

# First data on the genetic structure of *Trachemys scripta* populations in Sicily (Testudines: Emydidae)

Luca Vecchioni<sup>1)</sup> , Federico Marrone<sup>1)</sup> , Marco Arculeo<sup>1)</sup> , Melita Vamberger<sup>2)</sup> 

<sup>1)</sup> Università di Palermo, Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Via Archirafi, 18, 90123, Palermo, Italy

<sup>2)</sup> Senckenberg Naturhistorische Sammlungen Dresden, Museum für Tierkunde, Dresden, Germany

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**Abstract:** The pond slider *Trachemys scripta* is one of the most widespread alien turtle species in the world. Its unregulated trade and the consequent uncontrolled releases into the wild led to negative impacts on the native turtles of the invaded areas. In Italy, alien pond sliders are widely spread, and the occurrence of hatchlings and well-established populations is known in some areas. However, to date in Sicily, only a single female of *Trachemys scripta* laying eggs was reported. Besides that, nothing is known about the actual reproduction success and establishment of self-sustaining *T. scripta* populations in Sicily. Therefore, based on 14 previously characterised highly polymorphic microsatellite loci, we aimed to unravel if the alien turtles successfully reproduce in Sicily, in both natural and semi-natural areas (i.e., lake “Biviere di Gela” and “Parco d’Orléans”, respectively). Our results show a clear structuring of the two studied populations. Several full-sibling relationships between the sampled turtles were found, suggesting actual reproduction in the wild of the pond sliders in Sicily. However, further sampling is desirable since no parent-offspring relationships were observed in the studied populations. Due to our results, systematic monitoring of alien pond sliders in the whole of Sicily is crucial for better planning of mitigation strategies in order to protect the native biota of Sicilian inland waters.

**Keywords:** biological invasions, microsatellites, non-indigenous species, pond turtle

## INTRODUCTION

The loss of biodiversity caused by the presence of alien species can happen in multiple ways and is not always easily detectable. For example, the invaders might compete with native species for food resources and cause niche displacement [GUREVITCH, PADILLA 2004], or might prey on native species, altering the trophic structure of the invaded ecosystems [PEELER *et al.* 2011]. When alien species breed successfully in the invaded area, spread and establish viable populations, they are called invasive alien species (IAS). IAS are often a serious threat to native biodiversity and alter the local ecosystem [GUREVITCH, PADILLA 2004]. Moreover, IAS can carry parasites and pathogens which can infect the native species occurring in the invaded areas [SCARDINO *et al.* 2021; VECCHIONI *et al.* 2021].

To date, one of the most widespread species beyond its native distribution range is the pond slider *Trachemys scripta*

(Thunberg in Schoepff 1792), which is a native of the south-eastern United States [ERNST, LOVICH 2009; VAMBERGER *et al.* 2020]. In fact, due to a massive and low-regulated pet trade, the species was imported all over the world, especially into Europe, until a pet trade regulation, implemented in the late 90s, banned the importation into Europe (EU Regulation No. 2551/97). Despite this, a high number of captive individuals were released into the wild, and the spread of the species occurred [BRINGSØE 2006]. This has had negative impacts on native turtles, such as *Emys orbicularis* (L. 1758), which compete with the pond slider for food, basking, and nesting sites [PEARSON *et al.* 2015].

In Europe, the occurrence of *T. scripta* was recorded throughout the Mediterranean region [STANDFUSS *et al.* 2016]. These authors elucidated for the first time the invasive potential of the *Trachemys* populations in Slovenia and provided crucial knowledge for the conservation and management of the native European pond turtle. Recently, another study by TIETZ *et al.*

[2022] genetically confirmed the successful reproduction of the alien pond slider also in Germany.

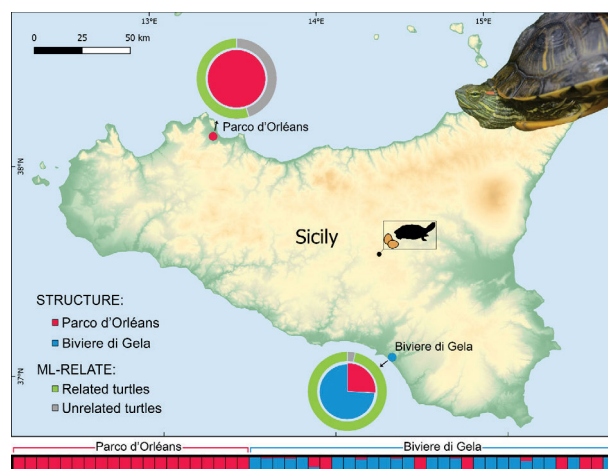
To date, the successful reproduction of the subspecies *T. scripta elegans* has been observed in the Italian peninsula [FICETOLA *et al.* 2009], but has been never genetically proven. Based on the results obtained by FICETOLA *et al.* [2009], it is likely that the bioclimatic conditions of Sicily, where sightings of feral individuals are frequent [CRESCENTE *et al.* 2014; MARRONE *et al.* 2015; SCARDINO *et al.* 2020], are suitable for its breeding. LIUZZO *et al.* [2020] reported the sighting of a female *T. scripta* laying eggs in Lago di Pergusa (Sicily, Italy). Besides that, nothing is known about its possible reproduction success and establishment of self-sustaining populations in Sicily, and information on the distribution of the species on the island is scant [BELLA, TURRISI 2005; LILLO 2008; VECCHIONI *et al.* 2022].

Since it is known that *Trachemys* negatively impacts the European pond turtle *Emys orbicularis*, we expect a similar negative impact also on the Sicilian pond turtle *Emys trinacris* (Fritz, Fattizzo, Guicking, Tripepi, Pennisi, Lenk, Joger, Wink 2005), a Sicilian endemic species listed as “endangered” in the Italian International Union for Conservation of Nature (IUCN) Red List of Threatened Species [RONDININI *et al.* 2013]. Following the previous work by STANDFUSS *et al.* [2016] we investigated, for the very first time, the pattern of genetic diversity and the invasiveness of alien pond slider *Trachemys scripta* from two locations in Sicily (Italy) using 14 microsatellite loci. The implementation of population genetic approaches in this study will allow us to investigate whether pond sliders successfully reproduce in Sicily and if they established viable populations. Our assumption is that reproduction in the wild occurs if closely related individuals are recorded, and that a viable population has established itself when at least some of the studied markers are in Hardy–Weinberg equilibrium (HWE) [STANDFUSS *et al.* 2016]. HWE cannot be achieved, if it is affected by the continuous release of non-native chelonians, i.e., by continuous input of new alleles in the population. Such information is crucial for the local authorities to manage this invasive taxon and to protect the threatened native species that occur in the region.

## MATERIALS AND METHODS

### SAMPLING, DNA EXTRACTION AND SELECTED LOCI AMPLIFICATION

Pond sliders were collected from May to August 2021 in two permanent water bodies: i) “Parco d’Orléans” (World Geodetic System 1984 – WGS84 geographical coordinates: 38.108961 N, 13.353098 E), located in Palermo, and ii) “Biviere di Gela” (WGS84 geographical coordinates: 37.01612 N, 14.349536 E), located close to Gela (province of Caltanissetta) (Fig. 1, Tab. 1). “Parco d’Orléans” is a zoological garden that includes a medium-sized artificial pond managed by Sicilian Region. Conversely, the “Biviere di Gela” is an Oriented Nature Reserve and a Ramsar site that extends over 1.2 km<sup>2</sup>, being one of the island’s largest coastal



**Fig. 1.** Genotypic structuring of 51 pond sliders (*Trachemys scripta*) from two sampling sites ( $K = 2$ ) using 13 microsatellite loci based on the Structure analysis; distinct clusters = colour-coded (red, blue); within each cluster, an individual turtle is represented by a vertical segment that reflects its ancestry; colours of pooled sampling sites in the map = Structure clusters; slice = turtles with mixed ancestries or conflicting cluster assignment (percentages); eggs-icon = the only specimen of *Trachemys scripta* found laying eggs in Lago di Pergusa, Sicily [LIUZZO *et al.* 2020]; source: own elaboration

**Table 1.** Genetic diversity indices of *Trachemys scripta* populations of “Parco d’Orléans” and “Biviere di Gela”

Locus	<i>Trachemys scripta</i> (“Parco d’Orléans”)								<i>Trachemys scripta</i> (“Biviere di Gela”)							
	$A_n$	$A_0$	$A_R$	$A_P$	$H_E$	$H_o$	$P$ -value	HWE	$A_n$	$A_0$	$A_R$	$A_P$	$H_E$	$H_o$	$P$ -value	HWE
Tsc108	13	x	12.60	4	0.91	0.94	0.45	✓	13	x	13.00	4	0.89	0.97	0.61	✓
Tsc169	9	x	10.59	2	0.86	0.71	0.06	✓	11	✓	9.00	4	0.84	0.67	0.05	x
Tsc241	7	x	4.00	3	0.77	0.58	0.02	x	4	x	6.68	-	0.59	0.55	0.43	✓
Tsc243	12	✓	13.57	2	0.92	0.75	0.01	x	14	x	11.78	4	0.89	0.94	0.27	✓
Tsc252	10	x	10.71	2	0.88	0.90	0.09	✓	11	x	9.68	3	0.86	0.94	0.30	✓
Tsc260	13	x	13.85	1	0.91	0.79	0.01	✓	14	✓	12.36	2	0.91	0.76	0.02	x
Tsc263	13	x	8.96	5	0.91	0.95	0.03	x	9	✓	12.55	1	0.79	0.50	0.00	x
Tsc288	15	x	17.19	3	0.89	0.85	0.27	✓	18	x	14.15	6	0.93	0.94	0.11	✓
Tsc297	6	✓	7.00	3	0.52	0.18	0.00	x	7	✓	6.00	4	0.54	0.19	0.00	x
Tsc299	13	x	11.85	3	0.91	0.90	0.67	✓	12	x	12.48	2	0.90	0.97	0.16	✓
Tsc302	9	x	13.58	1	0.86	0.94	0.92	✓	14	x	9.00	6	0.89	0.87	0.44	✓

cont. Tab. 1

Locus	<i>Trachemys scripta</i> ("Parco d'Orléans")								<i>Trachemys scripta</i> ("Biviere di Gela")							
	$A_n$	$A_0$	$A_R$	$A_P$	$H_E$	$H_o$	$P$ -value	HWE	$A_n$	$A_0$	$A_R$	$A_P$	$H_E$	$H_o$	$P$ -value	HWE
Tsc323	8	x	7.80	2	0.82	0.94	0.42	✓	8	x	8.00	2	0.83	0.90	0.06	✓
Tsc328	14	✓	10.72	3	0.94	0.89	0.42	✓	11	✓	13.74	–	0.77	0.66	0.14	✓
Tsc330	11	x	10.00	4	0.90	0.79	0.17	✓	10	x	10.76	3	0.69	0.70	0.25	✓
Total	153	3	10.89	–	–	–	–	9	156	5	10.66	–	–	–	–	10
Ø	10.9	–	–	–	0.85	0.80	–	–	11.1	–	–	–	0.80	0.75	–	–

Explanations:  $A_n$  = number of alleles;  $A_0$  = null alleles;  $A_R$  = allelic richness;  $A_P$  = private allele;  $H_E$  = expected heterozygosity;  $H_o$  = observed heterozygosity; HWE = Hardy-Weinberg equilibrium,  $p$ -value > 0.05 = HWE; Ø = average number; ✓ = yes, x = no.  
Source: own study.

lakes [BARONE *et al.* 2010]. According to BARONE *et al.* [2010], this area is characterised by a typical Mediterranean climate, which is classified as "semi-arid" following De Martonne's aridity index [DE MARTONNE 1926].

Turtles were captured using hoop traps baited with beef liver and mackerel fillet, identified *in situ* based on VAMBERGER *et al.* [2020], and marked with a triangular file on the marginal scutes of the carapace. In addition, the colouration and pattern of the head were observed to determine which subspecies the collected individual belonged to [VAMBERGER *et al.* 2020]. After having taken the morphometric measurements (body mass; carapace linear length, width and height; plastron linear length and width), and determined the age class, after VAMBERGER, KOS [2011], sex and subspecies of each caught turtle, a small tissue sample from the interdigital posterior membrane was cut off from each of them, fixed *in situ* in 96% ethanol and then stored in a freezer at  $-20^{\circ}\text{C}$ .

DNA extraction, PCRs and genotyping of 14 microsatellite loci, previously characterised by SIMISON *et al.* [2013] (Tab. 1), followed the protocols described in the works of STANDFUSS *et al.* [2016] and VAMBERGER *et al.* [2020]. The fragment lengths were determined on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster, CA, USA) using the GeneScan-600 LIZ Size Standard (Applied Biosystems, Foster, CA, USA) and the software Peak Scanner 1.0 (Life Technologies, Carlsbad, CA, USA).

### GENETIC DIVERSITY INDICES AND CLUSTER ANALYSIS

The microsatellite data were analysed by calculating population genetic diversity indices using Convert 1.31 [GLAUBITZ 2004] and Arlequin 3.5.1.2 [EXCOFFIER, LISCHER 2010]. Genetic cluster analysis was performed for *Trachemys scripta* from "Parco d'Orléans" and "Biviere di Gela" using an unsupervised Bayesian clustering approach, implemented in the software Structure 2.3.3 [HUBISZ *et al.* 2009; PRITCHARD *et al.* 2000] to analyse whether the two populations correspond to separate clusters and form established populations. Structure searches in the dataset for populations which are in Hardy-Weinberg equilibrium (HWE) and linkage equilibrium. In the analysis, we applied the admixture model and correlated allele frequencies and arbitrarily set the upper bound for calculations to  $K = 10$ . Because Micro-Checker 2.2.3 [VAN OOSTERHOUT *et al.* 2004] suggested the presence of null alleles (Tab. 1), data were corrected for null alleles according to FALUSH *et al.* [2007]. The most likely number of populations ( $K$ )

was determined by using the log probability of data ( $\ln P(D)$ ) (mean the likelihood of  $K$ ) values and the  $\Delta K$  method [EVANNO *et al.* 2005], implemented in the software Structure Harvester [EARL, VON HOLDT 2012]. We repeated calculations 10 times for each  $K$  using an MCMC chain of 750.000 generations for each run, including a burn-in of 250.000 generations. Population structuring and individual admixture were visualised using the software Distruct 1.1 [ROSENBERG 2004]. Following RANDI [2008], we categorised individuals with proportions of cluster membership below 80% as having mixed ancestries.

Principal component analyses (PCA) was run to examine population structuring based on microsatellite data using the package Adegenet [JOMBART 2008] for CRAN R 3.2.3. Compared to software based on population genetic hypotheses, PCA is less sensitive to sample size bias and not dependent on population genetic assumptions [PUECHMAILLE 2016].

### KINSHIP ANALYSIS

The most likely relationships between individuals of both study sites were determined using the maximum likelihood approach for pairwise relatedness estimation and computing Wright's coefficient ( $r$ ) of relatedness, as implemented in ML-Relate software [KALINOWSKI *et al.* 2006]. Moreover, previously detected null alleles were accommodated by ML-Relate. Kinship analysis was conducted for both *Trachemys scripta* Sicilian populations, i.e., "Parco d'Orléans" and "Biviere di Gela". The kinship analysis was conducted for all relationships available in the software (U – unrelated, HS – half-sibling, FS – full sibling and PO – parent-offspring). The confidence level for estimated relationships in ML-Relate was set at 95% by running 100,000 simulations. When ML-Relate suggested more than one relationship, we executed a specific hypothesis test for two a priori relationships by means of a likelihood ratio test.

### MORPHOMETRIC ANALYSES

Principal coordinates analysis (PCoA) plot, and analysis of similarities (ANOSIM), both based on the morphometric measurements, were performed using the software package Primer 6 [CLARKE, GORLEY 2006], by using the Bray-Curtis distance matrix. In addition, box-plot diagrams were made to graphically describe the distribution groups (i.e., males, females and subadults), based on the raw morphometric data, for both studied populations (Figs. S1–S3).

## RESULTS

### SAMPLING ACTIVITIES

Overall, 51 *Trachemys scripta* individuals were collected, 20 from “Parco d’Orléans” (14 females and 4 males) in a single sampling session of 14 days, and 31 from “Biviere di Gela” (2 females, 18 males and 11 subadults) (Tab. 1), in 4 sampling sessions over 56 days. In “Parco d’Orléans” we captured only adult turtles, 17 old adults and 3 middle-aged individuals. In “Biviere di Gela”, we collected 1 old adult, 4 middle-aged individuals, 15 young adults and 11 subadults (Tab. 1). In addition, three subspecies (*scripta*, *elegans* and *troostii* – Tab. 1) have been identified due to morphological characters as explained in the methods section. Besides that, three melanistic males could be caught.

### GENETIC DIVERSITY INDICES AND GENETIC CLUSTER ANALYSIS

The highest average number of alleles per locus ( $\emptyset$ ) was revealed for the “Biviere di Gela” population with a value of 11.1 (Tab. 1). In both *T. scripta* populations, Micro-Checker detected null alleles for six loci (Tab. 1). The numbers of private alleles ranged from one to five. Furthermore, in both studied *T. scripta* populations numerous loci were in Hardy-Weinberg equilibrium (HWE) (i.e., 9/14 and 10/14 in “Parco d’Orléans” and “Biviere di Gela”, respectively). In “Parco d’Orléans” population the observed heterozygosity ( $H_o$ ) values (range 0.18–0.95) were lower than the expected ones ( $H_E$ , range 0.52–0.94) as well as in “Biviere di Gela” ( $H_o$ , range 0.19–0.97;  $H_E$ , range 0.54–0.93) (Tab. 1).

Using 13 microsatellite loci out of 14 (we excluded the locus TSC297 because of scoring errors due to stuttering) and the correction for null alleles in Structure, we examined whether *T. scripta* from each location corresponds to a population in Hardy-Weinberg and linkage equilibrium. Ln P(D) values and the  $\Delta K$  method suggested  $K = 2$  being the most likely number of clusters (Fig. S4), each corresponding to the respective study site (Fig. 1). However, the genotype found in “Parco d’Orléans” (red cluster, Fig. 1) was observed in seven individuals from “Biviere di Gela” (blue cluster, Fig. 1). Also, the PCA revealed distinct clusters (i.e., the same as detected by the Structure analysis) for each *Trachemys scripta* population analysed (Fig. S5).

### KINSHIP ANALYSIS

Within the two analysed *Trachemys scripta* populations only HS and FS-relationships were detected (Figs. S6–S7). The kinship analysis of the “Parco d’Orléans” population detected an FS-relationship between two subspecies of *T. scripta*, i.e., *T. scripta scripta* (23221) and *T. scripta troostii* (23226) (both old-aged female) (Fig. S6).

### MORPHOMETRIC ANALYSES

PCoA revealed the occurrence of two well-separated groups, corresponding to the two studied sites (Fig. 2). In addition, analysis of similarities (ANOSIM) revealed significant differences between sites, with a value of global R of 0.724. Box-plot diagrams revealed the tendency towards higher values within the “Biviere di Gela” *Trachemys scripta* female groups than the ones of “Parco

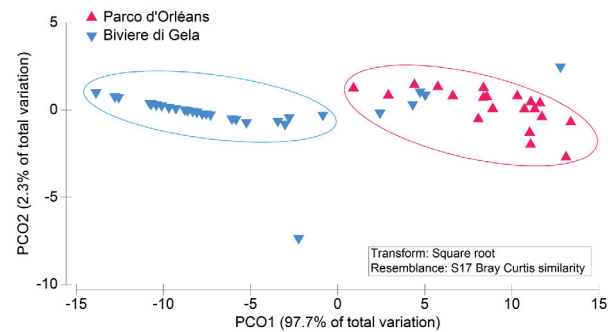


Fig. 2. Principal coordinates analysis (PCoA) plot based on the morphometric measurements (i.e., body mass; carapace linear length, width and height; plastron linear length and width) of *Trachemys scripta* individuals analysed in this study from “Parco d’Orléans” and “Biviere di Gela”; source: own study

d’Orléans”, even though only two females were found at “Biviere di Gela”. Conversely, when male groups were compared, between both populations, higher values were observed at “Parco d’Orléans” (Figs. S1–S3). Due to a lack of sampling of subadults at “Parco d’Orléans”, a comparison between the two study sites was not possible. Nevertheless, the produced box-plots showed a certain degree of consistency between the collected individuals within the same site (Figs. S1–S3).

## DISCUSSION

The pond slider *Trachemys scripta* is regarded in the European Union as an invasive species (IAS) due to its reproductive success and establishment of viable populations not only in the Mediterranean region [STANDFUSS *et al.* 2016] but also in more northern European countries like Germany [TIETZ *et al.* 2022]. It is known, that in some parts of the invaded region, it negatively impacts the native European pond turtle [STANDFUSS *et al.* 2016; JABLONSKI *et al.* 2017], however, the impact on local communities and the ecosystem is still unknown. For these reasons and because of the observation of several large populations of *T. scripta* in Sicily (personal observation), successful reproduction and establishment of viable population in Sicily is expected.

Our results are perfectly in line with our expectations since the software Structure suggests a clear structuring of the two analysed Sicilian populations into two respective clusters (Fig. 1), also supported by Hardy-Weinberg equilibrium found in most of the analysed microsatellite loci (Tab. 1). By comparing our genetic diversity indices (Tab. 1) with those reported by STANDFUSS *et al.* (2016) for the Slovenian populations of *T. scripta*, we observed similar values, suggesting that viable populations are currently settling in Sicily. This is also supported by several FS and HS relationships in both pond slider populations, even though we could not find any PO relationship due to the lack of sampling (Figs. S6–S7).

In addition, the morphological data suggest a good health status of the Sicilian populations compared with the native populations of *T. scripta* in the USA [ROSE, MANNING 1996]. In terms of body size, the average carapace linear length (CLL) of both studied Sicilian populations of *T. scripta* fits within the range of those native populations studied by ROSE and MANNING [1996], and PARKER [1984; 1990] for western Texas (i.e., males – CLL of 10.1–14.0 cm; females – CLL of 18.1–24.0 cm) and western

Mississippi (males – CLL of 17.1–19.0 cm; females – CLL 18.4–22.9 cm), respectively. In fact, at “Parco d’Orléans” ones, the average CLL for males was 18.3 cm, while for females – around 21.8 cm. Conversely, at “Biviere di Gela”, for both adult sexes, lower average CLL values were observed (12.6 cm for males and 20.7 cm for females). Also, the observed sex ratio of 0.4:1 (males: females), occurring at the “Parco d’Orléans”, falls approximately within the range of the native populations of the species (e.g., Texas – 0.53:1; Florida – 0.55–0.73:1) [ARESCO 2005; ROSE, MANNING 1996]. Conversely, at the “Biviere di Gela”, the sex ratio is unbalanced towards males with a ratio of 9:1. However, it should be considered that this value is strongly biased due to the fact that we only sampled two adult females. Therefore, we avoided making meaningful interpretations based on the sex ratio as suggested by THOMAS *et al.* [1999].

Unfortunately, we have a sampling bias in both studied sites, in terms of age structure and sex ratio, despite the extensive sampling effort over the whole sampling season. In fact, in “Biviere di Gela”, only two females could be sampled, while in “Parco d’Orléans” we have a complete sampling lack in subadults or young adults (Tab. 1).

## CONCLUSIONS

Although the import of *Trachemys scripta* s.l. has been banned for many years in Europe, and taking into account that EU Regulation 1143/2014 sets out the rules to prevent the introduction and spread of IAS (including eradication, control or containing them), rigorous monitoring should be carried out in order to assess the real distribution of this invasive species in the Sicilian territory. Particular attention should be paid to all those natural and semi-natural water bodies, where the species could negatively impact endemic species, e.g., the Sicilian pond turtle *Emys trinacris* [VECCHIONI *et al.* 2020], or those with declining populations at a regional level, e.g., the native freshwater crab *Potamon fluviatile* (Herbst 1785) [MARRONE *et al.* 2020; VECCHIONI *et al.* 2017], which are already threatened by the occurrence of a high number of alien and invasive species [MARRONE, NASELLI-FLORES 2015; NASELLI-FLORES, MARRONE 2019]. Furthermore, the ability of the pond slider to use a wide variety of food resources (omnivorous diet), as well as its predatory behaviour, makes it necessary to deepen the studies on its diet not only in Sicily but in the whole invaded range to elucidate the real damage that it can cause to native species and on natural ecosystems.

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## SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at <https://www.jwld.pl/files/Supplementary-material.pdf>

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