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Application of biomolecular techniques on tsetse fly puparia for species identification at larvipostion sites

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Abstract

Puparia are commonly found in tsetse fly larviposition sites during studies on larval ecology. This chitinous shell is representative of past or ongoing exploitation of these sites by tsetse flies. The morphological characteristics of the puparium are not sufficiently distinctive to allow identification of the species. This study explores the applicability of biomolecular techniques on empty puparia for tsetse fly species identification. Five techniques were compared for DNA extraction from tsetse fly puparia, 1/Chelex® 100 Resin, 2/CTAB, 3/Livak's protocol, 4/DEB + proteinase K and 5/QIAamp® DNA Mini kit, using two homogenisation methods (manual and automated). Using a combination of two primer pairs, Chelex, CTAB, and DEB + K proved the most efficient on fresh puparia with 90, 85, and 70% samples identified, respectively. Shifting from fresh to one- to nine-month-old puparia, the Chelex method gave the best result allowing species identification on puparia up to seven months old. The subsequent testing of the Chelex extraction protocol identified 152 (60%) of 252 field-collected puparia samples at species level. The results show that reliable genetic identification of tsetse flies species can be performed from empty puparia, what can prove of great interest for future ecological studies on larviposition sites. The Chelex technique was the most efficient for DNA extraction, though the age-limit of the samples stood at seven months, beyond which DNA degradation probably compromises the genetic analysis.

Introduction

Tsetse flies (Diptera: Glossinidae) are the cyclical vectors of African trypanosomes, the causative agents of Animal African Trypanosomosis (AAT) or nagana in wild and domestic animals, and Human African Trypanosomiasis (HAT) or sleeping sickness in humans (Solano *et al.*, 2010). Due to their distribution over 36 countries in sub-Saharan Africa, tsetse flies and trypanosomes impair the development of productive and sustainable agricultural systems in over ten million km^2 of sub-Saharan Africa (Cecchi *et al.*, 2008; Simarro *et al.*, 2012) leading to overall annual direct lost potential in livestock and crop production estimated at US$4.75 billion (Vreysen *et al.*, 2013).

Thirty-one species and subspecies of tsetse flies have been described in Africa (Solano *et al.*, 2010). Species identification used to be performed by direct observation of adult flies by highly experienced entomologists. Morphometrics studies of wing venation patterns indicate the presence of seven species from West Africa (Kaba *et al.*, 2017) and more recently wing interference patterns has been developed in combination with deep learning for tsetse identification (Cannet *et al.*, 2022). Molecular tools are also available: several PCR primer pairs have been developed targeting conserved regions in the mitochondrial or nuclear DNA sequences to identify tsetse species (Dyer *et al.*, 2008; Augustinos *et al.*, 2018) with the advantage of being applicable on both adult and immature individuals, unlike non-molecular methods.

Gravid tsetse females deposit a single larva at 10-day intervals in specific sites from which an adult will emerge around one month later. These sites are identified by the presence of pupae and puparia in the ground, a puparium being the chitinous outer shell that covers the pupae. Collection of tsetse fly immature stage provides important ecological information on tsetse larviposition habitat which is one of the less known aspects of their life cycle. Puparia are very similar between species and recent attempts to identify species based on puparium morphometrics have failed (Ta *et al.*, 2021). Thus, the species to which a pupa belongs is generally identified after incubation and emergence of the adult in an insectary, using the identification methods listed above. This is time-consuming and subject to rearing failure. At the end of the metamorphosis, the emerging adult fly leaves behind the hardened puparium and a thin exuvial membrane (the puparial shell and the prepupal skin) formed out

of the last two larval cuticles (Mazzanti *et al.*, 2010). To date, no tool has been developed to determine the species of the puparia present in natural larviposition sites, although such information would provide important insights into the reproductive ecology of tsetse flies.

In this study, we investigated whether a reliable molecular identification of tsetse species can be made out of DNA from puparia. Because a puparium contains little DNA, a successful PCR amplification would depend heavily upon the DNA extraction technique used (Young *et al.*, 2014). We thus evaluated and compared the performance of five DNA extraction methods, selected as the most widely used in insect genetic studies (Koella *et al.*, 1998; Alessandrini *et al.*, 2008; Onyango *et al.*, 2022), namely Chelex® 100 Resin (Walsh *et al.*, 1991), CTAB (Doyle, 1987), Livak's protocol (Livak, 1984), DEB + proteinase K (Cornel and Collins, 1996) and QIAamp® DNA Mini kit (QIAGEN, Hilden, Germany), this for subsequent molecular analyses on laboratory puparia of varying ages, as well as on field-collected ones.

Materials and methods

Puparia

Puparia used in this study originated from insectary and larviposition sites in the field.

Insectary

Puparia of *Glossina palpalis gambiensis* (Diptera: Glossinidae) species originating from the mass rearing colony of the Centre International de Recherche Développement sur l'Elevage en zone Subhumide (CIRDES) based in Bobo-Dioulasso, Burkina Faso (Gimonneau *et al.*, 2021) were used to determine the optimal DNA extraction protocol. In a first step, fresh puparia (collected less than 24 h after emergence of the adult) were used to compare the general ease of use and performances of five extraction protocols. In a second step, puparia of increasing ages were used to select the best protocol. It was assumed that the probability of degradation of the DNA present in the puparia increases with time. The puparia were obtained by monthly collection over nine months. At time 0 (T0), 100 fresh puparia were collected from the colony and buried in an autoclaved sand aluminium tray, and conserved in the insectary at 25 ± 2°C and 70 ± 10% relative humidity. Each month during nine months (T0 to T9), 6 puparia for subsequent experiments were retrieved from the tray by sifting, and stored individually in 1.5 ml tubes at −20°C.

Field

252 wild puparia collected in three larviposition sites in Folonzo (Comoé Province, Cascades Region, Burkina Faso), were identified with the best DNA extraction protocol as defined by experimentation on laboratory-generated puparia. These puparia had been extracted by sifting the soil, and transferred into 10 ml tubes and stored at −20°C.

Comparison of homogenisation methods for samples preparations

Two homogenisation techniques were compared. For each one, puparia were placed individually in 1.5 ml tubes. Manual homogenisation was carried out using a mini-pestle adapted to the tubes, with the puparia being homogenise under dry conditions for 5 min. Automated homogenisation was performed in a Qiagen TissueLyser II. For that purpose, 25 µl of distilled water and a 0.7 mm stainless steel ball were placed in each tube containing a puparium, and the mixture was homogenised at 20 Hz for 4 min. The lysis buffer used in each of the extraction techniques was added after homogenisation unless stated otherwise.

Comparison of DNA extraction protocols

Five DNA extraction protocols were compared on 10 fresh puparia obtained using manual or automated homogenisation as described above: Chelex® 100 Resin, CTAB, Livak's protocol, DEB + proteinase K and QIAamp® DNA Mini kit protocols (thereafter Chelex, CTAB, Livak's, DEB + K and Qiagen, respectively). After extraction, DNA was amplified with two different primer pairs designed out of ribosomal DNA sequences (Dyer *et al.*, 2008; Augustinos *et al.*, 2018), both targeting *G.p. gambiensis* and other species present in Burkina Faso with the exception of *Glossina medicorum* for which no primer is available (Table 1).

Based on statistical comparisons of the number of amplified samples for each primer pair used, only the three best DNA extraction protocols were retained for a second round of evaluation on puparia of varying ages (from fresh to nine months old). For each age category, two puparia were used to evaluate each protocol performances.

Finally, the best DNA extraction protocol, based on the previous experimentations, was used to identified glossina species from the 252 wild puparia collected in Folonzo.

Table 1. Primer pairs used to evaluate DNA extraction protocols

Primer names and sequences 5′-3′	Target species	Amplicon sizes	Reference
DiagFw/Rv_ITS1 (*Diag*) TGG ACT TCG GAT TAA GTA CAA CA TCA TTA TGC GCT ATT AAG GTA AGC	*G. palpalis gambiensis* *G. palpalis palpalis* *G. tachinoides*	168 241 and/or 320 221	Dyer *et al.* (2008)
GlossinaITS1_Fw/Rv_ITS1 (*Glos*) GTG ATC CAC CGC TTA GAG TGA GCA AAA GTT GAC CGA ACT TGA	*G. palpalis gambiensis* *G. tachinoides* *G. morsitans morsitans* *G. morsitans submorsitans*	543 597 775 800 + 150	Dyer *et al.* (2008) Augustinos *et al.* (2018)
	G. pallidipes *G. morsitans centralis* *G. fuscipes fuscipes* *G. brevipalpis*	920 800 + 150 618 778	

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DNA extraction protocols

Chelex method

Some 50 µl of a 5% w/v suspension of Chelex® 100 Resin (Bio-Rad, Hercules, CA, USA) in water was added to each 1.5 ml Eppendorf tube containing the homogenise material. Tubes were incubated at 56°C for 1 h, mixed by vortexing, and incubated again at 94°C for 30 min. Tubes were then spun at 12,000 rpm for 3 min and the supernatant transferred to a new tube, carefully avoiding pipetting resin or other debris (Walsh *et al.*, 1991). The supernatant containing the DNA was then stored at −20°C until use.

CTAB method

Some 200 µl of lysis buffer (2% w/v CTAB (Sigma-Aldrich, St Louis, USA), 1.4 M NaCl, 10 mM EDTA and 100 mM Tris HCl pH = 8 (Doyle, 1987)) was added to each 1.5 ml Eppendorf tube containing the homogenise material, and incubated at 65°C for 10 min. Some 200 µl of phenol-chloroform 1:1 was then added to the mixture, followed by centrifugation. The upper aqueous phase was then transferred to a new 1.5 ml tube. The DNA was precipitated by adding 200 µl of isopropanol to the tube, collected by centrifugation, and the pellet washed with 70% ethanol and left to dry at room temperature. The DNA was subsequently dissolved in 50 µl of TE buffer and stored at −20°C until use.

Livak's method

Livak's extraction was performed according to the protocol described by Livak (Livak, 1984). The Livak's buffer consists of 0.5% SDS, 160 mM NaCL, 120 mM EDTA, 10 mM Tris-HCL pH 8 and 60 mM sucrose. Briefly, 100 µl of Livak's buffer was added to each homogenise sample and incubated for 30 min at 65°C. Some 14 µl of 8 M ammonium acetate solution was then added to the mixture, which was incubated on ice for 30 min. After clarification of the mixture by centrifugation at 13,000 rpm for 20 min, the supernatant was transferred to a new tube, and the DNA precipitated by addition of 200 µl of 100% ethanol, followed by centrifugation at 13,000 rpm for 15 min. The resulting pellet was washed with 70% ethanol and then dried at room temperature. The DNA was finally dissolved in 50 µl of TE buffer and stored at −20°C until use.

DEB + proteinase K method

The DEB extraction buffer consists of 0.5% w/v SDS dissolved in 100 mM NaCl, 25 mM EDTA, and 10 mM Tris-HCl pH 8, to which 7 µl of proteinase K per 1.5 ml was added (Cornel and Collins, 1996). Puparia were homogenise in 100 µl of DEB + proteinase K and incubated at 55°C for 1 h. Some 13 µl of 8 M potassium acetate was then added to the mixture, the tube vortexed, incubated on ice for 30 min and centrifuged at 13,000 rpm for 15 min. The supernatant was transferred to another tube and the DNA precipitated by addition of 200 µl of 100% ethanol and incubated at −20°C for 30 min. The pellet was collected by centrifugation, washed with 70% ethanol and air-dried for 10 min. The resulting DNA was dissolved in 20 µl of TE buffer and stored at −20°C until use

QIAamp® DNA mini extraction kit

DNA was extracted using the QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) following the protocol described by the manufacturer, but adapted as follows. Each sample was pre-soaked in distilled water for 10 min before homogenisation. After adding 180 µl of ATL buffer and 20 µl of proteinase K to the mixture (step 3 of the protocol), samples were incubated overnight in a water bath at 56°C, before carrying on with step 4. The resulting DNA was eluted in 200 µl of AE eluent for use in PCR.

DNA amplification

Amplification was performed in a 25 µl reaction volume consisting of 2.5 µl of DNA and 22.5 µl of the mix containing 2.5 µl of 10X buffer with 25 mM MgCl2 (2.5 µM final), 1 µl of 10 mM dNTPs (400 µM final), 1 µl of each 10 µM sense/antisense primers (400 nM final), 0.1 µl of 5 unit μl^{-1} of Taq DNA polymerase (0.5 unit $reaction^{-1}$), 16.9 µl of distilled water. Two different primer pairs both targeting the ITS1 region, the 'DiagFw/Rv' and 'GlossinaITS1_Fw/Rv' thereafter named 'Diag' and 'Gloss' were used (Dyer *et al.*, 2008; Augustinos *et al.*, 2018). PCR cycles were an initial denaturation step at 95°C for 3 min followed by 40 cycles composed of a denaturation step at 95°C for 30 s, a hybridisation at 56°C for 1 min and an extension step at 72°C for 1 min with a final extension at 72°C for 5 min. For each DNA amplification, negative and positive (*G. p. gambiensis* DNA) controls were used. The amplicons, analysed on 2% agarose gel electrophoresis, were visualised under UV light and documented by E-Gel Imager. The two primer pairs used for amplification are detailed in Table 1.

Statistical analysis

The effect of homogenisation methods on PCR results was analysed with a generalised linear mixed model with a binomial family distribution. PCR results were set as response variable and homogenisation methods (manual or automated) as explanatory variable. DNA extraction protocols and primer pairs used were considered as random effect.

The effect of DNA extraction protocols and primer pairs used on PCR results was analysed with a generalised linear model with a binomial family distribution.

For each analysis, the best models were selected on the basis of the lowest Akaike information criterion (Burnham and Anderson 2002). R software (version 3.1.0) was used for all statistical analyses (R Core Team 2017).

Results

Comparison of five DNA extraction techniques on fresh puparia

Results of homogenisation comparison are shown in Table 2. Irrespective of the DNA extraction method and PCR used, automated homogenisation proved the most efficient (Tukey post-hoc test value: $Z = 5.692$, $P < 0.001$). Among the 100 samples tested per homogenisation technic, 66% led to positive PCR when homogenise with the TissueLyser whereas only 26% succeeded with manual homogenisation (Table 2). As a result, subsequent comparisons of DNA extraction protocols were based on PCR results obtained with automated homogenisation.

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Table 2. Results of two different PCRs applied on puparia whose DNA was extracted using five different methods and two homogenisation techniques

	PCR			
	Diag		*Glos*	
DNA extraction methods	Manual homogenisation	Automated homogenisation	Manual homogenisation	Automated homogenisation
Chelex	5	9	4	9
CTAB	5	10	3	7
DEB + K	1	7	0	7
Livak's	1	4	0	3
Qiagen	5	8	2	2
Total (%)	17 (34%)	38 (76%)	9 (18%)	28 (56%)

Ten fresh puparia were used for each evaluation. Chelex, CTAB, Livak's, DEB + K and Qiagen refers to: Chelex® 100 Resin, CTAB buffer, Livak's protocol, DEB + proteinase K and QIAamp® DNA Mini kit DNA extraction protocols. *Diag* and *Glos* refers to the two PCRs performed using primer pairs from Table 1. The numbers indicated in the table are the numbers of positive PCR obtained among 10 samples.

Regarding PCRs performance, the *Diag* PCR performed better than *Glos* (Tukey post hoc test value: $Z = 2.299$, $P = 0.021$) with 38 and 28 of positive results out of 50 samples, respectively (Table 2).

Regardless of the PCR used, the Chelex and CTAB DNA extraction protocols gave the best results with 90 and 85% positive samples respectively and outperformed Livak's that gave the lowest score (35% positive results; Tukey post hoc test: $P < 0.02$). DEB + K and Qiagen protocols (70 and 50% positive results respectively) gave intermediate results that were not different from the others extraction protocols (Tukey post hoc test: $P > 0.05$). Therefore, Chelex, CTAB and DEB + K were retained for a second evaluation on *G.p. gambiensis* puparia of varying ages using the *Diag* PCR.

Table 3. Results of PCR Diag applied on 2 puparia of different ages (0 to 9 months old) whose DNA was extracted using three different methods subsequent to automated homogenisation of the puparia

	DNA extraction methods		
Age in month	Chelex	CTAB	DEB + K
0	++	+	++
	++	+	++
1	++	+	–
	++	+	–
2	++	+	–
	++	+	–
3	++	–	–
	++	+	–
4	+	+	–
	+	–	–
5	–	–	–
	+	–	–
6	–	–	–
	+	–	–
7	–	–	–
	+	–	–
8	–	–	–
	–	–	–
9	–	–	–
	–	–	–

PCR was performed with *Diag* primers. Each line represents one PCR result. High intensity bands are represented by '++' and low intensity bands by '+', while '–' denotes the absence of a visible band.

Evaluation of the three best DNA extraction protocols on puparia of differing ages

Chelex-extracted samples allowed to amplify DNA of puparia aged up to seven months old; CTAB-extracted samples up to four months while DNA extracted using DEB + K protocol gave positive results only on fresh puparia (Table 3). Among the positive results, DNA extracted using Chelex led to high intensity PCR bands up to month 3 and low intensity bands thereafter, whereas PCR done on CTAB-extracted samples always showed low intensity bands, up to four months.

Because the Chelex extraction method allows to extract DNA from puparia up to 7 months old, it was selected to identify the species of field-collected puparia.

Evaluation of Diag PCR and Glos PCR on DNA extracted with Chelex out of field-collected puparia

Since automated homogenisation followed by DNA extraction using Chelex provided 90% of positive result (see Table 2), we decided to evaluated the two *Diag* and *Glos* PCRs on field collected puparia. Puparia collected in three larviposition sites were subjected to automated homogenisation and DNA extraction with Chelex. Among the 252 samples analysed, 152 (60%) were identified by *Diag* or *Glos* PCRs, among which 149 (98%) belonged to the subspecies *G. palpalis gambiensis* and 3 (2%) to *Glossina tachinoides* (Diptera: Glossinidae; Table 4).

Of the overall positive samples, the *Diag* PCR identified 95% of them while the *Glos* PCR only 44% (Table 4), most as *G.p. gambiensis*. *Diag* PCR also identified three *G. tachinoides* samples but *Glos* PCR did not. Of the 145 puparia identified by the *Diag* PCR, only 65 were also recognised by the *Glos* PCR.

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Table 4. Identification of tsetse fly species from wild puparia using *Diag* and *Glos* PCRs

			Species identified					
			G. palpalis gambiensis			*G. tachinoides*		
Larviposition site	Number of puparia analysed	Number of puparia identified	Diag	Glos	Diag/ Glos	Diag	Glos	Diag/ Glos
Site 1	154	73 (47%)	42	6	25	0	0	0
Site 2	58	47 (81%)	23	1	22	1	0	0
Site 3	40	32 (80%)	19	0	11	2	0	0
Total	252	152 (60%)	149			3		

Diag and *Glos* results represent the number of sample identified only by one of the two PCRs, whereas *Diag/Glos* results represent the number of samples identified by both PCRs.

Discussion

In this study, we evaluated five DNA extraction methods in order to investigate the possibility of identifying by PCR the species of tsetse flies from the evacuated puparia. Two specific sets of primers were used for this purpose.

The first experiment was designed to select the three best DNA extraction protocols amongst the five chosen for the study. The two PCRs (Diag & Glos) used amplify DNA from *G.p. gambiensis,* our workhorse species, but also target other tsetse species (Ji *et al.*, 2003; Dyer *et al.*, 2008; Augustinos *et al.*, 2018) of medical and veterinary importance present in West Africa namely *G. tachinoides*, *G. morsitans submorsitans* and *G. palpalis palpalis*. Chelex, CTAB and DEB + K DNA extraction protocols gave the best results on fresh puparia with more than 70% of PCR positive samples whereas Qiagen kit and Livak's protocol were at best reaching 50%. These mediocre and somewhat counter-intuitive results obtained with Qiagen kit and Livak's may stem from the relative low abundance in the puparia. Both protocols require several tube transfers and washing steps, increasing the risk of losing DNA. This observation is in accordance with other studies on optimisation of DNA extraction from old or low-quantity DNA samples (Rohland and Hofreiter, 2007; Gould *et al.*, 2011; Freitas *et al.*, 2014) that concluded that the ideal method to maximise DNA recovery ought to minimise purification steps. As far as commercial kits are concerned, economic considerations should not be overlooked as they are expensive (USD 3 to 10 per purification). A simple protocol with fewer purification steps and of low cost, namely buffer extraction techniques such as Chelex or CTAB, at equal performances, should therefore be preferred (de la Cruz-Ramos *et al.*, 2019).

Two homogenisations methods were also compared in order to optimise DNA extraction. Automated homogenisation with a Qiagen TissueLyser II was superior to manual homogenisation. This tool allowed a better crushing of samples and therefore increased DNA availability, especially in samples where little DNA is present. Although manual homogenisation can be used, if automated homogenisation is not available, it must be considered that it will lead to a significantly lower number of identified samples.

The three selected DNA extraction protocols, Chelex, CTAB and DEB + K, were then evaluated on *G.p. gambiensis* puparia of increasing ages (from fresh to nine months old). Chelex extraction generated enough DNA to identify species from puparia up to seven months old, CTAB up to four months, while DEB + K only worked for fresh samples. The reasons behind this large disparity between protocols are not clear, especially as far as DEB + K is concerned, a method specifically designed for chitinous samples (Campos and Gilbert, 2019). Indeed, the proteinase K used in the latter method is supposed to effectively assist in liberating the DNA from chitinous samples. Chelex method proved more performant than CTAB. In this kind of samples where DNA is present in very low amount, the main aspect is probably that a single purification step with hardly any pipetting steps or tube changes is carried out with Chelex, against several for CTAB, which multiplies by as much the risk of losing the DNA. In other studies, the Chelex method has proven very effective to extract DNA from several chitinous samples such as tsetse fly legs (Ravel *et al.*, 2007), mosquitoes (de la Cruz-Ramos *et al.*, 2019), or honey bees wings (Madella *et al.*, 2021) and as such been recommended by various authors (Gould *et al.*, 2011; Asghar *et al.*, 2015). Moreover, the Chelex extraction method presents several advantages: it is quick to perform (30 to 60 min), does not require multiple tube transfers, is safe, as it does not use toxic organic solvents, and is very cheap, which is an asset in low-income countries, or when large number of samples have to be processed. The main disadvantage is that it is unable to remove inhibitors, which can be detrimental to downstream processes other than classical PCR. Also, the presence of contaminants generally does not allow reliable DNA quantification. In our hands, the shelf-life of Chelex-purified DNA samples is rather short, limited to a couple of weeks, even when stored at −20°C. We shall leave this observation to the readers to ponder, but it should be taken in consideration for certain application beyond timely diagnosis or, in our case, species determination. A limitation to the comparison of extraction methods may be to have used different puparia. The best way to evaluate extraction methods would have been to compare the same puparia across methods. However, due to the small quantity of DNA present in puparia, and the difficulty to split a single puparium into five equal parts, we choose to compare different puparia obtained and conserved under controlled conditions. Another shortcoming could be the small sample size used to compare extraction methods. First, each method was compared based on PCR results of 20 puparia using automated homogenisation. Then the three best protocols were compared on puparia of varying ages (from fresh to nine months old) based on two puparia per ages. This low sample size has been deliberately chosen for convenience sake, given the large number of analyses to be carried out, as it was assumed that different puparia originating from controlled conditions in an insectary were likely to contain equivalent amount of DNA.

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Chelex protocol for DNA extraction was then used to identify tsetse fly species from field-collected puparia of unknown age. Puparia were collected on the river bank on the Comoe river, close to tree trunks in a sandy substrate covered by dead leaves (Salou *et al.*, 2022a). Among the 252 puparia collected from three larviposition sites, 152 (60%) were identified as belonging to two tsetse species: *G.p. gambiensis* (98%) and *G. tachinoides* (2%). The identification rate obtained from wild puparia (60%) was close to that obtained on laboratory puparia, from fresh to nine months old (13/20, 65%). It can therefore be assumed that wild puparia collected and identified were no older than seven months, the age beyond which it was not possible to detect DNA from laboratory puparia, although aging under laboratory conditions may not totally reflect aging under field conditions. For the 99 non-identified puparia, the DNA was probably too old or degraded to allow an efficient extraction.

From an ecological point of view, puparia identification confirms that both *G.p. gambiensis* and *G. tachinoides* are present along the river bank of the Comoe river and that share the same larviposition sites. This is in line with previous studies that showed both species coexisting in sympatry in Folonzo (Rayaissé *et al.*, 2009; Salou *et al.*, 2012, 2022a; Djohan *et al.*, 2015). Recently, Salou *et al.* (2022a), based on field-collected pupae emerged under insectary conditions, showed that *G.p. gambiensis* and *G. tachinoides* almost always share the same larviposition sites (85%, 11/13) in riverine forest galleries with *G.p. gambiensis* being the dominant species (84%). Similarly, even with only 2% of *G. tachinoides* puparia identified in this study, the presence of both species in the same larviposition site is in accordance with previous studies (Buxton, 1955; Salou *et al.*, 2022a) and suggests that both species are driven by similar factors to select larviposition sites (Gimonneau *et al.*, 2021). Among the 99 non-identified puparia, it cannot be excluded that some belong to the *G. medicorum* species, for which no ITS1-based primers are available. Nevertheless, it is very unlikely that *G. medicorum* represent a large part of the unidentified puparia as this species constitutes less than 0.5% of the total adult flies in the Folonzo game reserve (Salou *et al.*, 2022b) and has never been reported as sharing larviposition sites with *G.p. gambiensis* or *G. tachinoides*.

In conclusion, our results showed that reliable molecular identification of tsetse species can be performed from puparia up to seven months old using DNA extracted with Chelex, a technique that performed better than the others we tested. After that time, the progressive degradation of the DNA probably compromises the genetic analysis.

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