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Long Range mtDNA Sequencing for mtDNA Mixture Deconvolution

Presenters
Brian Young and Scott Tighe

Brian Young^{1,2,3}, Sophie Hadley⁴, Jeff Smith¹, Bhupinder Singh¹,
George Duncan⁵, Scott Tighe⁶

¹NicheVision Forensics, ²Syracuse University, ³Florida International University, ⁴University of Vermont, ⁵Nova Southeastern University

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Overview

We present the use of two technologies not yet widely used in mitochondrial DNA analysis:

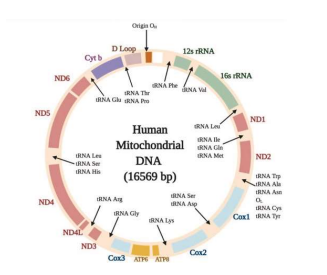
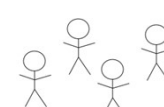
1. Long-range mtDNA PCR combined with Oxford Nanopore sequencing
2. Deconvolution of mixed mitochondrial samples

Through the combination of these technologies, we can derive mtDNA haplotypes for each individual contributor in a mixed sample.


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Human Mitochondrial DNA 16,571 BP 

Resolving mixed individuals with large overlapping sequences

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PCR Amplification and ONT Sequencing

4

Multiplex Long Range PCR

PCR Amplifications of Mitochondria with 1500 bp overlap
 Takara Biosciences GXL master mix
 6 Primer Pools 1500bp overlaps
 9 cell line mixes

Long Range PCR Takara GXL PCR
 25 ul GXL
 6 ul primer multiplex
 18 ul H2O
 1 ul DNA (1-3ng)

Type	17257 (L15)	17115 (L50)	17066 (L50)	Barcode
1 Single Source	2ul	---	---	1_single_source_17257
2 Single Source	---	2.8ul	---	2_single_source_17115
3 Single Source	---	---	2.2ul	3_single_source_17066
4 Mixed 3:1	3ul	1.4ul	---	4_mixed_3_1_17257+17115
5 Mixed 3:1	3ul	---	1.1ul	5_mixed_3_1_17257+17066
6 Mixed 3:1	---	4.2ul	1.1ul	6_mixed_3_1_17115+17066
7 Mixed 1:3	1ul	4.2ul	---	7_mixed_1_3_17257+17115
8 Mixed 1:1	1ul	1.4ul	---	8_mixed_1_1_17257+17115
9 Mixed 10:1	10ul	1.4ul	---	9_mixed_10_1_17257+17115

Oxford Nanopore Sequencing
 Ligation NDB114 barcoded Library
 Multiplex PCR Product
 GridION with R10.4 flow cells

The goal is even PCR amplification efficiency for effective secondary analysis and sequencing

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Nanopore Sequencing

- 40-100 Gb per flow cell P2
- Detect base modifications on native DNA
- Reads up to 2 megabases
 - Amplicon
 - Native DNA
 - DNA base modification detection
 - Direct RNA sequencing
 - RNA base modifications
 - Low input PCR barcoding
 - Short read sequencing
 - Adaptive Sampling Sequencing

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Increased Accuracy at 40x Coverage and New Duplex chemistry HD

MacKenzie M, Argyropoulos C. An Introduction to Nanopore Sequencing: Past, Present, and Future Considerations. *Micromachines (Basel)*. 2023 Feb 16;14(2):459. doi: 10.3390/mi14020459. PMID: 36838199; PMCID: PMC9966620.

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Can sequence just about anything...

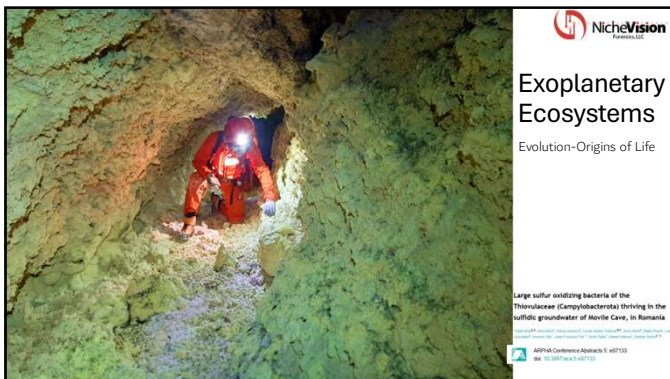
8



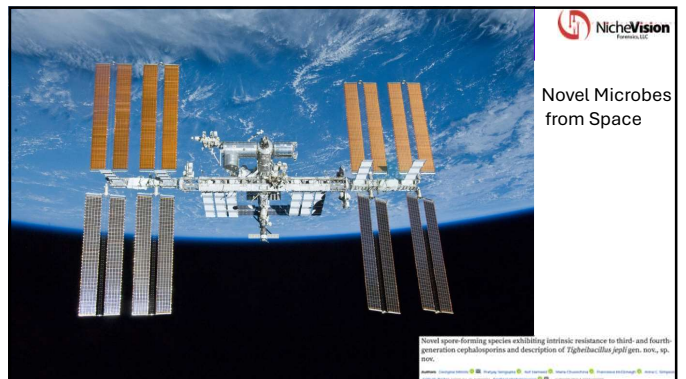
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11



12

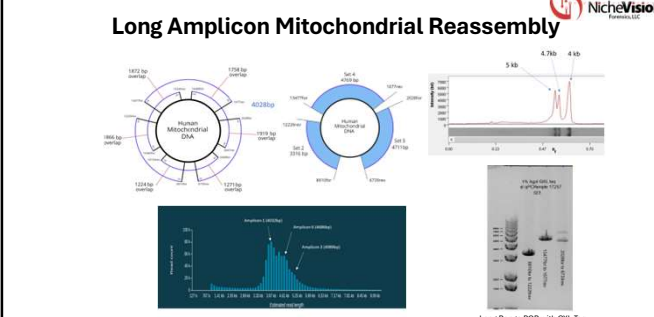
Unknown Infections



Organism	Read Count
<i>Escherichia coli</i>	11314 (42.07%)
<i>Klebsiella pneumoniae</i>	21628 (84.61%)
<i>Enterococcus faecium</i>	2056 (7.65%)
<i>Enterococcus casseliflavus</i>	1977 (7.35%)
<i>Morganella morganii</i>	1676 (6.22%)
<i>Enterococcus sp.</i>	816 (3.03%)
BDJ2_DV0187	469 (1.74%)
<i>Enterococcus faecalis</i>	469 (1.74%)
<i>Enterococcus sp.</i>	279 (1.04%)
306_DN0942	264 (0.98%)
<i>Clostridium sp. CAG-299</i>	264 (0.98%)
<i>Enterococcus invasi</i>	255 (0.95%)
<i>Klebsiella aerogenes</i>	222 (0.83%)
<i>Klebsiella oxytoca</i>	134 (0.5%)
<i>Clostridium parvigena</i>	133 (0.49%)
<i>Morganella sp.</i>	130 (0.48%)

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Long Amplicon Mitochondrial Reassembly



Read Length Histogram of the Amplicons

Long Range PCR with GXL Taq

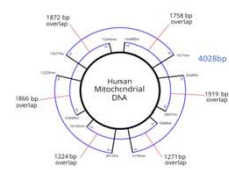
Brian Young NicheVision

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PCR of Mitochondria

Long Range PCR with Takara GXL taq

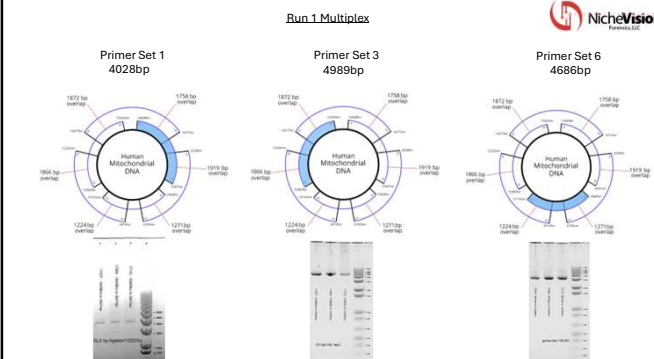
- Set 1: 16488for to 3947rev
- Set 2: 8910for to 12226rev
- Set 3: 10360for to 15349rev
- Set 4: 13477for to 1677rev
- Set 5: 2028for to 6739rev
- Set 6: 5468for to 10154rev



Primers : Amanda Ramos, Cristina Santos, Luis Alvarez, Ramon Nogue, Maria Pilar Atuja. Human mitochondrial DNA complete amplification and sequencing: A new validated primer set that prevents nuclear DNA sequences of mitochondrial origin co-amplification. Electrophoresis 2009, 30, 1587-1593.

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Run 1 Multiplex

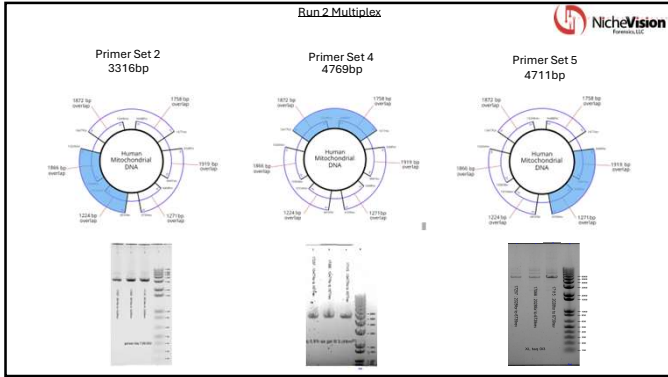


Primer Set 1 4028bp

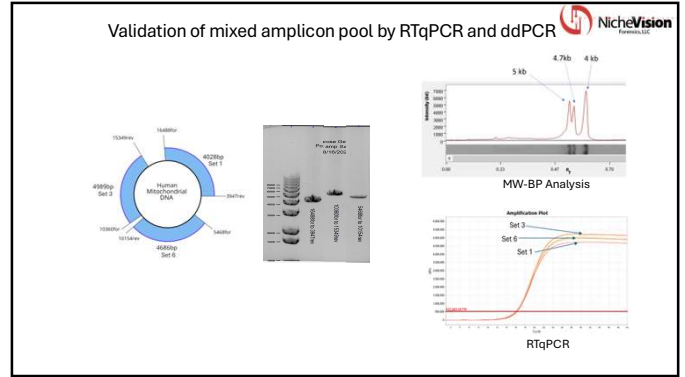
Primer Set 3 4989bp

Primer Set 6 4686bp

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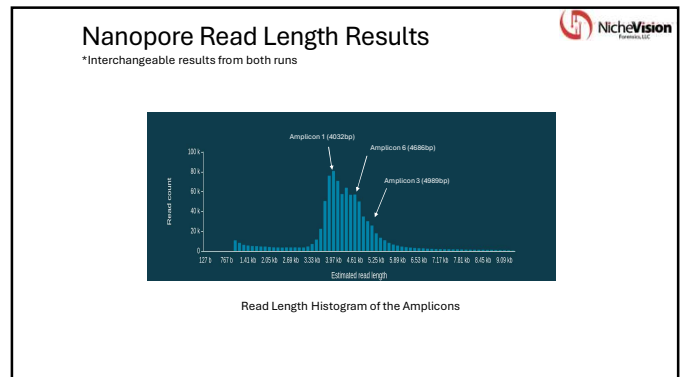
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Nanopore Read Depth by Sample – Run 2

In-Vitro Mixture	Type	Nanopore Barcode	Total Bases (Mb)	Total Reads	Barcode label
1	Single Source	1	383.66	89,680	1_single_source_17257
2	Single Source	2	328.94	78,950	2_single_source_17115
3	Single Source	3	276.29	64,490	3_single_source_17066
4	Mixed 3:1	4	440.1	103,850	4_mixed_3-1_17257v17115
5	Mixed 3:1	5	411.09	96,800	5_mixed_3-1_17257v17066
6	Mixed 3:1	6	451.16	107,050	6_mixed_3-1_17115v17066
7	Mixed 1:3	7	435.01	103,130	7_mixed_1-3_17257v17115
8	Mixed 1:1	8	409.63	97,250	8_mixed_1-1_17257v17115
9	Mixed 10:1	9	429.52	100,750	9_mixed_10-1_17257v17115



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Total Nanopore Output

Flow cells: 2

Total output: 6.92 Gbases


Total number of reads (> 1 Kb): 1.59 x 10⁶

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Mixed mtDNA Deconvolution and Individual Haplotyping

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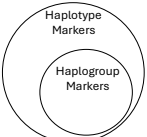


Definitions

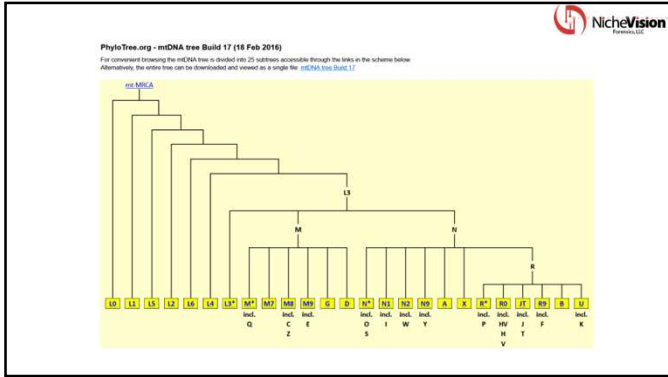
- **HAPLOGROUP:** a group of similar mtDNA sequences that share a common ancestor.
- **HAPLOTYPE:** the unique genetic sequence of an individual's mtDNA (i.e., their unique maternal genetic profile).



https://en.wikipedia.org/wiki/Human_mitochondrial_DNA_haplogroup



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Current mtDNA Analysis Workflow

- Current practice for mtDNA haplotyping requires single-source samples due to inability to deconvolute mixtures.
- Being able to deconvolute mtDNA mixtures will enable exploitation of mtDNA from crime scenes.

```

    graph LR
      A[Single Source Sample] --> B[Sanger or MPS Sequence]
      B --> C[Align to rCRS Reference]
      C --> D[Call Variants]
      D --> E[Haplotype List of Variants]
  
```

- Single hair
- Single bone/tooth

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Workflow for Mixed mtDNA Samples

>Classification of Reads by Maximum Likelihood

```

    graph LR
      A[Mixed Sample] --> B[Massively Parallel Sequencing]
      B --> C[Filter NUMTs]
      C --> D[Separate Reads by Contributor Using Phylogenetic Information]
  
```

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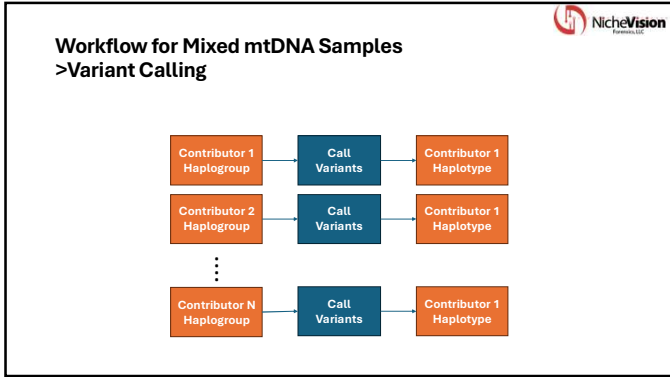
Workflow for Mixed mtDNA Samples

>Haplogrouping by Phylogenetic Analysis

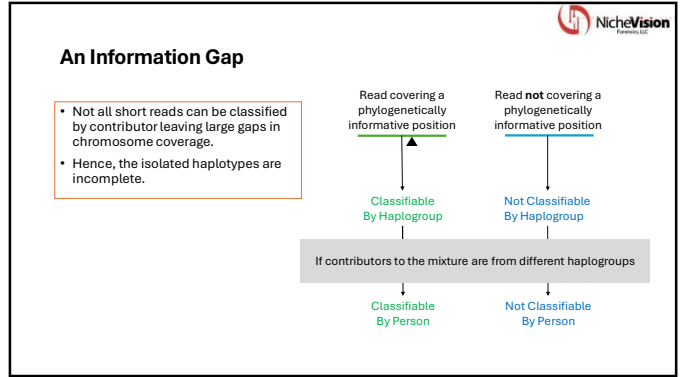
```

    graph LR
      A[Separate Reads by Contributor Using Phylogenetic Information] --> B[Probabilistic Phylogenetic Analysis]
      B --> C[Contributor 1 Haplogroup]
      B --> D[Contributor 2 Haplogroup]
      B --> E[...]
      B --> F[Contributor N Haplogroup]
  
```

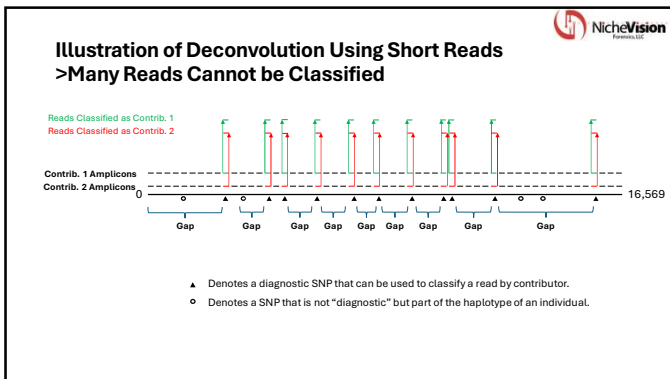
28



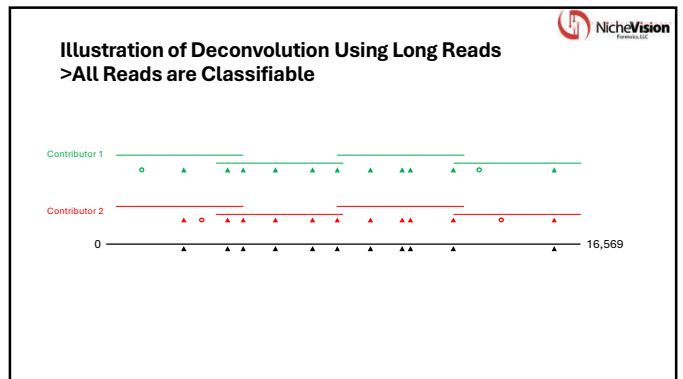
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Samples Used for Creating Mixtures

Accession	Coriell Population	Haplogroup (Estimated by MixtureAceMT)	Expected Haplotype ^{1,2}	
			Tree Span	Variant Calls
NA17257	Caucasian	U5b2b1b	US -> Leaf	<p>758 101 2620 388 10 314 10 7500 14588 111 27088 34910 47860 54884 7088 7700 88880 8478 18880 114870 11613 11738 11820 12380 12724 13680 14207 14870 15810 16410 17100 18180 19200 20100 21000 22000 23000 24000 25000 26000 27000 28000 29000 30000 31000 32000 33000 34000 35000 36000 37000 38000 39000 40000 41000 42000 43000 44000 45000 46000 47000 48000 49000 50000 51000 52000 53000 54000 55000 56000 57000 58000 59000 60000 61000 62000 63000 64000 65000 66000 67000 68000 69000 70000 71000 72000 73000 74000 75000 76000 77000 78000 79000 80000 81000 82000 83000 84000 85000 86000 87000 88000 89000 90000 91000 92000 93000 94000 95000 96000 97000 98000 99000 100000</p>
NA17115	African American	L1b1a3	L1b -> Leaf	<p>758 1400 1807 1817 1820 1822 1824 1826 1828 1830 1832 1834 1836 1838 1840 1842 1844 1846 1848 1850 1852 1854 1856 1858 1860 1862 1864 1866 1868 1870 1872 1874 1876 1878 1880 1882 1884 1886 1888 1890 1892 1894 1896 1898 1900 1902 1904 1906 1908 1910 1912 1914 1916 1918 1920 1922 1924 1926 1928 1930 1932 1934 1936 1938 1940 1942 1944 1946 1948 1950 1952 1954 1956 1958 1960 1962 1964 1966 1968 1970 1972 1974 1976 1978 1980 1982 1984 1986 1988 1990 1992 1994 1996 1998 2000 2002 2004 2006 2008 2010 2012 2014 2016 2018 2020 2022 2024 2026 2028 2030 2032 2034 2036 2038 2040 2042 2044 2046 2048 2050 2052 2054 2056 2058 2060 2062 2064 2066 2068 2070 2072 2074 2076 2078 2080 2082 2084 2086 2088 2090 2092 2094 2096 2098 2100 2102 2104 2106 2108 2110 2112 2114 2116 2118 2120 2122 2124 2126 2128 2130 2132 2134 2136 2138 2140 2142 2144 2146 2148 2150 2152 2154 2156 2158 2160 2162 2164 2166 2168 2170 2172 2174 2176 2178 2180 2182 2184 2186 2188 2190 2192 2194 2196 2198 2200 2202 2204 2206 2208 2210 2212 2214 2216 2218 2220 2222 2224 2226 2228 2230 2232 2234 2236 2238 2240 2242 2244 2246 2248 2250 2252 2254 2256 2258 2260 2262 2264 2266 2268 2270 2272 2274 2276 2278 2280 2282 2284 2286 2288 2290 2292 2294 2296 2298 2300 2302 2304 2306 2308 2310 2312 2314 2316 2318 2320 2322 2324 2326 2328 2330 2332 2334 2336 2338 2340 2342 2344 2346 2348 2350 2352 2354 2356 2358 2360 2362 2364 2366 2368 2370 2372 2374 2376 2378 2380 2382 2384 2386 2388 2390 2392 2394 2396 2398 2400 2402 2404 2406 2408 2410 2412 2414 2416 2418 2420 2422 2424 2426 2428 2430 2432 2434 2436 2438 2440 2442 2444 2446 2448 2450 2452 2454 2456 2458 2460 2462 2464 2466 2468 2470 2472 2474 2476 2478 2480 2482 2484 2486 2488 2490 2492 2494 2496 2498 2500 2502 2504 2506 2508 2510 2512 2514 2516 2518 2520 2522 2524 2526 2528 2530 2532 2534 2536 2538 2540 2542 2544 2546 2548 2550 2552 2554 2556 2558 2560 2562 2564 2566 2568 2570 2572 2574 2576 2578 2580 2582 2584 2586 2588 2590 2592 2594 2596 2598 2600 2602 2604 2606 2608 2610 2612 2614 2616 2618 2620 2622 2624 2626 2628 2630 2632 2634 2636 2638 2640 2642 2644 2646 2648 2650 2652 2654 2656 2658 2660 2662 2664 2666 2668 2670 2672 2674 2676 2678 2680 2682 2684 2686 2688 2690 2692 2694 2696 2698 2700 2702 2704 2706 2708 2710 2712 2714 2716 2718 2720 2722 2724 2726 2728 2730 2732 2734 2736 2738 2740 2742 2744 2746 2748 2750 2752 2754 2756 2758 2760 2762 2764 2766 2768 2770 2772 2774 2776 2778 2780 2782 2784 2786 2788 2790 2792 2794 2796 2798 2800 2802 2804 2806 2808 2810 2812 2814 2816 2818 2820 2822 2824 2826 2828 2830 2832 2834 2836 2838 2840 2842 2844 2846 2848 2850 2852 2854 2856 2858 2860 2862 2864 2866 2868 2870 2872 2874 2876 2878 2880 2882 2884 2886 2888 2890 2892 2894 2896 2898 2900 2902 2904 2906 2908 2910 2912 2914 2916 2918 2920 2922 2924 2926 2928 2930 2932 2934 2936 2938 2940 2942 2944 2946 2948 2950 2952 2954 2956 2958 2960 2962 2964 2966 2968 2970 2972 2974 2976 2978 2980 2982 2984 2986 2988 2990 2992 2994 2996 2998 3000 3002 3004 3006 3008 3010 3012 3014 3016 3018 3020 3022 3024 3026 3028 3030 3032 3034 3036 3038 3040 3042 3044 3046 3048 3050 3052 3054 3056 3058 3060 3062 3064 3066 3068 3070 3072 3074 3076 3078 3080 3082 3084 3086 3088 3090 3092 3094 3096 3098 3100 3102 3104 3106 3108 3110 3112 3114 3116 3118 3120 3122 3124 3126 3128 3130 3132 3134 3136 3138 3140 3142 3144 3146 3148 3150 3152 3154 3156 3158 3160 3162 3164 3166 3168 3170 3172 3174 3176 3178 3180 3182 3184 3186 3188 3190 3192 3194 3196 3198 3200 3202 3204 3206 3208 3210 3212 3214 3216 3218 3220 3222 3224 3226 3228 3230 3232 3234 3236 3238 3240 3242 3244 3246 3248 3250 3252 3254 3256 3258 3260 3262 3264 3266 3268 3270 3272 3274 3276 3278 3280 3282 3284 3286 3288 3290 3292 3294 3296 3298 3300 3302 3304 3306 3308 3310 3312 3314 3316 3318 3320 3322 3324 3326 3328 3330 3332 3334 3336 3338 3340 3342 3344 3346 3348 3350 3352 3354 3356 3358 3360 3362 3364 3366 3368 3370 3372 3374 3376 3378 3380 3382 3384 3386 3388 3390 3392 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4994 4996 4998 5000 5002 5004 5006 5008 5010 5012 5014 5016 5018 5020 5022 5024 5026 5028 5030 5032 5034 5036 5038 5040 5042 5044 5046 5048 5050 5052 5054 5056 5058 5060 5062 5064 5066 5068 5070 5072 5074 5076 5078 5080 5082 5084 5086 5088 5090 5092 5094 5096 5098 5100 5102 5104 5106 5108 5110 5112 5114 5116 5118 5120 5122 5124 5126 5128 5130 5132 5134 5136 5138 5140 5142 5144 5146 5148 5150 5152 5154 5156 5158 5160 5162 5164 5166 5168 5170 5172 5174 5176 5178 5180 5182 5184 5186 5188 5190 5192 5194 5196 5198 5200 5202 5204 5206 5208 5210 5212 5214 5216 5218 5220 5222 5224 5226 5228 5230 5232 5234 5236 5238 5240 5242 5244 5246 5248 5250 5252 5254 5256 5258 5260 5262 5264 5266 5268 5270 5272 5274 5276 5278 5280 5282 5284 5286 5288 5290 5292 5294 5296 5298 5300 5302 5304 5306 5308 5310 5312 5314 5316 5318 5320 5322 5324 5326 5328 5330 5332 5334 5336 5338 5340 5342 5344 5346 5348 5350 5352 5354 5356 5358 5360 5362 5364 5366 5368 5370 5372 5374 5376 5378 5380 5382 5384 5386 5388 5390 5392 5394 5396 5398 5400 5402 5404 5406 5408 5410 5412 5414 5416 5418 5420 5422 5424 5426 5428 5430 5432 5434 5436 5438 5440 5442 5444 5446 5448 5450 5452 5454 5456 5458 5460 5462 5464 5466 5468 5470 5472 5474 5476 5478 5480 5482 5484 5486 5488 5490 5492 5494 5496 5498 5500 5502 5504 5506 5508 5510 5512 5514 5516 5518 5520 5522 5524 5526 5528 5530 5532 5534 5536 5538 5540 5542 5544 5546 5548 5550 5552 5554 5556 5558 5560 5562 5564 5566 5568 5570 5572 5574 5576 5578 5580 5582 5584 5586 5588 5590 5592 5594 5596 5598 5600 5602 5604 5606 5608 5610 5612 5614 5616 5618 5620 5622 5624 5626 5628 5630 5632 5634 5636 5638 5640 5642 5644 5646 5648 5650 5652 5654 5656 5658 5660 5662 5664 5666 5668 5670 5672 5674 5676 5678 5680 5682 5684 5686 5688 5690 5692 5694 5696 5698 5700 5702 5704 5706 5708 5710 5712 5714 5716 5718 5720 5722 5724 5726 5728 5730 5732 5734 5736 5738 5740 5742 5744 5746 5748 5750 5752 5754 5756 5758 5760 5762 5764 5766 5768 5770 5772 5774 5776 5778 5780 5782 5784 5786 5788 5790 5792 5794 5796 5798 5800 5802 5804 5806 5808 5810 5812 5814 5816 5818 5820 5822 5824 5826 5828 5830 5832 5834 5836 5838 5840 5842 5844 5846 5848 5850 5852 5854 5856 5858 5860 5862 5864 5866 5868 5870 5872 5874 5876 5878 5880 5882 5884 5886 5888 5890 5892 5894 5896 5898 5900 5902 5904 5906 5908 5910 5912 5914 5916 5918 5920 5922 5924 5926 5928 5930 5932 5934 5936 5938 5940 5942 5944 5946 5948 5950 5952 5954 5956 5958 5960 5962 5964 5966 5968 5970 5972 5974 5976 5978 5980 5982 5984 5986 5988 5990 5992 5994 5996 5998 6000 6002 6004 6006 6008 6010 6012 6014 6016 6018 6020 6022 6024 6026 6028 6030 6032 6034 6036 6038 6040 6042 6044 6046 6048 6050 6052 6054 6056 6058 6060 6062 6064 6066 6068 6070 6072 6074 6076 6078 6080 6082 6084 6086 6088 6090 6092 6094 6096 6098 6100 6102 6104 6106 6108 6110 6112 6114 6116 6118 6120 6122 6124 6126 6128 6130 6132 6134 6136 6138 6140 6142 6144 6146 6148 6150 6152 6154 6156 6158 6160 6162 6164 6166 6168 6170 6172 6174 6176 6178 6180 6182 6184 6186</p>

**3:1 mixture
NA17257:NA17115
CAU:AA**

**Haplogroup-informative
SNP calls are Concordant
With Expected**

**Additional private variant
calls that define the
haplotype are not shown.**

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Conclusions

- Long range sequencing by Oxford Nanopore Technologies (ONT) combined with probabilistic deconvolution holds promise for mtDNA analysis of mixed samples.
- Here, the mtDNA genome was covered by 6 amplicons to accommodate slightly degraded DNA. However, entire mtDNA chromosomes can possibly be sequenced. If done on mixed samples, the mixture will be "deconvoluted by sequencing", and the haplotypes will be "phased by sequencing".

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**Long Range mtDNA Sequencing for mtDNA
Mixture Deconvolution**

Brian Young^{1,2,3}, Sophia Hadley⁴, Jeff Smith¹, Bhupinder Singh¹,
George Duncan⁵, Scott Tighe⁴

¹NicheVision Forensics, ²Syracuse University, ³Florida International University, ⁴University of Vermont, ⁵Nova Southeastern University

Green Mountain DNA Conference 2024

Brian@nichevision.com
Scott.Tighe@uvm.edu

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