciation in dragonflies

The results of our study unravelled two new dragonfly species which at the organismal level appeared to be 'cryptic' species. To our knowledge, this is the first detection of speciation in dragonflies that are distributed in the same region but lack morphological differences as well as obvious differences in their genitalia, and hence, lacking in visual reproductive barriers.

Odonates in general are not supposed to evolve 'cryptic' species. Their methods of communicating are not based on invisible mechanisms such as smells or sounds, which are believed to be a major driving force for cryptic speciation (Bickford *et al.* 2007). Nevertheless, their unique reproductive behaviour and fast reaction to environmental change can promote speciation processes without accompanying morphological changes (Kirkpatrick & Ravigne 2002; McPeek & Gavrilets 2006; Svensson *et al.* 2006). Their complex reproductive system and a variety of sperm competition mechanisms may allow the fast evolution of reproductive barriers via strong sexual selection (Waage 1979; Arnqvist *et al.* 2000; Córdoba-Aguilar *et al.* 2003; Cordero Rivera *et al.* 2004). Furthermore, their fast reaction to environmental changes allows fast ecological shifts.

There are examples of morphologically similar dragonfly species e.g. in the genus *Brachythemis* that have only recently been separated as distinct species (Dijkstra & Matushkina 2009). But in these dragonfly species detailed morphological analyses finally revealed clear differences. In the presented study no immediately obvious differences in morphology were found between the two new *Trithemis* species and without the inclusion of genetic data, the species would have remained

Table 5 Summary of diagnostic characters used in the taxonomic circle to proof the discovery of two new species. Shown are the diagnostic characters discriminating the true *T. stictica* from the two newly discovered *T.* spec. nov (clade 2) and (clade 3). Sequence divergence (Seq. div., %), number of variable nucleotide positions distinguishing all individuals of one species from all individuals of the others (diagnostics), significant morphological traits (length of hindwing [HW], length of the base of hindwing [Bs Hw], length of abdomen [AbdL], length of abdomen segment 4 [S4], distal penis segment [Cornuti]), F_{st}-values, and a simplified description of differences in ecological and biogeographical patterns (details see text). Significant *P*-values are displayed with $*P < 0.05$, $**P < 0.001$, and $***P < 0.0001$

	DNA				Morphology				Reproductive isolation					
	Seq. div.		Diagnostics		Size parameters				Cornuti shape differences	F_{st}				
	ND1	CO1	ND1	CO1	Hw	Bs Hw	AbdL	S4		ND1	COI		Ecology	Geography
T. stictica/ Clade 2	9.0	7.9	26	43	**	**	–	–	Significant	0.960	0.984	*T. stictica*	Open habitat	Widespread
T. stictica/ Clade 3	8.5	8.3	27	43	***	***	***	***	Significant	0.944	0.966	Clade 2	Swamp-like habitats	Caprivi region
Clade 2/ Clade 3	5.0	5.7	13	28	–	–	**	**	Weak	0.906	0.921	Clade 3	Fast running water	Caprivi region

undetected. This example shows how important it is to combine different disciplines to determine species boundaries in modern taxonomy. A modern taxonomic system can be derived from both quantitative data and expert opinion. Integration of datasets from different disciplines into one character based matrix ultimately allows species discovery and species assignment in a more objective way.

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This work is part of a long term study on biodiversity and global change using African odonates as model system. The study represents a part of Sandra Damm's doctoral thesis. The author is interested in conservation and population genetic studies of odonates, including phylogeography and biodiversity, with special emphasis on speciation processes and evolutionary adaptations. Bernd Schierwater's research covers (i) integrative approaches to the ecology and evolution of basal metazoans, (ii) evolutionary and applied genomics of Placozoa, (iii) Placozoa biology and systematics, and (iv) new approaches to conservation ecology. Heike Hadrys' research comprises the evolutionary ecology of odonates, new approaches to DNA barcoding for conservation and biodiversity, and phylogenomic approaches to the systematic of insects.

Supporting information

Additional supporting information may be found in the online version of this article.

Table SI Variable nucleotide positions (vnp's) discriminating *T. stictica* and the two new species *T.* spec. nov. (C2) and *T.* spec. nov. (C3)

Fig. SI Maximum Parsimony tree (NDl) of all individuals sampled under the species name of *T. stictica.* Included are posterior probabilities and bootstrap values. A clustering of the individuals into three separate clades is highly supported. Clade 1 consists of individuals of the real *T. stictica,* and Clade 2 and Clade 3 are the putative new species. Locality abbreviations are congruent with Table 1.