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Mitochondrial DNA Sequencing of Middle Neolithic Human Remains of Ling-Ding Site II: Implication for the Social Structure and the Origin of Northeast Coast Taiwaneses

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Abstract

There is a consensus that gene flow characterizing modern Mainland Chinese arrived in Taiwan during the last 400 years, mostly from East China. However, primary genetic studies of ancient human remains of the middle Neolithic era, revealing inconsistencies between the archaic genes profile and that of modern Mainland Chinese, raised debates about the time of arrival of modern Chinese in Taiwan.

To resolve this problem, this study focuses on the analysis of 3000 years BP human remains excavated from the Neolithic east coast archeological Ling-Ding site II near Hualien in Taiwan. The mitochondrial DNA (mtDNA) recovered from five archeological human remains was analyzed to elucidate their genealogy, and to characterize their genetic relationship with the present-day aboriginal and non-aboriginal people of Taiwan.

Five mtDNA haplogroups were characterized from the Ling-Ding site II skeletons, C4a2, N9a1, B4c1b2a, Z, and B4b. Except for mtDNA haplogroups B4c1b2a, commonly seen among the present-day central Taiwan Aborigines and scarce in the heavily sinicised Taiwan western plain tribes, all other haplogroups were common to urban Taiwanese and modern Mainland Chinese. It is proposed that a middle Neolithic gene flow, characterizing Modern Mainland East Asians, was introduced to Taiwan by settlers who reached the East coast of Taiwan in Hualien (Ling-Ding site II) and co-habited with Taiwan Mountain tribe Aborigines. The findings of this study may be relevant for the understanding of the middle Neolithic peopling of Taiwan by non-Austronesian speakers.

Keywords: Ancient DNA; Mitochondrial DNA; Human origins; Taiwan; Molecular genetics; Austronesian speakers

Introduction

With 23 million inhabitants, Taiwan today is populated by two main groups of peoples, the non-Taiwan Aborigines (non-TwA, 97.5%) and the Taiwan Mountain tribe Aborigines (TwA, 2.4%). The great majority of non-TwA today are urban and descendants of Minnan (73.5%) and Hakka (17.5%) from the East and Southeast Coast of China, other non-TwA live in the western plains (~6.5%). The great majority of non-TwA migrated to Tawain in the past 400 years. Most of the TwA (2.5%) live on the Taiwan Mountain Range and the east coast of Taiwan. Their languages belong to the Austronesian language family that started in Taiwan 6000 years ago and expanded across Island Southeast Asia (ISEA), Oceania, and the Indian Ocean in the last 3000 years [1,2]. A few groups of these Austronesian speakers, the Taiwan plain tribes (TwPlt), settled in the western plain of Taiwan, they are now heavily sinicised and most of their original languages are presently extinct [3-5].

Past genetic studies, using Histoleucocyte antigens (HLA), non-recombining Y-chromosome (NRY), mitochondrial DNA (mtDNA), and complete Human genome using Affymetrix Human Origins SNP, have shown that non-TwA and TwA have clearly distinct genetic profiles, and the TwPlt are heavily mixed with non-TwA (approximately 75% to 90%) [6-10]. In addition, more than 85% of the maternal lineages in TwA are nested within mtDNA haplogroups B4a1, B5a, F1a1, F3b, E1a1, and M7 [9,11-14]. According to genetic and linguistic studies, these haplogroups were acquired in Taiwan from Austronesian speaking agriculturists southeast Asia in early Neolithic [1,2]. In contrast, non-TwA groups and TwPlts, have a lower frequency of these mtDNA haplogroups, instead, most of their profile is represented by descendants of mtDNA haplogroups A, C, D4, G, M9, M10 and Z which are commonly seen among the present-day populations of continental East Asia. It is generally believed that these haplogroups represent gene flow over the past 400 years from expanding continental East Asian populations, such as Minnan and Hakka. The finding of Mainland Asia genetic characteristics among ancient human remains from other archeological sites in Taiwan [15] has been the subject of debates among archeologists and anthropologists.

How much of the initial Neolithic genetic changeover in Taiwan coincided with a substantial gene flow from China is still unknown. The degree of genetic relationships between the ancient and modern

populations of Taiwan, and whether there is genetic continuity between them remain to be determined. In this study, we compared the mtDNA genetic makeup of living aboriginal and non-aboriginal native of Taiwan to the genetic profile obtained from five ancient human remains of the Lin-Ding archeological site II, in Hualien on the East coast of Taiwan, dating back to the middle Neolithic era 3,000 years BP. This analysis should help anthropologists and archaeologists explain the genetic origin of the Taiwanese, and address matters that cannot be treated with traditional methods.

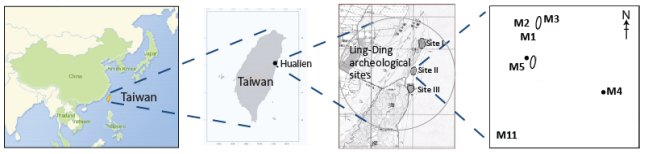

Figure 1: Location of ancient human remains of the archeological Ling-Ding site II in Hualien.

Materials and Methods

Samples

Ling-Ding Site II in Hualien on the east coast of Taiwan (Figure 1) provided 6 different ancient human skeletal remains of which 8 teeth samples were suitable for DNA analysis. Radiocarbon dating, using accelerator mass spectroscopy (AMS) of one tooth LD-M11, indicated an age estimate of 2950~3160 years (Table 1). Accordingly, using artifacts, stratigraphic and chronological relationship to LD-M11, other samples on site II were assigned to a mid-Neolithic age most likely older than 2950 years BP.

Silica-Based ancient DNA purification

Ancient DNA (aDNA) extracts and handling of the teeth were carried out in a laboratory dedicated exclusively for ancient human remains where no modern human DNA preparation or polymerase chain reaction (PCR) has ever been performed. aDNA purification was carried out according to the protocol described by Rohland and Hofreiter [16] with minor modifications. Briefly, teeth pulp was grounded to powder (0.1~0.05 g) using a bleached dental drill and was added to an extraction buffer (0.5 M EDTA, 1% Sodium Diodecil Sulphate, 0.25 mg/ml proteinase K, pH 8.0) for approximately 24 hrs at 55°C. After centrifugation at 3500 × g for 15 minutes, the supernatant was transferred to a 1 ml binding buffer (5 M GuSCN, 25 mM NaCl, 50 mM Tris) supplemented with a 100 µl silica suspension and set on rotation for 3 h at room temperature. The pellet was washed twice in buffer (50% alcohol, 0.1M NaCl, 1 mM EDTA, 10 mM Tris), air-dried for 15 min, dissolved in distilled H_2O for ten minutes at 56°C and stored at -20°C.

PCR amplification of Mitochondrial DNA

Each 20 µl amplification reaction contained 2 µl of DNA extract, 60 mM Tris-SO_4 (pH 8.9), 18 mM Ammonium Sulfate, Bovine Serum Albumin 10 µg, 2 mM $MgSO_4$, 0.2 mM dNTPs and 0.5 unit of Taq polymerase (Platinum Taq High Fidelity; Invitrogen, USA). Negative controls were included for every set of PCR reactions. A modern DNA positive control was finally added to each set of reactions outside the aDNA PCR-setup laboratory. Seven overlapping segments covering the entire hypervariable segment-I (HVS-1) of the mtDNA control region were amplified using primer sequence pairs listed in Table 2.

Beta Sample number	Human remains	Measured radiocarbon age (years BP)	13C/12C (o/oo)	Conventional age (years BP)	Calibrated years BP (2-Sigma)
Beta-373818	LD-M11	2950 ± 30	12.2	3160 ± 30	3450-3345(95%)
	LD-M1	na			
	LD-M2	na			
	LD-M3	na			
	LD-M4	na			
	LD-M5	na			
na: not applicable					

Table 1: Ling-Ding radiocarbon dating of ancient human remains.

Determination of additional informative mutations used to obtain more accurate haplogroup assignments was obtained from the sequencing of pertinent segments of the coding region using primer pairs 8, 9, 10, 11, and 12 (Table 2) chronologically.

Sequencing was performed using Big Dye terminator kit (ABI, Taiwan) in a final volume of 10 µl containing 2.5x Ready Reaction Premix, 5x BigDye sequencing buffer, 3.2 pmol primers and 2 µl template. PCR conditions were as follows: 30 cycles of heat treatment at 96°C for 20 s; 50°C for 20 s, and 60°C for 60 s. A purification step using a G50 Sephadex column (Pharmacia, Taiwan) was performed before the final run on an automated DNA sequencer (ABI model 3730). Negative controls were included in both stages of the assay (extraction and PCR) to assess for the potential contamination. All relevant personnel in this study had their mtDNA typed. The two researchers who took the samples from the Ling-Ding site had mtDNA matching haplogroups H and M10b, and all aDNA work was carried out by JYH who matched mtDNA haplogroup N9a10.

Primer pairs	Primer Names	Forward Primer (5' to 3')	Primer Names	Reverse Primer (5' to 3')	Amplified regions
1	L15995	TCCACCATTAGCACCCAAAG	H16118	TTTATGGTACCGTACAATATTC	15995-16118
2	L16079	GTATTGACTCACCCATCAAC	H16196	TGCTGTACTTGCTTGTAAGC	16079-16196
3	L16176	GTACATAAAAACCCAATCCAC	H16329	TGACTGTAATGTGCTATGTAC	16176-16329
4	L16250	TCACACATCAACTGCAACTC	H16404	ATATTGATTTCACGGAGGATG	16250-16404
5	L16358	ACATTACAGTCAAATCCCTTC	H16483	AGATGTCGGATACAGTTCAC	16358-16483
6	L16449	ACAAGAGTGCTACTCTCCTC	H21	AGCTCCCGTGAGTGGTTAAT	16449-21
7	L16542	AAAGCCTAAATAGCCCACAC	H114	AGACAGATACTGCGACATAG	16542-114
8	L143	AGTATCTGTCTTTGATTCCTG	H268	TTTGTTATGATGTCTGTGTGG	143-268
9	L5972	ACACTATACCTATTATTCGGC	H6081	ATTATTACAAATGCATGGGC	5972-6081
10	L10280	AAATTGCCCTCCTTTTACCC	H10431	TCATAATTTAATGAGTCGAAATC	10280-10431
11	L10779	AATGCTAAAACTAATCGTCCC	H10903	TTGGGGAACAGCTAAATAGG	10779-10903
12	L14535	AACCCATATAACCTCCCCC	H14698	TGGTTTTTCATATCATTGGTC	14535-14698

Table 2: Primer sequences used for aDNA amplification of the HVS1control region and other relevant coding regions.

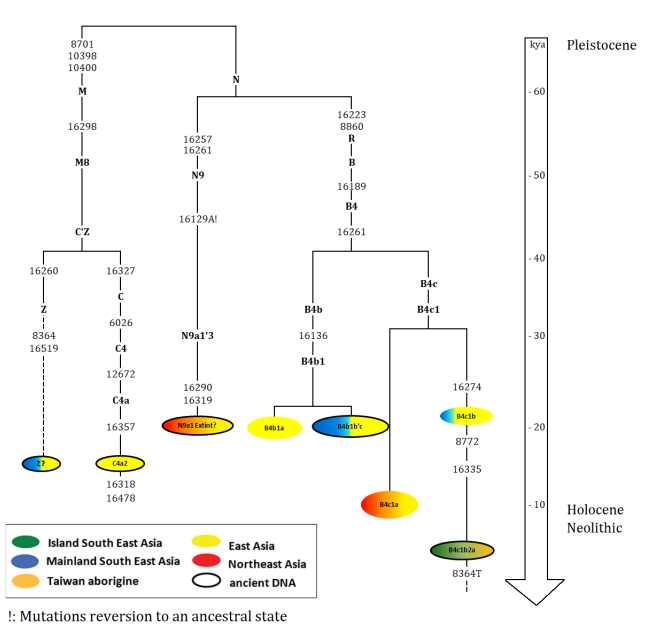

Figure 2: Phylogenetic tree restricted to Ling-Ding site II results (modified from Brandao's et al. [11]). Shadings represent the geographic relative distribution of mtDNA haplogroups across Asian regions. Black circles shadings indicate haplogroups detected in the ancient human remains of Ling-Ding Site II. Nucleotide positions are only those indicated in Table 3.

Results

Five of the six ancient human remains from the Ling-Ding archeological site (Figure 1) produced successful mtDNA extracts. Further, on the basis of remains surrounding each burial, specimens that were not carbon dated were categorized as belonging to the same period as specimen LD-M11 (approximately 3000 years BP) (Table 1) and their genetic structure was anticipated to represent the characteristics of Taiwan people in middle Neolithic time.

C4a2 sample LD-M1

Haplogroup C (Figure 2 and Table 3) is a descendant of Haplogroup M. The C4a2 subtype originated approximately 15,000 years BP [17] and branches of this clade, such as C4a2a, are commonly seen in the Evenki and Tuva of Siberia [18]. Further, population dispersals of C4a2 from these regions resulted in the presence of C4a2b and C4a2c in the Himalayas, Tibet and Indian regions [19]. C4a2 has not been seen among Austronesian speaking groups, such as TwA, the Philippines or Indonesia [20,21], and the subtype seen in this study is a new type that is only seen at low frequency (<1%) among non-TwA (Lin M, personal communication). Accordingly, the presence of C4a2 in any Taiwan individual will associate to a continental East Asian origin.

N9a1 sample LD-M2

Branches of haplogroup N9a (Figure 2 and Table 3) are mostly seen in Mongolia (2.1%), northeastern China (2.8%), Korea (2.1%), Japan (4.6%) [18], and Mainland southeastern Asia (MSEA) (1.5–4.5%) [22]. The type seen in Taiwan (N9a10) (2-7%) is unique to Amis, Puyuma, Atayal, and Toroko, and scarce among non-TwA (<1%). It has not been reported in Mainland China. The N9a1 type found in ancient human remain LD-M2 (Table 3) has not been described elsewhere [17]. According to Phylotree [17], it appears to be an extinct branch of N9a1 and cannot be confidently given a label of continental East Asian or TwA origin.

	Nucleotide positions																													
	Coding region									Control region (HVS1)																				
	6	8	8	8	8	9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	0	3	7	7	8	8	0	0	2	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	
	2	6	0	7	6	2	3	4	6	1	1	2	2	2	2	2	2	2	3	3	3	3	3	3	3	4	5			
	6	4	1	2	0	4	9	0	7	3	8	2	5	6	6	7	9	9	1	1	1	2	3	5	5	9	7	1		
Sample ID							8	0	2	6	9	3	7	0	1	4	0	8	1	8	9	7	5	5	7	0	8	9		Haplogroups
CRS	G	A	A	T	A	T	A	C	A	T	T	C	C	C	C	G	C	T	T	A	G	C	A	C	T	G	C	T		
LD-M1	A	-	-	-	-	-	G	T	G			T						C		G		T			C		T	C	C4a2	
LD-M2	-	-	-	-	-		-	-	-	-	-	T	T		T		T				A						-	-	N9a1 (Extinct)	
LD-M4	-	G	-	C	G	-	-	-	-							A							G				-	-	B4c1b2a	
LD-M5	-	G	G		T	-	G	T	-			T		T				C									-	-	Z	
LD-M11	-	-	-	-	-	-			-	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B4b1

CRS: Cambridge Reference Sequence; Blanks: Nucleotide position identical to CRS; negative sign (-): nucleotide position not tested

Table 3: Mitochondrial sequence variations in five ancient human remains of Ling-Ding site II and haplogroup assignments.

B4c1b2a sample LD-M4

Haplogroup B4c1b2a (Figure 2 and Table 3) is seen in most Austronesian speaking groups. Variants of B4c1b2a have been seen in the Philippines, Orchid Island, western ISEA, and Malaysia [11]. In Taiwan, among Atayal, Yami, Puyuma, and Tsou, it is seen as haplogroup B4c1b2a2a and is characterized by two extra nucleotides, T195C and G15301A that differentiate it from its sister types seen among non-TwA and other groups from South and East China [17]. The quality of aDNA retrieved from LD-M4 did not allow further determination than B4c1b2a, and accordingly, the type could not be associated to either, a non-TwA or TwA ancestry.

Z sample LD-M5

Haplogroup Z (Figure 2 and Table 3), most likely arose in Siberia and central Asia 15,800 ± 4,400 years BP [23]. Subtypes of Z are seen at low frequency (<2%) throughout East Asia, among non-TwA, and the Philippines. Here, the finding of Z in ancient remain LD-M5 is most likely associated to a continental East Asian ancestry.

B4b sample LD-M11

Most members of the B4b (Figure 2 and Table 3) clade are seen in Mainland Southeast Asia (Indochina), Taiwan and the Philippines [9,11-13], but also in Japan and Siberia [24]. Subtypes of B4b in non-TwA are principally represented by subtypes of B4b1b, B4b1c, and B4b1a1. Conversely, in Austronesian speaking groups, it is seen as subtypes of B4b1a2. The sole carbon dated specimen of the Ling-Ding site II, LD-M11 (Table 1 and 3) could only be assigned to haplogroup B4b1. A TwA or a Mainland East Asian label could not be characterized.

Discussion

In this study, we applied molecular genetics on ancient human remains of the middle Neolithic archaeological Lin-Ding site II of Hualien on the east coast of Taiwan in search for a better understanding of the origins and affinities of the autochthonous populations of Taiwan. We primarily analyzed the polymorphisms of mtDNA coding-regions (nps 8,000-9,000 and 10,000-11,000) and control region HVSI (nps 16,030 to 16,490), and then used other pertinent SNPs of the coding region for proper haplogroup assignments. Dedicated ancient DNA laboratory, contamination controls and mtDNA typing of archaeologists and laboratory researchers participating in the research project were done to ascertain the authenticity of all ancient specimen analyzed.

The mtDNA sequences of five ancient human remains were obtained. All sequences assignments, B4c1b2a, B4b, C4a2, N9a1, and Z (Figure 2), had a distinct geographical association with populations of continental East Asia and revealed information about their prehistoric source of ethnicity back to more than 15,000 years BP. Two sequences, C4a2 and Z (LD-M1 and LD-M5 respectively) commonly seen in modern continental East Asians, suggested the presence of such people in the middle Neolithic on the East coast of Taiwan. This profile is not as clearly defined by the three other haplogroups, namely B4c1b2a, B4b1, and N9a1 since the depth of the sequencing attained was not sufficient to assign confidently these haplogroups to a TwA or non-TwA specificity (Table 3). Most likely, B4c1b2a was representing a TwA as the frequency of this haplogroup is twice higher among northern and central Taiwan Aborigines [9,12] than southern TwA or non-TwA. Conversely, B4b1 and N9a1 are more commonly seen among non-TwA, and consequently, their presence among present-day TwA is usually regarded as the result of continental East Asian gene flow. Although the haplogroups mentioned above do not represent the polymorphism profile expected from the whole analysis of the burial site, the estimated dating of the Ling-Ding site, 3,160 years BP, represent a minimum of 60 generations of 25 years [25,26], and the polymorphism represented by these mtDNA haplogroups indicates that middle Neolithic settlers from the continental East Asia, arrived there as a group composed of a significant number of non-TwA females and males.

Finding expectation

The determination, in Ling-Ding Site II, of two possible ethnically distinct gene pools, one associated with a 3,000 years BP continental origin bearing the same genetic profile as present-days continental East Asians, and the other being the result of a Taiwan Aboriginal genetic expansion, requires the analysis of more ancient remains to be evidenced. Nonetheless, the presence of continental East Asian settlers on the East coast of Taiwan and the possibility of their cohabitation with nearby TwA can confidently be ascertained and opens new insights about their social organization, collaboration, and genetic sinicisation. This is supported by the archaeology report of Ling-Ding site [27] which propose a hierarchy in the distribution of burials between rich and poor individuals based on the presence of fine chorded ware, stones relics, fine jades artifacts, and the information given by the structural and elaborate arrangement of the burials (Figure 1).

Did these ancient non-TwA settlers circumnavigate Taiwan or walk to Ling-Ding site II?

Migration routes of Formosan speakers within Taiwan reported by Paul Li [5] suggest historical migrations associated with specific tribes. Most are short distance displacements of tribes finally reaching their present-day settlement in the Mountain range. Only the Amis covered long coastal marches to finally reach Hualien, and the Sediq tribe a cross range migration to reach the northern region of Hualien. These two migrations routes can represent the routes followed by the continental East Asian settlers of Ling-Ding. Alternatively, these middle Neolithic Mainlanders had navigation skills and technology that could have permitted them to circumnavigate the Island.

Their arrival 3,000 years BP intriguingly corresponds to the "out of Taiwan diaspora (OOT) [1] of which gene flow also includes mtDNA polymorphism of modern Southeast Asians and South Chinese [28]. It is possible that it is the presence of ancient non-TwA settlers who triggered the OOT.

Why more non-TwA than TwA in Ling-Ding site II?

Before the twentieth century, Taiwan Aborigines were more commonly burying their deads in the house or in the village graveyard without coffins [29]. Conversely, burial with coffins or urn in a community site by Mainland East Asians is the general practice [30]. The high level of non-TwA genetic profile found in the Ling-Ding site II is therefore expected, and should probably be also expected in any other well-structured middle Neolithic burial sites throughout Taiwan.

Culture and genetic profile

As proposed above, the Ling-Ding archaeological site represents a middle Neolithic colonization event of Taiwan by non-TwA and characterizes an East coast region composed of genetically mixed ethnic groups with a complex social organization. It also supports previous finding of Mainland associated mtDNA lineages in other archaeological sites of Taiwan [15,31]. Furthermore, the traces of Fine Corded Ware Culture found in Ling-ding site II (also known as the Red Corded Ware Culture or Huagangshan culture) brings a supporting element for a proposed association between Fine Corded ware and the Longshanoid culture of Mainland China [15,32].

Finally, the primary state of this project makes it necessary to exercise caution when interpreting these findings. In the future, greater sample size studies of complete genome analysis of any extant populations and ancient human remains in Taiwan, will allow a) to differentiate with more confidence those traits or lineages associated to prehistory from those associated to the last 400 years of Taiwan written history, b) to construct a more complete picture of the Taiwanese human prehistory and allowing retracing migration according to space and time, c) to determine better genetic association between archaeological cultures and genetic, d) to define the relationship of ancient human remains to modern human groups in Taiwan.

Conclusion

While still in its infant stage, the results obtained here were most surprising, first, because there is a strong public belief that the genetic of all ancient human remains of archaeological sites in Taiwan are associated with Taiwan mountain tribe Aborigines, and second, because out of five ancient human remains dating ~3000 years BP, it was unexpected to find two possible mountain tribes associated haplogroups and three mainland-Asia associated haplogroups, therefore moving the historical sinicization of autochthonous Taiwanese into a prehistorical landscape.

New insights into the prehistorical population relationships between Taiwan and Continental Asia have been shown, indicating various possible population dispersals in the middle Neolithic period, and while greatly helping our understanding on the demography of Taiwan, it brings new lights on our understanding of human migration in Taiwan and island Southeast Asia.

Conflict of Interest Statement

To my knowledge I declare that no economic interest or any conflict of interest exists.

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Authors' Contributions

The project was conceived and designed by JYH and JAT and JYH performed the laboratory work. JYH and JT performed data analysis and drafted the manuscript. All other authors gave useful contribution on the analysis of data and text of the manuscript. All authors have read and approved the final version of the manuscript.

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