Characterization of newly isolated and culture-adapted *T. equiperdum*

Keisuke Suganuma[a], Narantsatsral Sandagdorj[b], Battur Banzragch[b], Battsetseg Badgar[b], Noboru Inoue[a]

[a] OIE Reference Lab. (Surra), National Research center for Protozoan Diseases, Obihiro Univ. of Agricult. & Vet. Med., Japan
[b] Institute of Veterinary Medicine, Mongolian Univ. of Life Science, Mongolia

Mongolian State University of Agriculture

Obihiro University of Agriculture and Veterinary Medicine

World Organisation for Animal Health

20171218, 19 2nd Int. Conf. on NTTAT @Belgium

Dourine cases in Mongolia

Lip, ear and motor paralysis; swelling of genital organ and accumulation of smegma; emaciation and death

T. equiperdum strains were isolated many years ago

T. equiperdum strains

Strain	Country	Host	Isolation
BoTat1.1	Morocco	Horse	1924
STIB 8181	China	Horse	1979
OVI	South Africa	Horse	1977
ATCC30019	France	Horse	1903?
ATCC30023	France	Horse	1903?
Am. stabilate	America?	Horse	?
Can. stabilate	Canada?	Horse	?
AnTat 4.1	?	?	?
Alfort	?	Horse	1949?
Hamburg	?	?	?
SVP	?	?	?
TERU 2259	?	?	?
Dodola	Ethiopia	Horse	2010
TeAp-N/D1	Venezuela	Horse	2009

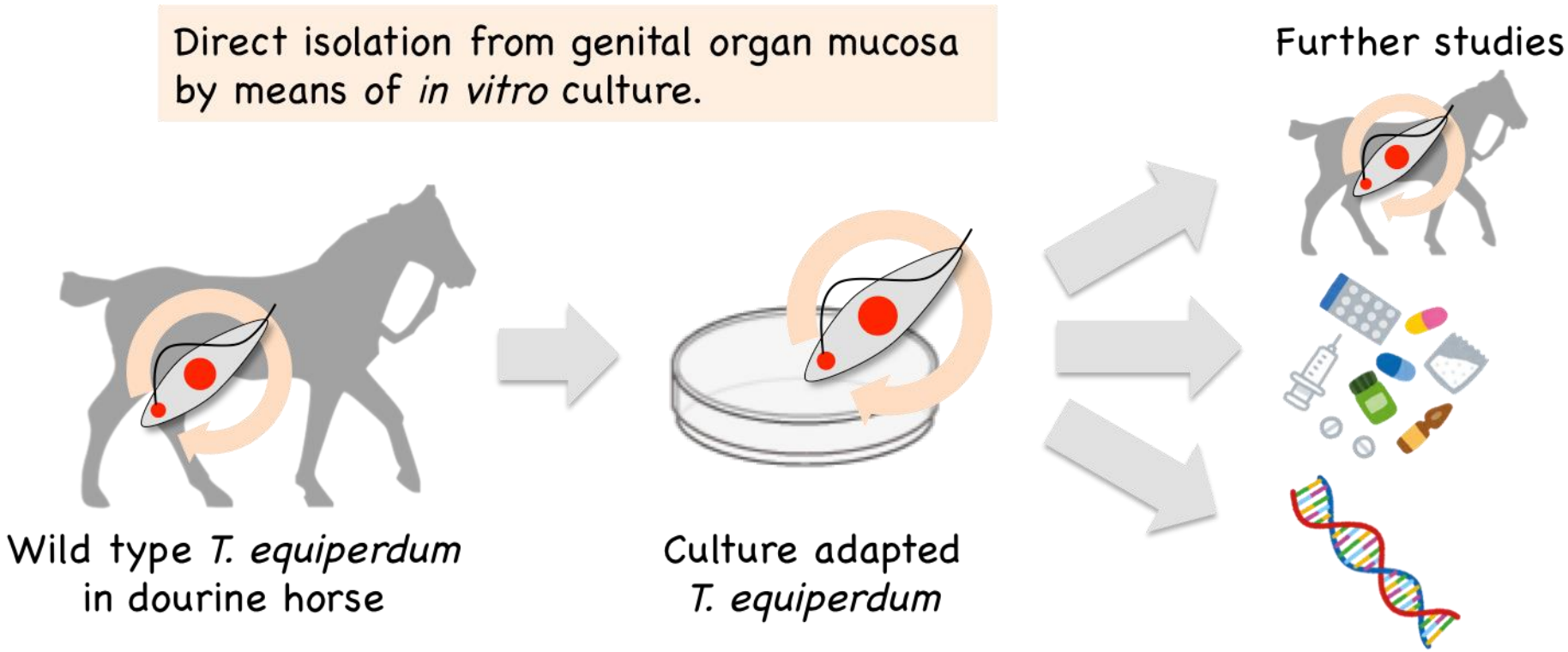
Modified from Claes *et al.*, 2005

Number of *T. equiperdum* strains were isolated and utilized for research.

Most of them were isolated over 40 years.

In addition, these strains lost host specificity and infectivity to horse via genital organ...

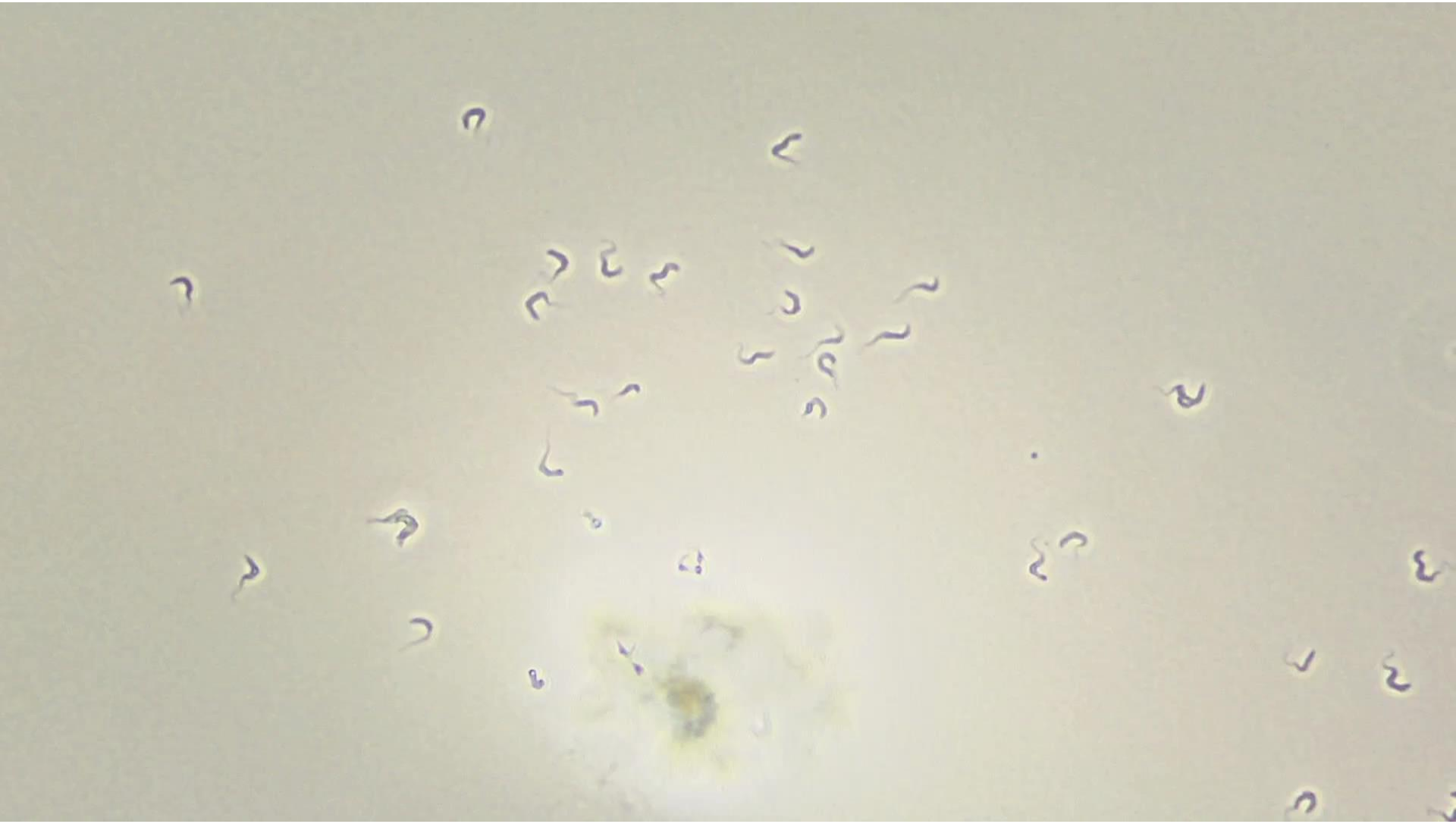
Research strategy and objective of our study



Objective

- ✓ Molecular characterization of new *T. equiperdum* strains which were directly isolated and culture-adapted from genital organ mucosa of dourine horses.

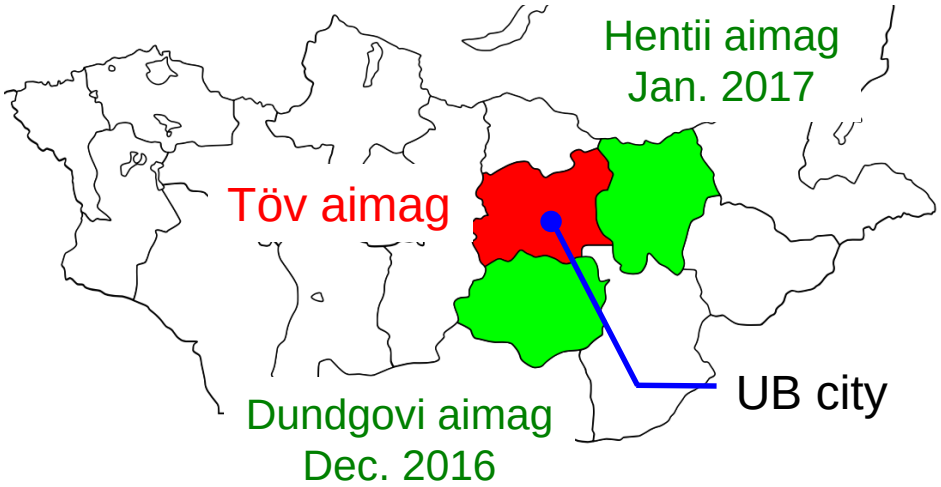
T. equiperdum IVM-t1 strain was isolated from urogenital tract of dourine horse using soft agarose medium



(Suganuma *et al*., 2016, Parasites and Vectors & 1st Int. Conf. on NTTAT by Dr. Igarashi)

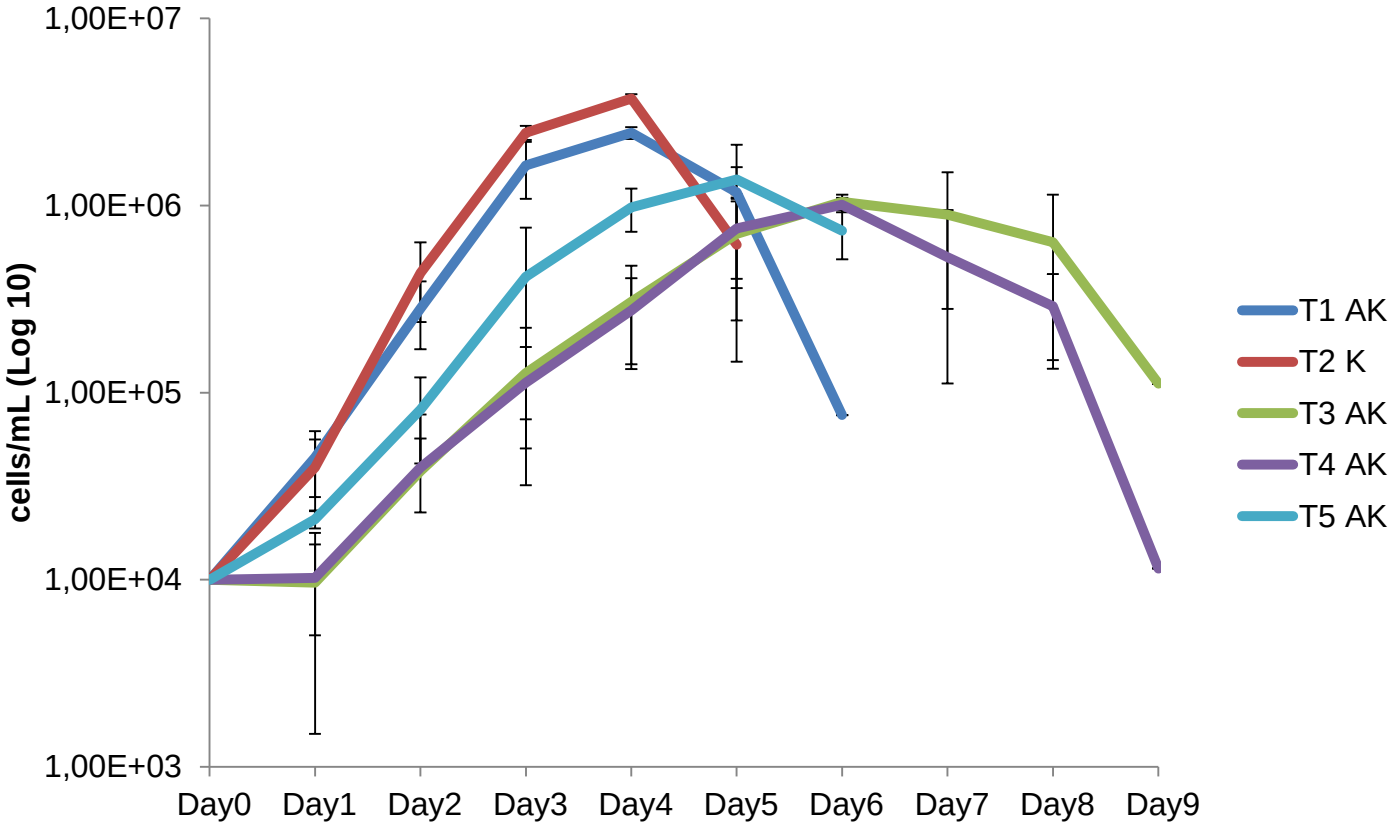
Five new *T. equiperdum* isolates from Monglia

Strain name	Isolated date	Sex, Age	Isolated lesion	Farm location (aimag)	Ref. & Remarks
IVM-t1	April, 2015	male, 7	Urogenital tract swab	Töv	Suganuma & Narantsatsral *et al*., 2016
IVM-t2	April, 2016	male, ?	Urogenital tract swab	Töv	
IVM-t3 (D3)	December, 2016	female, 9	Cerebrospinal fluid (CSF)	Dundgovi	
IVM-t4 (Kh4)	January, 2017	male, 8	Urogenital tract swab	Hentii	
IVM-t5	March, 2017	Thoroughbred stallion, 6	Urogenital tract swab	Töv, Batsumber county	Horse imported from Russia



Five new *T. equiperdum* strains were directly isolated from urogenital tract or cerebrospinal fluid and culture adapted using soft agarose medium.

Growth curve of culture-adapted *T. equiperdum*

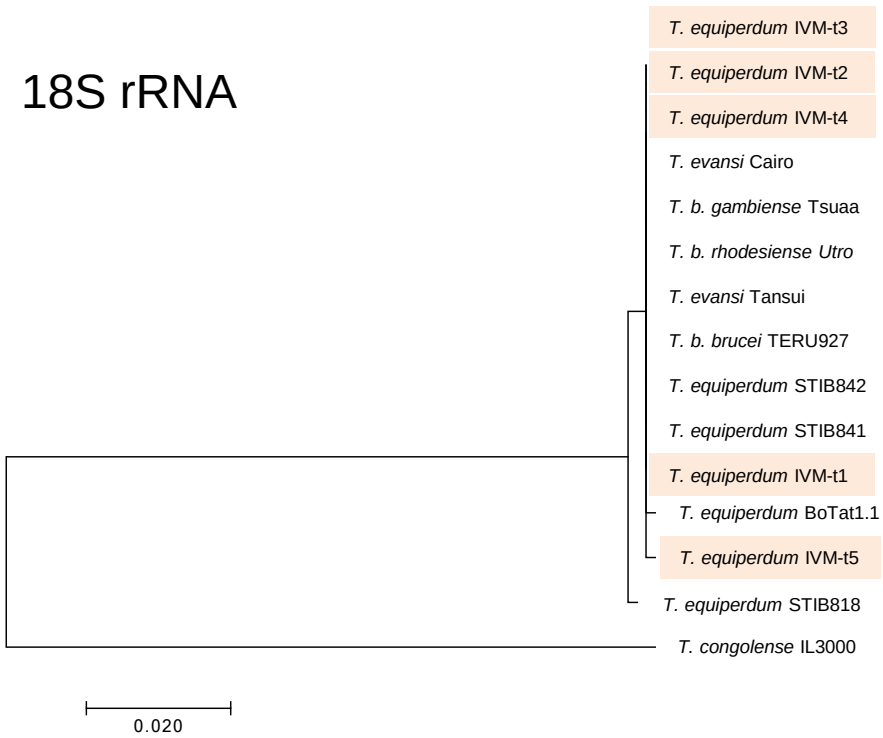


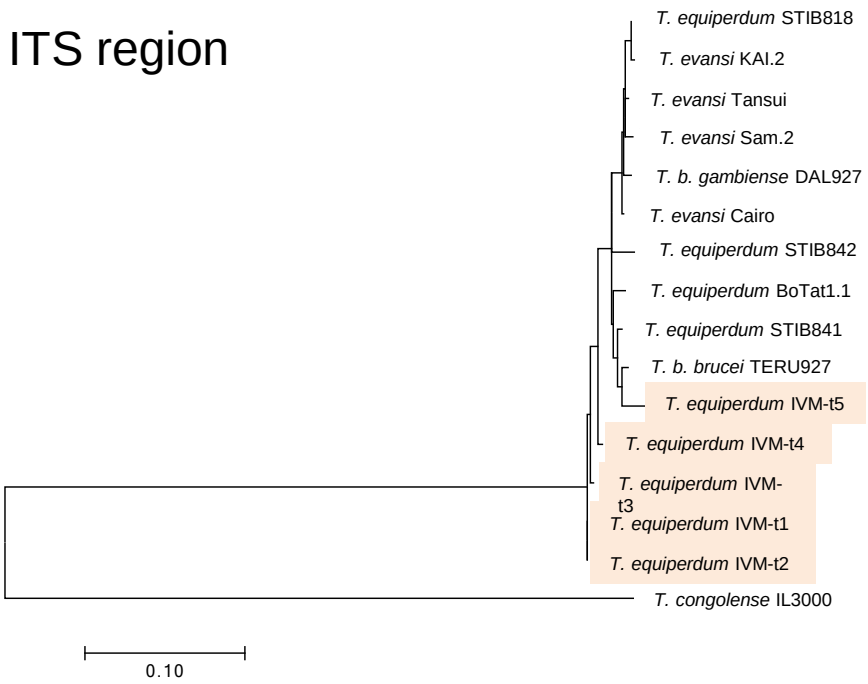
Doubling times
(mean ± SD hours)
T1: 10.0 ± 0.85
T2: 9.1 ± 0.14
T3: 19.7 ± 1.67
T4: 19.5 ± 1.86
T5: 14.1 ± 2.44

Liquid (1% FCeM supplemented HMI-9, Suganuma *et al*., 2017) culture-adapted *T. equiperdum.*
Experiments repeated 3 times, Mean ± SD cells/mL

The doubling times of t1 and t2 were significantly shorter than that of t4 and t5 ($p < 0.05$).

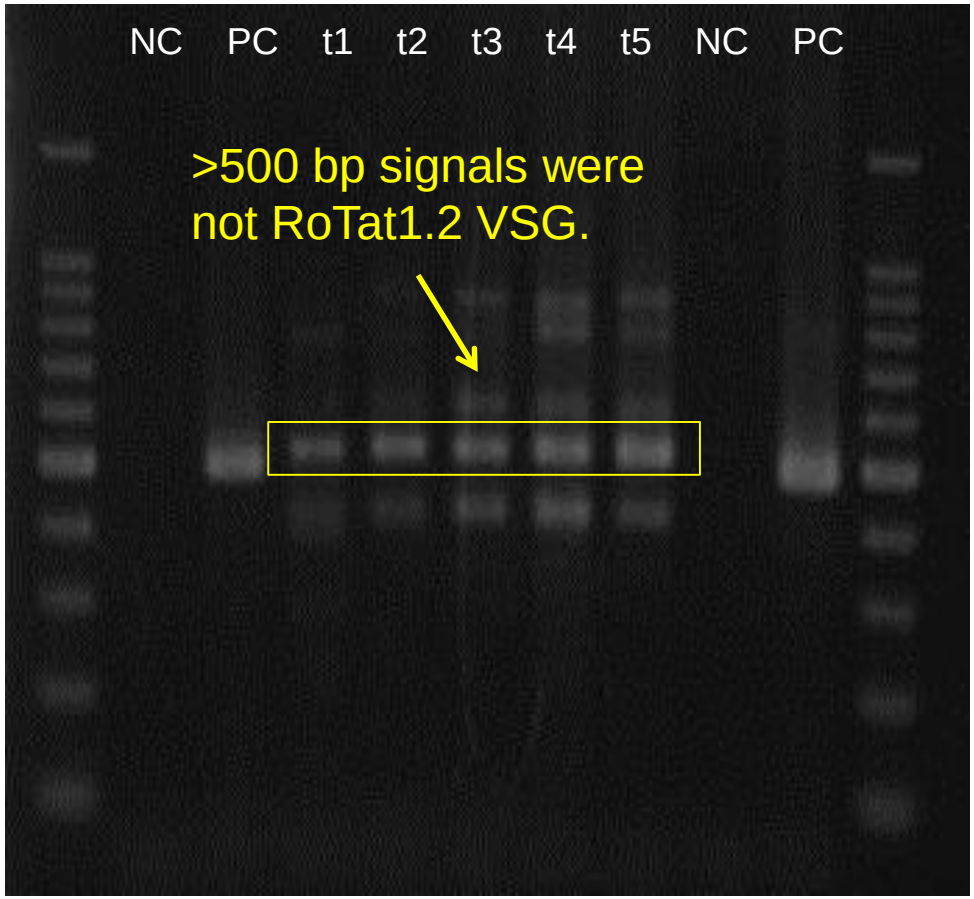
Phylogenetic analysis based on 18S rRNA and ITS





The new *T. equiperdum* strains could not be genetically distinguished from *T. brucei* and *T. evansi* based on 18S rRNA and ITS sequences.

RoTat 1.2 VSG PCR was negative



Primer: ILO7957/ILO8091

PC: Positive control (*T. evansi* IL1695 DNA, 488 bp)
NC: Negative control (DW)
t1-t5: *T. equierdum* IVM-t1, t2, t3, t4, t5

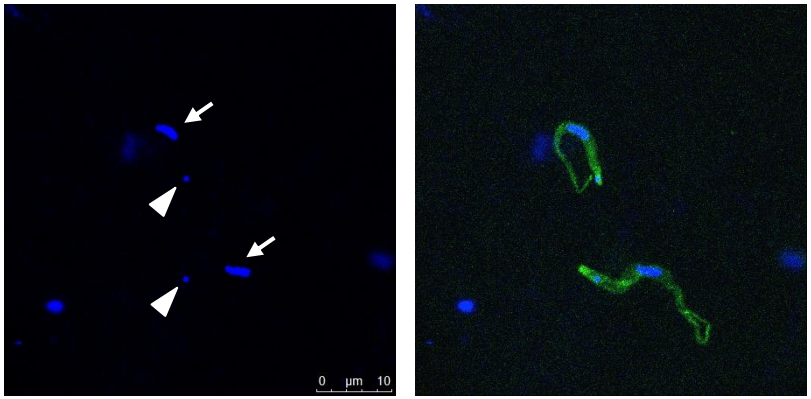
>500 bp signals in *T. equiperdum* isolates were not VSG (sequences were confirmed)

RoTat 1.2 VSG PCR (488 bp) was negative in all *T. equiperdum* strains

Loss of kinetoplast during long term cultivation (?)

T. equiperdum IVM-t1

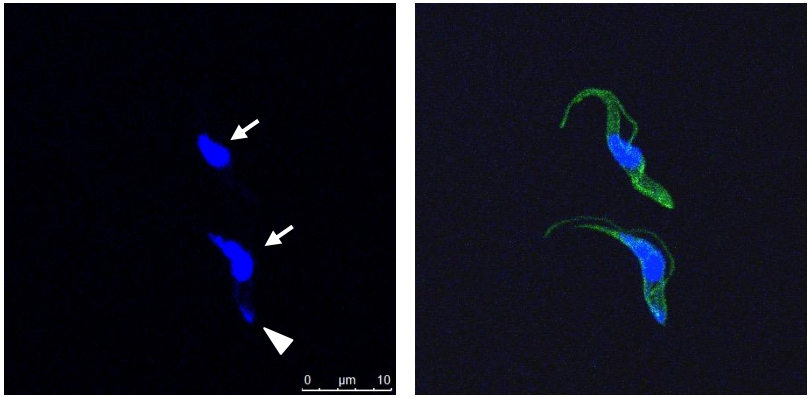
WT



K: 99.37%, AK: 0.63%

Cultivation > 2.5 years

Culture adapted



K: 4.0%, AK: 96.0% (≈ 1.0 y culture)
(AK 100%, > 2.5 y culture)

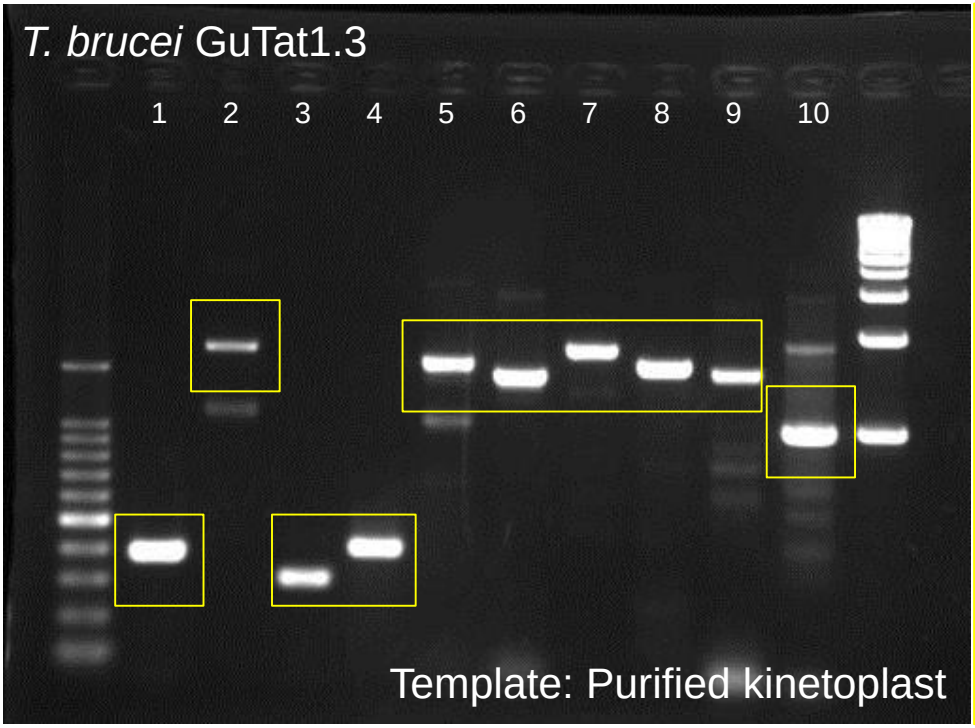
Arrow: Nucleus, Arrowhead: Kinetoplast (Suganuma *et al.*, 2016)

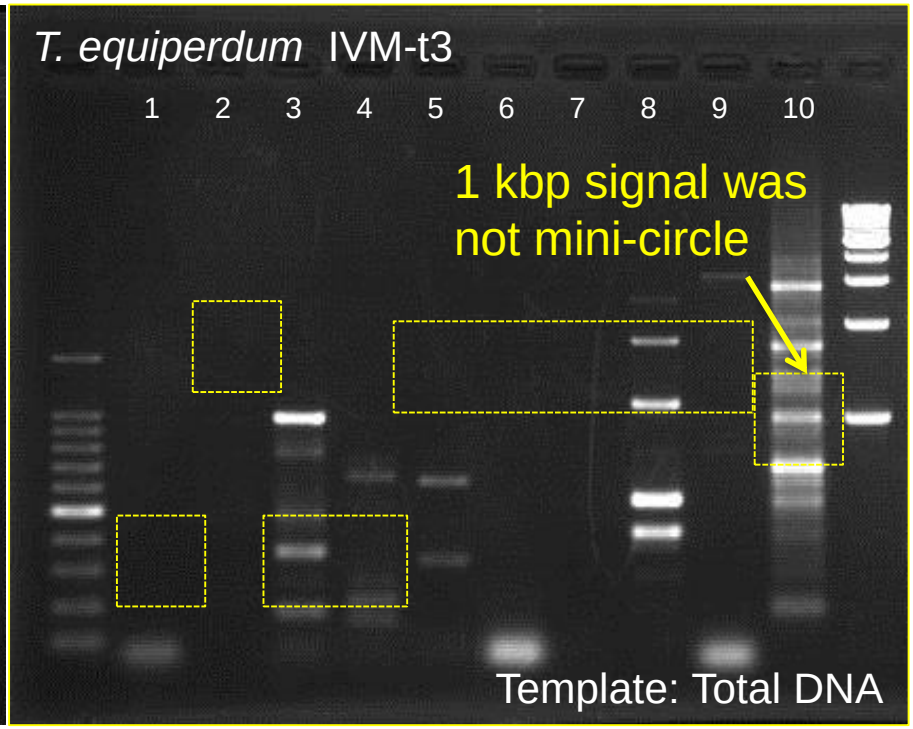
	T3	T4	T5
WT @Mongolia	K: 90.3% AK: 9.7%	K: 84.9% AK: 15.1%	K: 84.9% AK: 15.1%
Cultivation period	≈ 1 year	≈ 1year	≈ 9 months
Culture adapted @Obihiro	K: 0% AK: 100%	K: 0% AK: 100%	K: 0% AK: 100%

Culture-adapted *T. equiperdum* IVM-t1, t3, t4 and t5 strains lost kinetoplast:

Akinetoplastic

Loss of kinetoplast during long term cultivation (?)

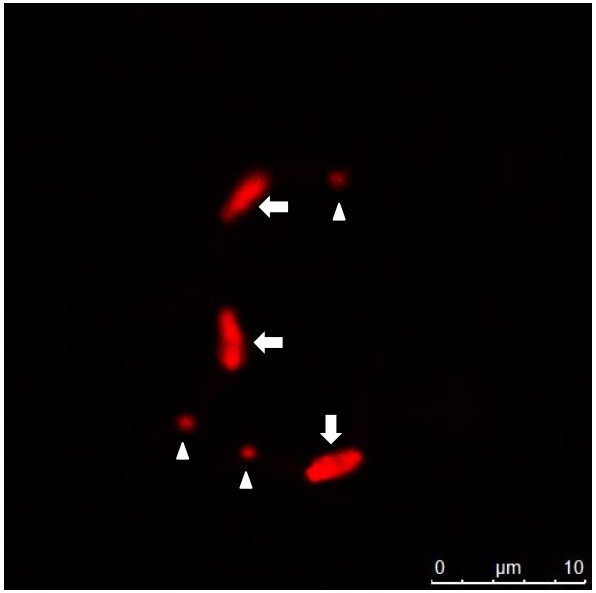


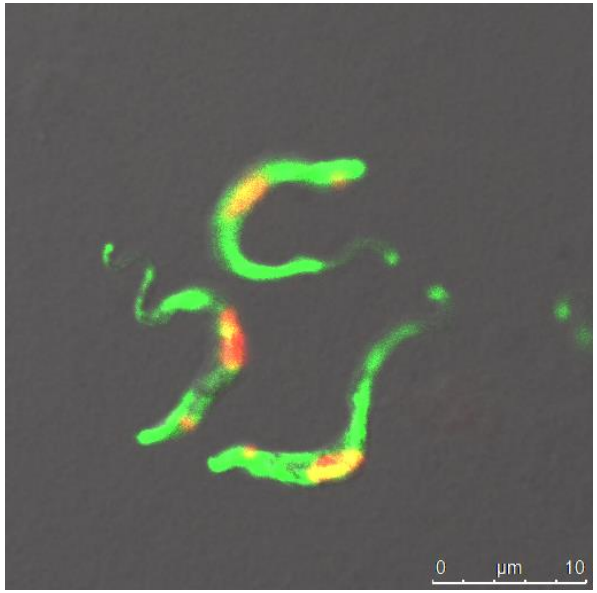


	Maxicircle									Mini
No.	1	2	3	4	5	6	7	8	9	10
Target (Primer)	ND7	Cox2	A6	ND5	12S rRNA	ND7-Cyb	MURF1-ND1	MURF2-Cox1	ND4-ND5	Mini A/B
No.	1	2	3	4	5	6	7	8	9	10
Size (bp)	383	1,747	299	395	1,597	1,450	1,779	1,551	1,515	1,000
Tbb	+	+	+	+	+	+	+	+	+	+
t1, t3, t4, t5	-	-	-	-	-	-	-	-	-	-

Culture-adapted IVM-t2 strain is kinetoplastic

Culture adapted *T. equiperdum* IVM-t2
> 1.5 years cultivation

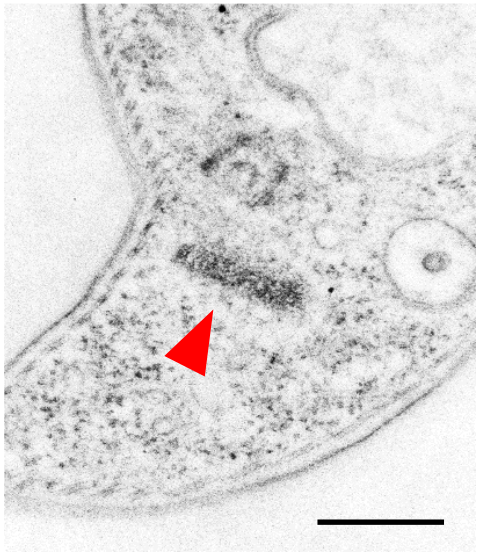


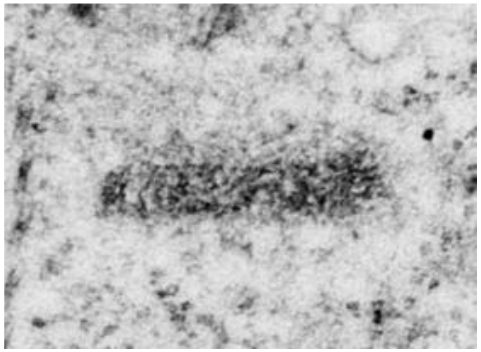


K: 93.0%, AK: 7.0%

Arrow: Nucleus
Arrowhead: Kinetoplast

Ultrastructure of kinetoplast





Bar: 500 nm
Photo by Dr. Kondho

The majority of culture adapted IVM-t2 strain contained kinetoplast:

Kinetoplastic

Kinetoplast analysis of culture-adapted IVM-t2 strain

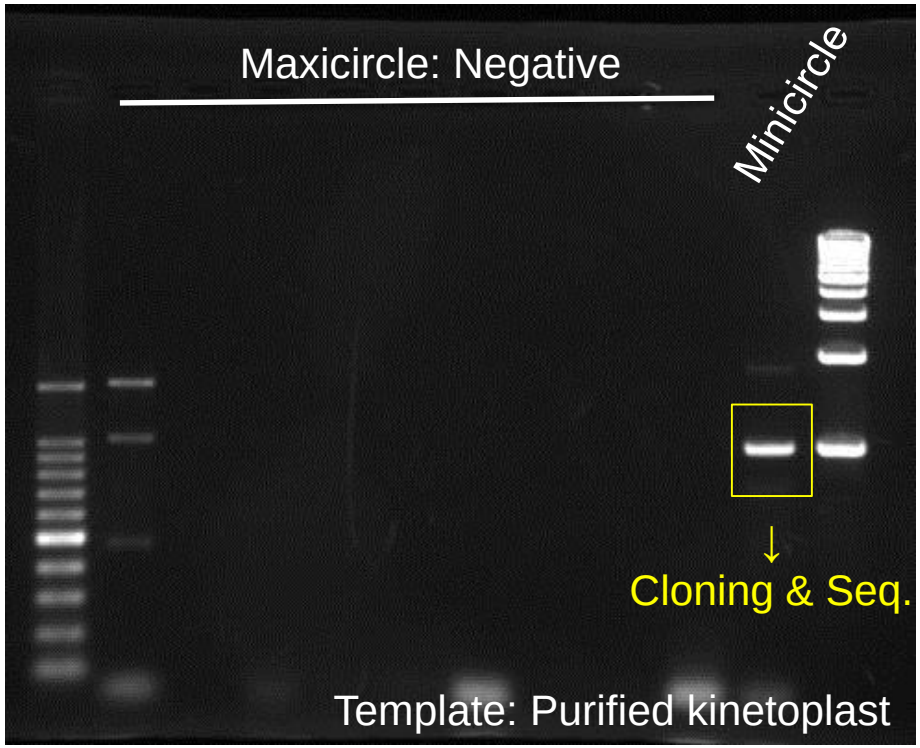
The maxicircle analyses by PCR

- Maxicircle PCR (9 PCRs) → Negative

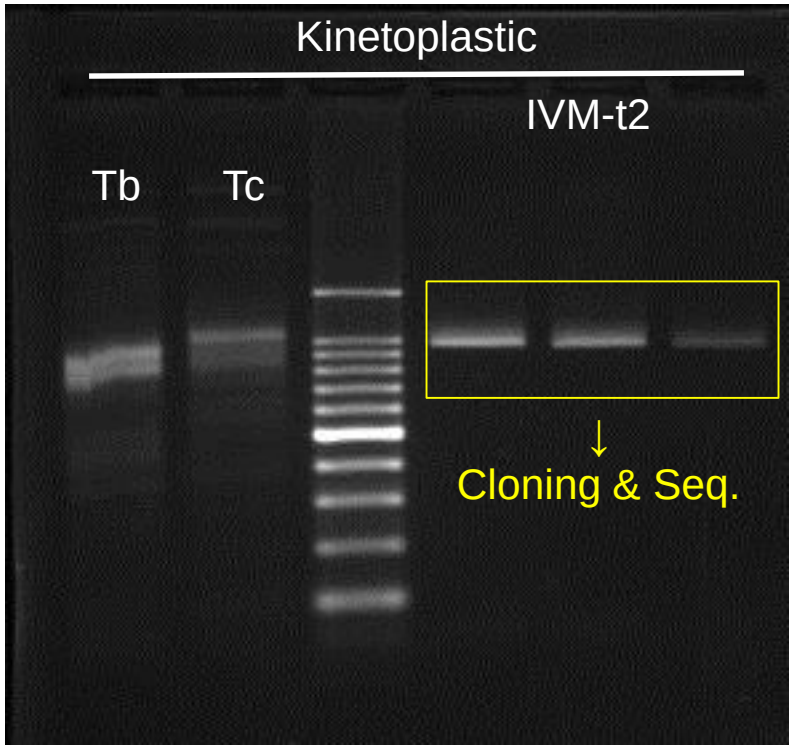
The minicircle analyses

- by restriction enzyme treatment of purified kinetoplast
  → Single band, homogeneous sequence → Cloning & Seq.
- by PCR → Positive → Cloning & Seq.

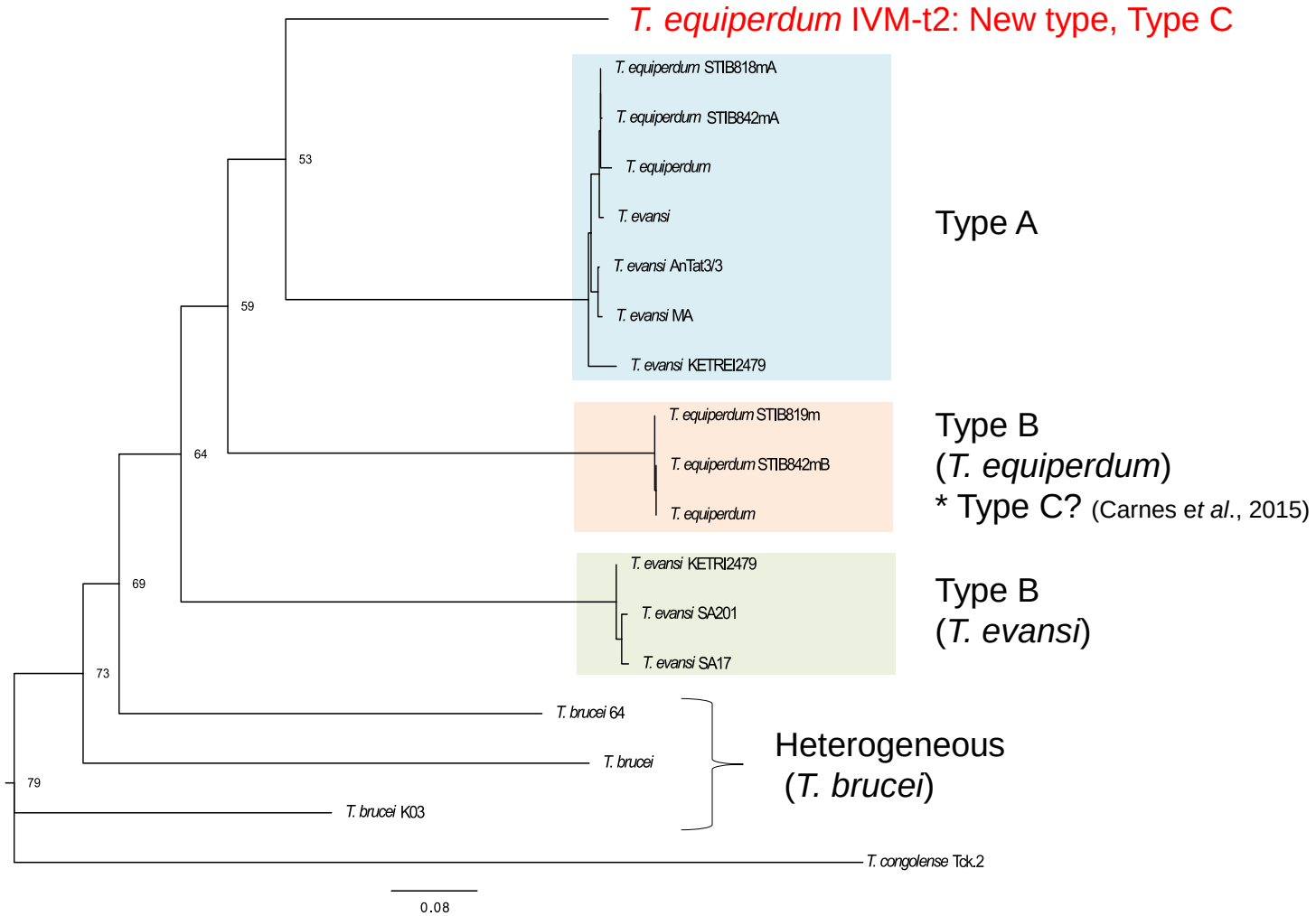
Maxicircle and minicircle PCR



Digested by *Taq* αI



Grouping among *Trypanozoon* based on minicircle sequence



The minicircle of *T. equiperdum* and *T. evansi* were reported type A or type B

While, the minicircle sequence in *T. equiperdum* IVM-t2 was totally different from type A/B, “named type C”

Summary of the characterization of *T. equiperdum*

		IVM-t1	IVM-t2	IVM-t3	IVM-t4	IVM-t5
Origin		Urogenital tract	Urogenital tract	CSF	Urogenital tract	Urogenital tract
Cultivation period		> 2.5 years	> 1.5 years	≈ 1 year	≈ 1year	≈ 9 months
Growth speed		Fast (≈ 9 hours)	Fast (≈ 10 hours)	Slow (≈ 20 hours)	Slow (≈ 20 hours)	Moderate (≈ 15 hours)
18S rRNA		*Trypanozoon* (Cannot identify species)				
ITS						
RoTat1.2		Negative				
Kinetoplast	DNA stain	WT: K → Cult.: AK	WT: K → Cult.: K	WT: K → Cult.: AK	WT: K → Cult.: AK	WT: K → Cult.: AK
	Mini circle	N	Homo, Type C (New type)	N	N	N
	Maxi circle	N*	N	N	N	N
	TEM	N	P	N	N	N

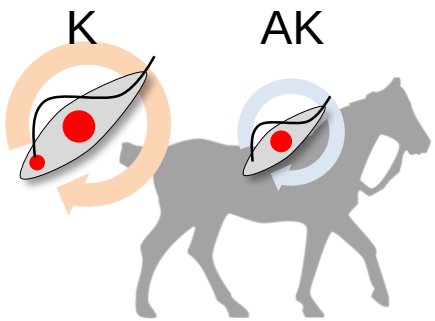
P: Positive, N: Negative, * Maxicircle was partially conserved in IVM-t1 (Suganuma *et al.*, 2016)

Summary of the characterization of *T. equiperdum*

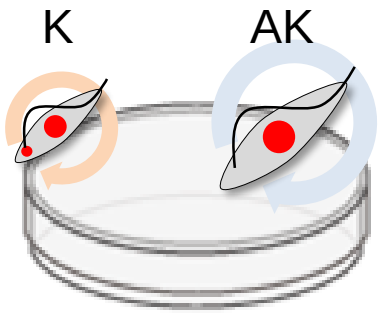
		IVM-t1	IVM-t2	IVM-t3	IVM-t4	IVM-t5
Origin		Urogenital tract	Urogenital tract	CSF	Urogenital tract	Urogenital tract
Cultivation period		> 2.5 years	> 1.5 years	≈ 1 year	≈ 1year	≈ 9 months
Growth speed		Fast (≈ 9 hours)	Fast (≈ 10 hours)	Slow (≈ 20 hours)	Slow (≈ 20 hours)	Moderate (≈ 15 hours)
18S rRNA		*Trypanozoon* (Cannot identify species)				
ITS						
RoTat1.2		Negative				
Kinetoplast	DNA stain	WT: K → Cult.: AK	WT: K → Cult.: K	WT: K → Cult.: AK	WT: K → Cult.: AK	WT: K → Cult.: AK
	Mini circle	N	Homo, Type C (New type)	N	N	N
	Maxi circle	N*	N	N	N	N
	TEM	N	P	N	N	N

P: Positive, N: Negative, * Maxicircle was partially conserved in IVM-t1 (Suganuma *et al.*, 2016)

Why does many of *T. equiperdum* strains lose kinetoplast during *in vitro* cultivation?



in vitro cultivation



Kinetoplast "positive" selection pressure in horse.

⇩

Kinetoplastic is major population in host

No kinetoplast "positive" pressure *in vitro*

⇩

Selected akinetoplastic trypanosomes?

- ✓ Careful observation of kinetoplastic and akinetoplastic populations in IVM-t2 strain during cultivation.
- ✓ Sequence analyses for ATP synthase-γ subunit and topoisomerase II of akinetoplastic and kinetoplastic strains.

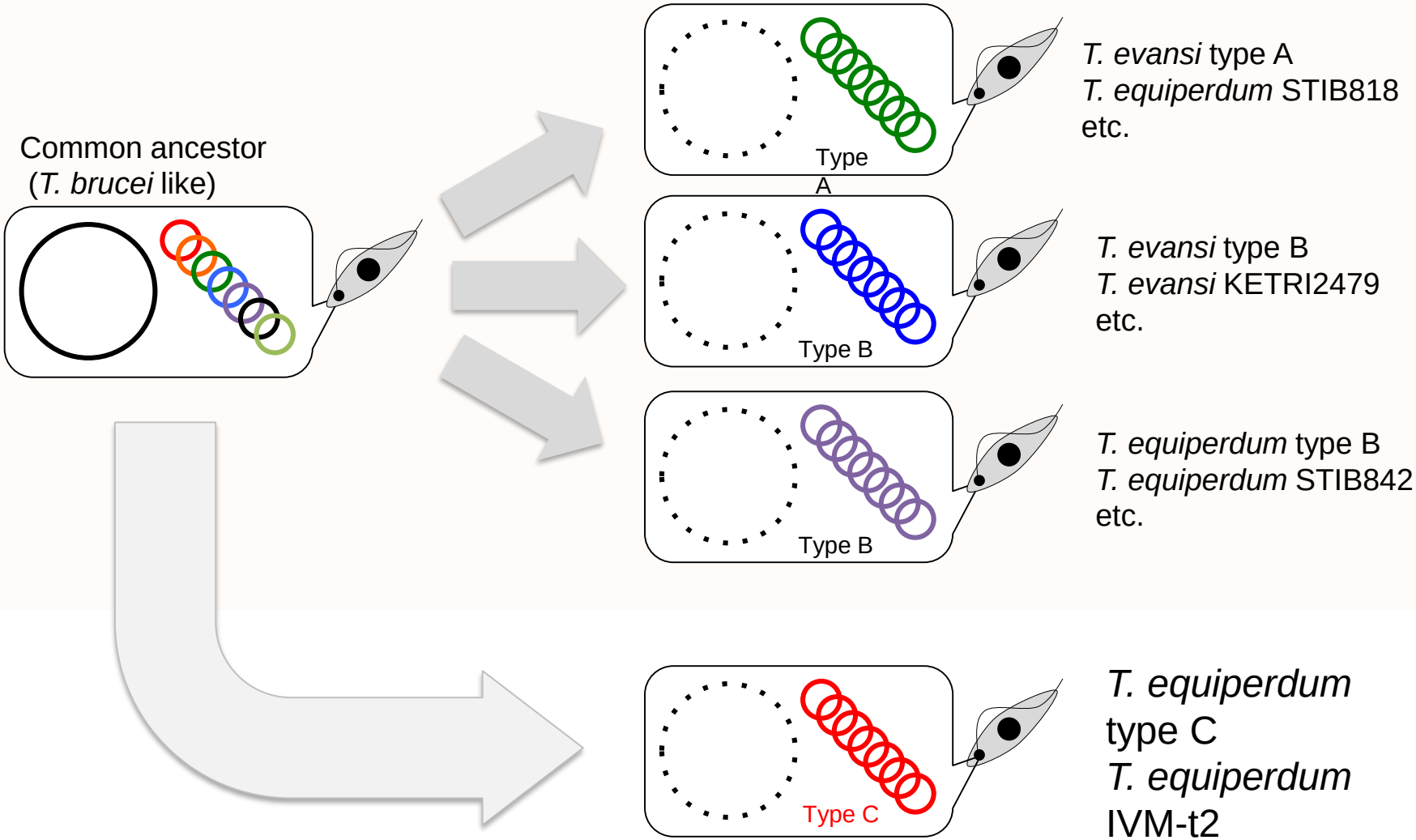
Summary of the characterization of *T. equiperdum*

		IVM-t1	IVM-t2	IVM-t3	IVM-t4	IVM-t5
Origin		Urogenital tract	Urogenital tract	CSF	Urogenital tract	Urogenital tract
Cultivation period		> 2.5 years	> 1.5 years	≈ 1 year	≈ 1year	≈ 9 months
Growth speed		Fast (≈ 9 hours)	Fast (≈ 10 hours)	Slow (≈ 20 hours)	Slow (≈ 20 hours)	Moderate (≈ 15 hours)
18S rRNA		*Trypanozoon* (Cannot identify species)				
ITS						
RoTat1.2		Negative				
Kinetoplast	DNA stain	WT: K → Cult.: AK	WT: K → Cult.: K	WT: K → Cult.: AK	WT: K → Cult.: AK	WT: K → Cult.: AK
	Mini circle	N	Homo, Type C (New type)	N	N	N
	Maxi circle	N*	N	N	N	N
	TEM	N	P	N	N	N

P: Positive, N: Negative, * Maxicircle was partially conserved in IVM-t1 (Suganuma *et al.*, 2016)

Unique minicircle sequence might indicate independent evolution of *T. equipedum* IVM-t2

Independent evolution of *T. evansi* & *T. equiperdum* (Carnes *et al*., 2015, Cuypers *et al*., 2017)



The type of minicircle will be utilized as genetic marker for evolution.

Conclusion

Five new culture-adapted *T. equiperdum* strains were established.

Independent evolution of *T. equiperdum* Mongolia (IVM-t2 strain) from common ancestor was suggested by it’s unique minicircle sequence (Type C).

Future plans

Whole genome and transcriptome analyses of these strains.

In vitro trypanocidal drug screening.

Experimental infection to horse via genital organ and treatment of dourine.

Acknowledgements

Researchers and Staff of NRCPD and IVM

JICA/AMED SATREPS

JSPS

Mongolian farmers and local veterinarians

Supplement

The comparison of minicircle sequences

T. equiperdum IVM-t2															
T. equiperdum STIB818mB	0.69														
T. equiperdum STIB842mB	0.69	0													
T. equiperdum	0.69	0	0												
T. evansi MA	0.6	0.68	0.68	0.68											
T. evansi AnTat3/3	0.6	0.68	0.68	0.68	0.01										
T. evansi KETRI2472	0.62	0.7	0.7	0.7	0.04	0.03									
T. evansi	0.6	0.69	0.69	0.69	0.03	0.03	0.04								
T. equiperdum STIB842mA	0.6	0.68	0.68	0.68	0.03	0.02	0.04	0.01							
T. equiperdum	0.61	0.69	0.69	0.69	0.04	0.04	0.05	0.02	0.01						
T. equiperdum STIB818mA	0.6	0.69	0.69	0.69	0.03	0.02	0.04	0.01	0	0.01					
T. evansi KETRI2479	0.73	0.86	0.86	0.86	0.69	0.69	0.71	0.71	0.71	0.72	0.71				
T. evansi SA201	0.75	0.89	0.89	0.89	0.7	0.7	0.74	0.73	0.72	0.74	0.72	0.01			
T. evansi SA17	0.76	0.88	0.88	0.88	0.71	0.71	0.74	0.73	0.73	0.74	0.73	0.01	0.01		
T. brucei 64	0.7	0.77	0.77	0.77	0.73	0.73	0.73	0.72	0.72	0.72	0.72	0.75	0.76	0.77	
T. brucei	0.91	0.93	0.93	0.93	0.8	0.81	0.83	0.81	0.8	0.82	0.8	0.85	0.86	0.87	0.87
T. brucei K03	0.71	0.74	0.74	0.74	0.78	0.79	0.81	0.79	0.79	0.79	0.78	0.81	0.83	0.83	0.72
T. congolense Tck.2	1.28	1.28	1.28	1.28	1.28	1.27	1.3	1.29	1.27	1.3	1.27	1.33	1.33	1.34	1.33

Type B (*T. equiperdum*)

Type A

Type B (*T. evansi*)

Type C minicircle of *T. equiperdum* IVM-t2 was different from Type A and B minicircle.