Primate Speciation Links to Massive and Directional Shrinkage of the Dinucleotide Short Tandem Repeat Compartment.

Masoud Arabfard

Baqiyatallah University of Medical Sciences

Mahmood Salesi

Baqiyatallah University of Medical Sciences

Iman Arabipour

Islamic Azad University, Tehran

Reza Najafipour

Qazvin University of Medical Sciences

Ahmad Delbari

University of Social Welfare and Rehabilitation Sciences

Hamid Reza Khorram Khorshid

Hope Generation Foundation, Iran

Mina Ohadi (✉ ohadi.mina@yahoo.com)

University of Social Welfare and Rehabilitation Sciences

Research Article

Keywords: dinucleotide, short tandem repeat, directional, primate, mouse, speciation

Posted Date: January 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-114156/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The evolutionary trend of short tandem repeats (STRs) at the crossroads of speciation remains largely elusive and attributed to random evolution for the most part. Here we investigated the dinucleotide STR compartment in primate speciation. We selected six species, which shared sequential chronological ancestors, including mouse, macaque, gorilla, chimpanzee, bonobo, and human, and collected three sets of data on the abundance of all classes of dinucleotide STRs (≥ 6 -repeats) for three regions of every chromosome, each region spanning 10 Mb of DNA. In all three datasets, we found consistent directional shrinkage of the dinucleotide STR compartment in all the primate species selected vs. mouse, as follows: mouse>macaque>great apes. The >20-repeat STRs were the most significantly affected as a result of this shrinkage. We propose that massive and directional shrinkage of the dinucleotide STR compartment had a decisive link with primate speciation. This is a prime instance of massive directional STR trend in multiple speciation.

Introduction

With an exceptionally high degree of polymorphism and plasticity, short tandem repeats (STRs) are a spectacular source of variation required for speciation and evolution¹⁻³. The impact of STRs on speciation is supported by their various functional implications, such as gene expression, alternative splicing, and translation³⁻¹⁰.

While a limited number of studies indicate that purifying selection and drift can shape the structure of STRs at the inter- and intra-species levels¹¹⁻¹⁶, the global trend of STR evolution at the crossroads of primate speciation remains largely unknown.

The most common STRs in the human genome are dinucleotide repeats¹⁷. Here we analyzed the evolutionary trend of this category of STRs in six selected species encompassing rodent, Old World monkey, and great apes.

Materials And Methods

Extraction of STRs from genomic sequences.

The abundance of dinucleotide STRs was studied in six selected species, which shared sequential chronological ancestors, including mouse, macaque, gorilla, chimpanzee, bonobo, and human. We designed a software package in C# environment (Suppl. 1) (<https://github.com/arabfard/Di-Finder>). All possibilities of dinucleotide motifs, consisting of AC, AG, AT, CA, CG, CT, GA, GC, GT, TA, TC, and TG, which were ≥ 6 -repeats were included (Suppl. 2). The written program was based on perfect (pure) STRs. By using the REST API service¹⁸ from Ensemble 101 (<https://asia.ensembl.org>)^{19,20}, data of three regions of every chromosome, each region spanning 10 Megabase (Mb) of genomic DNA, were accessed in the six species (Fig. 1). In each chromosome, the STR abundances were calculated and compared on a

chromosome-to-chromosome basis (Table 1, Suppl. 2). Subsequent to collecting the entire data, we also differentiated the STRs based on their length into two classes of 6-20 repeats and >20-repeats, and studied their abundance in the selected species. Finally, the data of the selected regions of the chromosomes in the six species were aggregated and analyzed.

Statistical analysis

The dinucleotide STR abundance trend in the six selected species was compared across datasets 1, 2, and 3, by correlation coefficient and repeated measurements analysis (Table 2). The Pearson correlation was conducted on the average of STR abundances, consisting of AC, AG, AT, CA, CG, CT, GA, GC, GT, TA, TC, and TG.

Results

Directional shrinkage of all classes of dinucleotide STRs in primates vs. mouse.

In three independent analyses, we studied the way dinucleotide STRs were distributed in respect of their abundance across mouse and primates. The observed trend was strikingly decremental in the five primates vs. mouse (Fig. 2). That trend was consistently replicated in datasets 1, 2, and 3 ($p=0.80$) (Table 2). The AC class of STRs had the most abundance difference in mouse vs. all primates, followed by TG dinucleotides.

The overall STR counts observed in macaque were an intermediate between mouse and the great apes (Table 2, Fig. 3), which matched the phylogenetic distance of these species.

The >20-repeat dinucleotide STRs were more dramatically affected by shrinkage.

The >20-repeat compartment was the most dramatically affected in respect of shrinkage in primates vs. mouse (Fig. 4). While in mouse, this compartment was on average 10-fold larger than in the five selected primate species, the 6-20 repeat compartment was 2-fold larger.

Discussion

It is largely unknown whether at the crossroads of speciation, STRs evolved as a result of purifying selection, genetic drift, and/or in a directional manner. Our approach to deciphering the above was simple, and yet robust. We selected species that shared sequential chronological ancestors, and investigated all possible dinucleotide STRs of all possible lengths ≥ 6 -repeats. Our analysis revealed a directional trend in the abundance of dinucleotide STRs during primate speciation.

Dinucleotide STRs are the most abundant class of STRs in the vertebrate genomes, and their global pattern of abundance may shed light on a vastly unknown aspect of evolutionary biology. The replicated trends observed in our three datasets seem to be independent of the genome size of the selected species. Mouse has the highest abundance of STRs in comparison to the five selected primate species, and yet

its genome is smaller than all those species. This finding is in line with the previous reports of lack of relationship between genome size and abundance of STRs^{21,22}.

An alternative hypothesis to the directional shrinkage of the dinucleotide STR compartment in primates is that this compartment has actually expanded in mouse. However, the observation that the overall STR counts in macaque were an intermediate between mouse and the great ape species studied (rodent>Old World monkey>great apes) (Fig. 5), supports the shrinkage hypothesis for at least part of the trend observed in our study. Indeed, while both hypotheses may stand in evolutionary terms, directional shrinkage is a likely explanation based on the species selected. It is possible that there is a mathematical threshold required for the abundance of STRs in various species. This is in line with the hypothesis that STRs function as scaffolds for biological computers²³. In mouse, the overall number of dinucleotide STRs never declined to the numbers observed in the five primates.

Certain STRs located in the core promoters have been subject to contraction in the process of human and non-human primate evolution²⁴. A number of those STRs are identical in formula in primates vs. non-primates, and the genes linked to those STRs are involved in characteristics that have diverged primates from other mammals, such as craniofacial development, neurogenesis, and spine morphogenesis. It is likely that those STRs functioned as evolutionary switch codes for primate speciation. In line with the above, structural variants are enriched near genes that diverged in expression across great apes^{25,26}. It is speculated that STR variants are more likely than single-nucleotide variants to have epistatic interactions, which can have significant consequences in complex traits, in human as well as model organisms^{27,28}. Future studies such as large-scale genome-editing of STRs²⁹ in embryonic stem cells and investigation of their differentiation into various cell lineages may be candidate approaches to investigate how the massive and dramatically diverged trend of dinucleotide STRs links to primate speciation and evolution.

Limitations

Although this study covered over 600 Mb of genomic DNA in each of the six selected species, it can be extended to the entire genome and additional species.

Conclusion

In conclusion, we propose that massive and directional shrinkage of the dinucleotide STR compartment links to, and probably has a determining impact on primate speciation. This is a prime instance of directional STR trend in multiple speciation.

Declarations

Acknowledgments

This research was funded by the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran.

Statement of Ethics

Not applicable. This was an in silico study, and animals and humans were not directly involved in the study.

Conflict of Interest Statement

The authors have no conflict of interest to declare.

Funding Sources

This research was funded by the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran.

Data Availability

All raw data are available at: https://figshare.com/articles/dataset/Di-Finder_Data/13603274.

References

- 1 Mohammadparast, S., Bayat, H., Biglarian, A. & Ohadi, M. Exceptional expansion and conservation of a CT-repeat complex in the core promoter of PAXBP1 in primates. *Am. J. Primatol.* **76**, 747-756 (2014).
- 2 Bushehri, A., Barez, M. R. M., Mansouri, S. K., Biglarian, A. & Ohadi, M. Genome-wide identification of human-and primate-specific core promoter short tandem repeats. *Gene* **587**, 83-90 (2016).
- 3 Nikkhah, M., Rezazadeh, M., Khorshid, H. R. K., Biglarian, A. & Ohadi, M. An exceptionally long CA-repeat in the core promoter of SCGB2B2 links with the evolution of apes and Old World monkeys. *Gene* **576**, 109-114 (2016).
- 4 Jakubosky, D. *et al.* Properties of structural variants and short tandem repeats associated with gene expression and complex traits. *Nature Communications* **11**, 1-15 (2020).
- 5 Valipour, E. *et al.* Polymorphic core promoter GA-repeats alter gene expression of the early embryonic developmental genes. *Gene* **531**, 175-179 (2013).
- 6 Ranathunge, C. *et al.* Transcribed microsatellite allele lengths are often correlated with gene expression in natural sunflower populations. *Mol. Ecol.* (2020).
- 7 Press, M. O., Hall, A. N., Morton, E. A. & Queitsch, C. Substitutions are boring: Some arguments about parallel mutations and high mutation rates. *Trends Genet.* **35**, 253-264 (2019).

- 8 Fotsing, S. F. *et al.* The impact of short tandem repeat variation on gene expression. *Nat. Genet.* **51**, 1652-1659 (2019).
- 9 Arabfard, M., Kavousi, K., Delbari, A. & Ohadi, M. Link between short tandem repeats and translation initiation site selection. *Human genomics* **12**, 47 (2018).
- 10 Yap, K. *et al.* A short tandem repeat-enriched RNA assembles a nuclear compartment to control alternative splicing and promote cell survival. *Mol. Cell* **72**, 525-540 (2018).
- 11 Srivastava, S., Avvaru, A. K., Sowpati, D. T. & Mishra, R. K. Patterns of microsatellite distribution across eukaryotic genomes. *BMC Genomics* **20**, 153 (2019).
- 12 Pavlova, A. *et al.* Purifying selection and genetic drift shaped Pleistocene evolution of the mitochondrial genome in an endangered Australian freshwater fish. *Heredity (Edinb.)* **118**, 466-476 (2017).
- 13 Jorde, P. E. *et al.* Genetically distinct populations of northern shrimp, *Pandalus borealis*, in the North Atlantic: adaptation to different temperatures as an isolation factor. *Mol. Ecol.* **24**, 1742-1757 (2015).
- 14 Legrand, D., Chenel, T., Campagne, C., Lachaise, D. & Cariou, M. L. Inter-island divergence within *Drosophila mauritiana*, a species of the *D. simulans* complex: Past history and/or speciation in progress? *Mol. Ecol.* **20**, 2787-2804 (2011).
- 15 Sun, G. *et al.* Global genetic variation at nine short tandem repeat loci and implications on forensic genetics. *Eur. J. Hum. Genet.* **11**, 39-49 (2003).
- 16 Abe, H. & Gemmell, N. J. Evolutionary footprints of short tandem repeats in avian promoters. *Sci. Rep.* **6**, 1-11 (2016).
- 17 Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. (2001).
- 18 Yates, A. *et al.* The Ensembl REST API: Ensembl data for any language. *Bioinformatics* **31**, 143-145 (2015).
- 19 Zerbino, D. R. *et al.* Ensembl 2018. *Nucleic Acids Res.* **46**, D754-D761 (2018).
- 20 Howe, K. L. *et al.* Ensembl genomes 2020—enabling non-vertebrate genomic research. *Nucleic Acids Res.* **48**, D689-D695 (2020).
- 21 Neff, B. D. & Gross, M. R. Microsatellite evolution in vertebrates: inference from AC dinucleotide repeats. *Evolution* **55**, 1717-1733 (2001).
- 22 Park, J. Y. *et al.* Evolutionary constraints over microsatellite abundance in larger mammals as a potential mechanism against carcinogenic burden. *Sci. Rep.* **6**, 1-5 (2016).

- 23 Herbert, A. Simple Repeats as Building Blocks for Genetic Computers. *Trends Genet.* **36**(10):739-750. (2020).
- 24 Ohadi, M. *et al.* Core promoter short tandem repeats as evolutionary switch codes for primate speciation. *Am. J. Primatol.* **77**, 34-43 (2015).
- 25 Kronenberg, Z. N. *et al.* High-resolution comparative analysis of great ape genomes. *Science* **360** (2018).
26. Bilgin Sonay, T. *et al.* Tandem repeat variation in human and great ape populations and its impact on gene expression divergence. *Genome Res.* **25**, 1591–1599 (2015).
- 27 Press, M. O. & Queitsch, C. Variability in a short tandem repeat mediates complex epistatic interactions in *Arabidopsis thaliana*. *Genetics* **205**, 455-464 (2017).
- 28 Press, M. O., Carlson, K. D. & Queitsch, C. The overdue promise of short tandem repeat variation for heritability. *Trends Genet.* **30**, 504-512 (2014).
- 29 Smith, C. J. *et al.* Enabling large-scale genome editing at repetitive elements by reducing DNA nicking. *Nucleic Acids Res.* **48**, 5183-5195 (2020).

Tables

Table 1: Chromosome-by-chromosome study of dinucleotide STR abundance (count) exemplified in Dataset 2*.

Human																			
Motif	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	111	133	223	203	208	238	216	207	154	168	150	26	21	5	201	161	92	173	192
AG	84	76	84	84	81	62	76	73	61	60	79	4	13	2	85	80	41	115	75
AT	84	103	195	188	139	170	197	173	160	122	156	31	17	0	118	135	71	149	136
CA	84	75	109	101	104	113	110	92	112	84	74	18	10	3	110	88	48	72	91
CG	2	1	1	0	0	0	0	1	0	1	1	0	1	0	0	2	0	4	2
CT	37	47	37	48	40	30	40	25	38	41	40	0	5	0	44	41	19	47	52
GA	64	35	66	96	58	41	43	51	60	45	48	4	5	0	71	42	20	77	55
GC	3	1	0	3	6	2	3	3	1	4	1	0	0	0	2	4	0	4	1
GT	99	104	112	135	125	92	110	104	108	95	108	16	12	3	99	113	58	102	104
TA	59	98	161	141	116	162	196	153	159	110	125	16	35	5	107	159	53	132	110
TC	81	106	107	103	102	85	71	87	81	105	79	9	29	1	102	74	48	106	101
TG	144	158	180	187	197	175	169	203	161	153	184	26	22	0	184	160	92	148	172
Human Total	852	937	1275	1289	1176	1170	1231	1172	1095	988	1045	150	170	19	1123	1059	542	1129	1091
Chimpanzee																			
Motif	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	115	143	211	203	207	243	234	198	154	153	154	177	185	180	177	154	144	165	12
AG	92	61	98	84	79	49	74	62	72	61	64	70	60	71	91	86	65	114	0
AT	85	98	199	157	140	140	173	184	159	119	157	155	180	148	130	135	160	159	0
CA	86	86	103	141	103	100	103	84	93	88	79	99	89	94	109	99	111	82	0
CG	2	1	0	0	3	0	0	4	1	1	0	0	1	2	1	4	2	3	0
CT	48	46	38	43	36	37	41	25	43	46	41	30	33	32	48	53	30	54	0
GA	55	40	62	56	57	47	34	43	61	33	45	44	46	42	60	42	42	71	0
GC	1	2	1	3	6	0	0	2	2	0	2	0	1	1	2	4	2	2	0
GT	96	101	108	106	131	92	114	95	103	99	96	139	82	105	107	150	103	90	0
TA	61	103	146	130	119	139	181	163	126	111	149	124	133	120	110	119	142	132	0
TC	78	91	104	106	103	76	67	73	75	104	89	83	75	80	102	105	81	121	0
TG	167	163	169	187	191	177	150	171	151	160	184	184	183	182	182	153	161	151	0
Chimpanzee Total	886	935	1239	1216	1175	1100	1171	1104	1040	975	1060	1105	1068	1057	1119	1104	1043	1144	12
Bonobo																			
Motif	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	106	142	214	174	205	243	216	194	141	151	143	0	19	0	142	132	132	135	176
AG	67	66	96	79	82	45	70	61	57	62	77	0	14	0	64	73	60	90	70
AT	71	105	183	131	133	155	136	169	145	112	141	0	25	0	77	101	161	113	133
CA	75	83	106	103	108	104	105	89	88	89	81	0	9	0	87	89	111	64	92
CG	1	0	2	0	3	0	1	1	0	1	1	0	0	0	0	2	2	3	1
CT	39	47	40	41	43	37	41	27	45	40	36	0	4	0	29	40	36	49	50
GA	48	30	61	52	49	39	42	36	62	37	42	0	8	0	46	31	43	52	55
GC	2	1	0	2	4	0	1	1	0	1	0	0	0	0	1	2	1	3	0
GT	82	92	112	104	130	93	118	94	101	96	103	0	15	0	73	132	88	88	92
TA	49	95	140	112	104	133	132	141	105	102	143	0	15	0	72	82	114	87	100
TC	69	91	111	108	93	81	65	79	66	78	78	0	11	0	60	90	68	98	101
TG	131	165	176	188	180	168	162	175	137	153	171	0	22	0	120	108	161	131	154
Bonobo Total	740	917	1241	1094	1134	1098	1089	1067	947	922	1016	0	142	0	771	882	977	913	1024
Gorilla																			
Motif	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	102	140	225	195	233	226	234	178	136	143	154	166	187	156	182	173	119	146	176
AG	86	57	83	88	80	50	69	73	67	56	78	58	65	69	79	80	48	89	78
AT	73	98	185	141	115	158	148	134	139	113	138	146	161	145	106	128	122	123	131
CA	88	80	156	90	87	100	104	93	87	91	71	89	87	79	95	75	66	73	86

CG	1	1	1	1	1	0	1	1	0	1	2	0	0	1	0	3	1	2	1
CT	38	43	32	50	43	34	42	39	32	36	33	28	41	39	38	41	18	41	35
GA	58	38	57	62	51	48	45	51	53	40	50	41	38	42	72	49	27	63	50
GC	2	2	1	0	3	0	1	0	1	0	1	0	0	2	0	2	1	3	1
GT	84	100	114	120	114	81	136	81	102	100	93	113	92	118	86	125	77	91	93
TA	45	91	148	126	124	133	173	127	136	103	154	115	148	119	96	158	108	120	102
TC	81	81	106	98	92	77	59	68	69	93	69	68	68	72	93	81	59	105	89
TG	139	170	179	197	206	174	188	161	152	148	181	175	173	154	161	178	123	144	154
Gorilla Total	797	901	1287	1168	1149	1081	1200	1006	974	924	1024	999	1060	996	1008	1093	769	1000	996
Macaque																			
Motif	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	228	194	228	313	216	185	240	185	207	187	242	193	179	158	176	195	282	174	190
AG	104	115	68	93	98	64	73	78	93	81	89	87	66	75	89	100	86	104	86
AT	121	144	140	162	170	127	144	137	118	167	125	113	128	107	109	122	188	132	126
CA	122	125	86	112	104	105	88	98	122	80	113	71	77	99	115	112	125	97	97
CG	1	2	1	0	2	2	0	2	2	3	0	0	1	4	3	0	1	0	2
CT	46	53	42	67	52	51	42	47	57	30	51	35	42	39	40	34	47	96	54
GA	51	59	50	70	71	60	56	59	61	38	47	44	41	44	49	40	60	74	101
GC	4	0	2	4	2	3	1	3	6	0	1	2	4	4	4	1	1	2	2
GT	92	148	119	155	112	102	96	125	119	82	109	89	94	90	108	108	114	92	105
TA	93	101	107	126	124	107	111	103	89	105	89	95	82	77	93	101	154	84	96
TC	121	124	79	133	110	94	69	107	77	66	95	84	88	72	89	67	97	175	98
TG	195	199	184	204	222	184	169	237	172	206	205	177	180	140	177	173	264	146	242
Macaque Total	1178	1264	1106	1439	1283	1084	1089	1181	1123	1045	1166	990	982	909	1052	1053	1419	1176	1199
Mouse																			
Core	Chr1	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Chr11	Chr12	Chr13	Chr14	Chr15	Chr16	Chr17	Chr18	Chr19	ChrX
AC	485	353	455	468	525	297	612	449	602	447	583	542	485	575	473	505	589	516	470
AG	214	189	216	172	193	200	186	188	239	200	214	185	179	226	174	165	178	199	189
AT	163	247	201	260	241	158	144	188	202	212	143	176	143	329	112	163	182	190	183
CA	268	199	249	245	292	233	404	309	295	242	324	262	311	266	314	262	329	278	272
CG	8	4	10	3	4	3	8	2	4	4	2	3	5	1	9	5	6	7	6
CT	139	96	138	115	149	136	128	134	162	118	198	137	133	130	124	130	120	138	149
GA	165	154	160	153	180	170	187	158	225	155	226	183	148	185	162	153	186	202	203
GC	4	3	10	5	4	8	6	8	9	1	1	9	8	4	5	4	8	3	8
GT	294	227	291	214	249	198	448	311	375	259	359	333	324	317	329	317	328	361	272
TA	141	172	180	190	220	90	118	181	176	176	138	165	108	245	92	128	185	186	156
TC	251	188	234	236	276	198	257	245	264	246	319	286	250	266	200	230	256	295	278
TG	433	365	462	438	467	323	551	411	620	455	647	494	434	493	463	460	523	518	438
Mouse Total	2565	2197	2606	2499	2800	2014	3049	2584	3173	2515	3154	2775	2528	3037	2457	2522	2890	2893	2624

*The entire datasets are provided as Suppl. 2.

Table 2. Repeated measurements analysis of datasets 1, 2, and 3.

	Human	Chimpanzee	Bonobo	Gorilla	Macaque	Mouse
Dataset 1	1515.92	1456.92	1255.25	1567.25	2025.50	3007.83
Dataset 2	1459.42	1629.42	1331.17	1619.33	1811.50	4240.17
Dataset 3	1751.67	1691.92	1581.42	1636.50	1780.17	4545.50
p-value	0.801					

Correlation between dataset 1 and 2: 0.960418, $p=0.002319$

Correlation between datasets 1 and 3: 0.935812, $p=0.006048$

Correlation between datasets 2 and 3: 0.992976, $p=0.000074$

Figures

Figure 1

Schematic representation of data collection of the dinucleotide STR compartment. All chromosomes were screened across the six selected species in three datasets 1, 2, and 3. Only one chromosome is depicted as an example.

Figure 2

Abundance of the various dinucleotide STR classes across the six selected species. All classes of dinucleotide STRs were consistently more abundant in mouse vs. all five primates studied.

Figure 3

Massive shrinkage of the dinucleotide STR compartment in primates, replicated in datasets 1, 2, and 3.

Figure 4

Abundance of dinucleotide STRs across mouse and primates based on length. We differentiated dinucleotide STRs based on length in two classes of 6-20-repeats and >20-repeats. While both classes were shrunk in primates vs. mouse, the >20-repeat compartment was more dramatically affected.

Figure 5

Proposed trend of dinucleotide STRs in primate speciation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Suppl.1.DiFinder.pdf](#)
- [Suppl.2..pdf](#)