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Microsatellite genotype matches of eastern Australian humpback whales to Area V feeding and breeding grounds

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ABSTRACT

Recent mitochondrial DNA analyses have determined Eastern Australian humpback whales to be one of 3 distinct sub-stocks within IWC BS-E. Using microsatellite genotypes (up to 12 microsatellite loci, mtDNA sequence data and molecular sex identification) from Eastern Australia (n=734 unique individuals), South Pacific Islands (Oceania, n=1086 unique individuals) and Antarctic feeding Areas I-VI (n=175 unique individuals), we detected migratory interchange between humpback whales in Eastern Australia and New Caledonia (n=11) and Eastern Australia and Tonga (n=1). Migratory interchange was also detected between Eastern Australia and summer feeding grounds in Antarctic Area V (n=3). There were no whales from Eastern Australia detected to move outside the boundaries of Area V (130°E-170°W). Given that the IUCN has listed the humpback whales from Oceania as endangered, these results have implications for the management of humpback whales in Eastern Australia and Oceania (Areas V and VI), because individuals from different Breeding sub-stocks appear to be mixing on both the breeding and feeding grounds. Additionally, this study shows that a technique used to make microsatellite genotypes directly comparable between research groups is useful for conducting large-scale genotype matching for investigating migratory interchange of humpback whales.

INTRODUCTION

Recent genetic analyses have suggested that at least 3 distinct sub-stocks comprise the International Whaling Commissions (IWC) Breeding Stock E (BS-E) (E1: Eastern Australia (EA), E2: New Caledonia, and E3: Tonga) based on mitochondrial DNA differentiation (Garrigue *et al*., 2006: Olavarria *et al*., 2006; 2007). These sub-stocks were traditionally grouped as a single management unit based on movement data provided by Discovery marking, which also linked the majority of whales from the 3 sub-stocks to Antarctic Area V feeding grounds (130°E - 170°W) (Chittleborough, 1959; Dawbin, 1959; Dawbin, 1964; Chittleborough, 1965; Dawbin, 1966). Differences in the recovery rates of the BS-E sub-stocks however, have recently led the IUCN to list humpback whales from islands in the South Pacific (Oceania) including New Caledonia and Tonga as endangered (Childerhouse *et al*., 2008). Given that the Government of Japan proposes to add humpback whales to their list of targeted species for harvesting in Antarctic waters under scientific permit, information on the migratory interchange between EA and sites within Oceania are vital because the whales from the endangered sub-stocks may share feeding grounds with the more abundant E1 sub-stock.

Discovery marking provided the first data on movement of individual whales between EA and Oceania with a tag recovered between EA and Fiji (Dawbin, 1959). Additionally, tag recoveries were documented between EA and the migratory corridors of Norfolk Island and New Zealand (Dawbin, 1959; 1964; 1966). Discovery marking has also provided links between EA humpback whales and Antarctic feeding grounds to the south, with the

majority of tag recoveries being made between EA and Antarctic Area V, however a small number of tags also linked EA whales to Areas IV and VI (Dawbin, 1964; Chittleborough, 1965; Paton & Clapham, 2006). More recently photo identification has detected movements of individual humpback whales between EA and New Caledonia (Garrigue *et al*., 2000; Garrigue *et al*., 2007) and EA and Tonga (Burns, 2010), as well as further evidence linking EA to Antarctic Area V feeding grounds (Kaufman *et al*., 1990; Rock *et al*., 2006; Franklin *et al*., 2008b). Additionally, satellite tagging has revealed movements of humpback whales from EA to Antarctic Areas IV & V (Gales *et al*., 2009).

Analyses of migratory interchange of humpback whales using microsatellite genotyping has been limited to within research groups in the Southern Hemisphere, due to issues with inconsistent microsatellite scoring when matching between laboratories because of differences in genotyping platforms and techniques (e.g. dye labels, primer modifications or in-lane size standards) used by each research group (Mansfield *et al*., 1996; Lazaruk *et al*., 1998; Haberl & Tautz, 1999; Harker, 2001; LaHood *et al*., 2002; Pasqualotto *et al*., 2007). Microsatellite standardisation between the Southern Cross University Whale Research Centre (SCUWRC) and the South Pacific Whale Research Consortium (SPWRC) (Anderson *et al*., 2003; Anderson, 2010) has enabled microsatellite genotyping to now be directly comparable between the two research groups, so that migratory interchange of humpback whales can be detected on a larger-scale.

Here we use microsatellite genotypes from Eastern Australia (SCUWRC), breeding grounds in Oceania (SPWRC) and feeding grounds in Antarctic Areas I-VI (SPWRC), to investigate humpback whale migratory interchange.

METHODS

In total, n=1545 sloughed skin samples were collected from humpback whales during the austral winters of 1996-2004 at three sites Byron Bay, Hervey Bay and Ballina along the east coast of Australia (Figure 1; Table 1). Tissue digestions were conducted using Proteinase K, with genomic DNA extracted using the silica-based method of Elphinstone *et al*. (2003). All samples were amplified at up to 13 microsatellite loci: EV1, EV14, EV21, EV37, EV94, EV96, EV104 (Valsecchi & Amos, 1996), GATA417, GATA28, GATA53 (Palsbøll *et al*., 1997a), 464/465 (Schlötterer *et al*., 1991), GT211, GT575 (Bérubé *et al*., 2000) (Table 2). Due to low amplification success during earlier analyses (Anderson *et al*., 2001), primers for seven loci (EV14, EV21, EV37, EV94, EV96, EV104, GATA417 – Table 2) had been redesigned from the original published sequences using the program NAROLIGO (Rychlik & Rhoads, 1989), so as to reduce the incidence of non-amplification and non-specific binding. Loci with redesigned primers are referred to with an asterisk (*) following the locus name e.g. EV14*. Each locus was amplified individually for each sample in 96 well microtitre plates. Loci were pooled into 2 sets for genotyping on an ABI 310 capillary (Applied Biosystems) (Table 2). A fragment of the mitochondrial DNA control region (800 base-pairs) was sequenced for all samples collected between 2002-2003 and molecular sexing was conducted for all samples using the protocols outlined in Olavarria *et al*. (2006).

Samples that amplified at less than 6 loci were removed from the dataset because they did not contain enough information to be reliably used for individual identification. The Probability of Identity (PI – Paetkau *et al*., 1995) for the 6 least informative loci plus molecular sex identification was determined to be $PI=3.98 \times 10^{-5}$, which equates to 1 chance in 25,000 that 2 whales sampled from EA would have matching genotypes by chance. Considering the EA population was estimated to be 7090 (Noad *et al*., 2006) in 2004 when sample collection concluded in EA, 6 loci plus a molecular sex ID were considered to be sufficient to differentiate between individuals with a high degree of certainty. Genotype matching was conducted using the program CERVUS (Marshall *et al*., 1998). Duplicate samples were removed from the EA dataset to produce a final Eastern Australian genotype dataset of n=734 unique individuals.

Genotype data from Oceania (n=1086 unique individuals) and Antarctica (n=175 unique individuals) were provided by the SPWRC (Steel *et al*., 2008), including samples contributed from SPWRC members, the International Decade of Cetacean Research and Southern Ocean Whale Ecosystem Research (IDCR/SOWER) of the IWC, the Chilean Antarctic Institute (INACH) and the Southern Ocean Global Ocean Ecosystems Dynamics (SO-GLOBEC). Standardisation of microsatellite genotypes between research groups was conducted as per Anderson *et al*., (2003) and Anderson (2010), so that the microsatellite genotypes of Steel *et al*. (2008) were directly comparable to the Eastern Australian dataset.

Genotype matching between Eastern Australia and both the Oceania breeding grounds and Antarctic feeding grounds were conducted using the program CERVUS, with search criteria limited to a minimum of 8 loci matching and up to 3 loci mismatching, in order to account for potential genotyping errors (Marshall *et al*., 1998; Hoffman & Amos, 2005; Morin *et al*., 2010). Where available mtDNA and/or molecular sex identifications were also used to provide support to genotype matches.

RESULTS

Matching between n= 734 individual Eastern Australian humpback whales, n=175 individuals sampled from Antarctic feeding grounds (Areas I-VI) and n=1086 individuals from Oceania (Table 3) (New Caledonia, Tonga, Cook Islands and French Polynesia) revealed a total of n=15 genotype matches (Table 4 & 5). The number of matching loci ranged between 8 - 12 with PI < 2.92×10^{-7} (Table 4). One case of a 'soft match' was detected between two genotypes (Samples 99A51519 and 01BBE01) at 1 locus (EV94) i.e. one sample was a homozygote for one allele of the other sample. Considering these two samples match exactly at 10 loci (PI=3.35×10^{-11}, which equates to less than 1 chance in 29 trillion), it is highly likely that the cause of the 'soft match' at locus EV94 is allelic dropout in the Antarctic sample and the two genotypes are therefore likely to represent a true genotype match.

Thirteen of the 15 matches were supported by molecular sex identification, while 5 were supported by mtDNA sequence data (Table 4). There were no instances of conflicting sex or mtDNA data between any matched samples. In addition at least 4 of the genotype matches between EA and New Caledonia are supported by photo ID matches that were collected at the time of genetic sampling (W. Franklin & C. Garrigue pers comm.).

No genotype matches were detected between Eastern Australia and breeding or feeding sites outside of Area V. Specifically migratory connections were detected between Eastern Australia and New Caledonia (n=11), Eastern Australia and Tonga (n=1) and Eastern Australia and Antarctic Area V (n=3) (Figure 2).

DISCUSSION

This study has detected the migratory interchange of (n=11) individual humpback whales between Eastern Australia and New Caledonia, increasing the total number of known movements between the two sites to n=23, when combined with photo identification matches (Garrigue *et al*., 2000; Garrigue *et al*., 2007; W. Franklin & C. Garrigue pers. comm.). In addition, the total number of recorded movements between Eastern Australia and Tonga have now doubled (to n=2) with the addition of our genotype match, to a photo ID match of a whale photographed in both Ballina (EA, Figure 1) and Tonga (Burns, 2010).

Genotype matches detected in this study have also highlighted the wide dispersal of Eastern Australian humpback whales in the Antarctic Area V feeding ground, with one whale sampled toward the western boundary of Area V in 1999 and 2 whales sampled near the eastern border of Area V in 1992 (Figure 2). The genotype matches between Eastern Australia and eastern Area V feeding grounds, support recent photo ID matches that detected movements of humpback whales between Eastern Australia and an area near the Balleny Islands (67°S, 163°E) (Franklin *et al*., 2008b).

Recent mtDNA analyses have suggested that BS-E is comprised of at least 3 differentiated sub-stocks (E1: Eastern Australia. E2: New Caledonia and E3: Tonga) (Garrigue *et al*., 2006; Olavarria *et al*., 2006; 2007). The degree of interchange that appears to be occurring between these sub-stocks is therefore unexpected. However, closer analyses of the results from this study show that a large proportion of interchange can be attributed to movements of males. This may explain the differences in the mtDNA differentiation and the movements observed between sub-stocks, because mtDNA is passed on to the next generation through females only. Similarly the differences in mtDNA differentiation and observed interchange could be the result of juveniles moving between sites, since they do not contribute to gene flow.

Differences in recovery rates of Eastern Australian and Oceania humpback whales since the last commercial whaling period have led the IUCN to declare Oceania sub-stocks as endangered (Childerhouse *et al*., 2008). This study however, demonstrates that migratory interchange does not only occur between Eastern Australia and Oceania breeding grounds, but that members of the Eastern Australian and other BS-E sub-stocks are also likely to share common feeding grounds, with a genotype match of a whale from New Caledonia found in Antarctic Area V (62°26S, 171°5W, Steel *et al*., 2008), in addition to a Discovery mark recovered between Antarctic Area V and Tonga (66°37S, 174°48E, Dawbin, 1959). These results have implications to the future management of these humpback whale sub-stocks, particularly given that the Government of Japan proposes to take humpback whales under scientific permit in Antarctic waters, where members of the more abundant Eastern Australian humpback whale sub-stock potentially mix with whales from the endangered Oceania sub-stocks.

Confirmation of the accuracy of our genotype matches using photo ID shows that microsatellite genotype data can successfully be standardized between research groups using the same loci, even when they are using different genotyping platforms and/or different techniques such as modified primers. The results of this study show that collaboration between research groups can detect migratory interchange of humpback whales on a larger-scale using existing data produced by independent research groups.

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Figure 1: Map of 3 sites (Hervey Bay, Byron Bay and Ballina) in Eastern Australia where sloughed skin samples were collected from humpback whales.

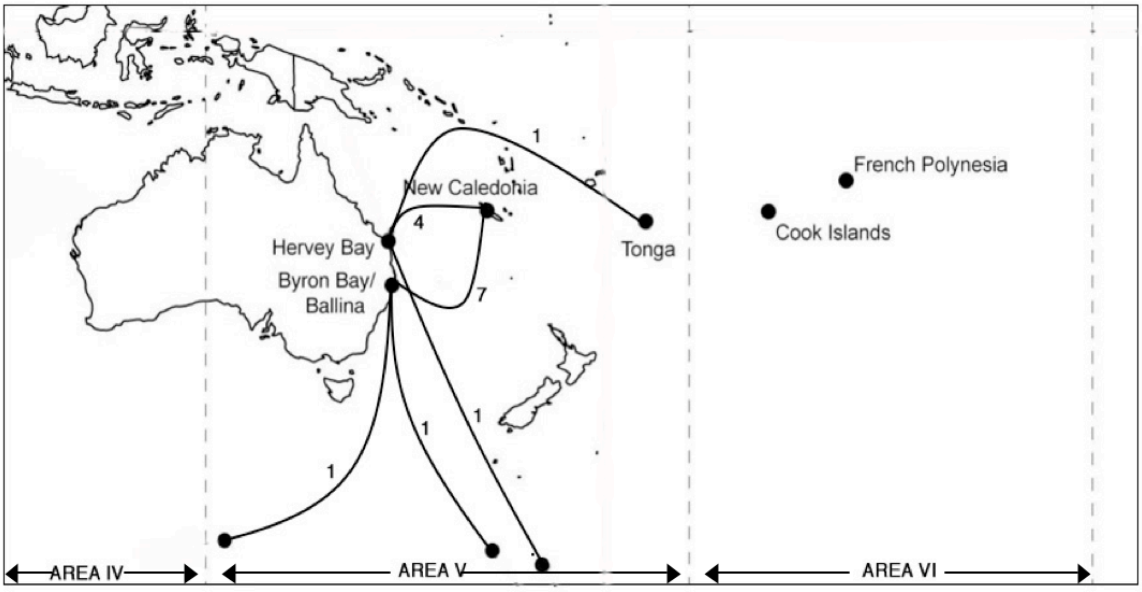

Figure 2: Map illustrating the 15 migratory interchanges of individual humpback whales detected in this study using microsatellite genotyping. Broken lines indicate IWC management area boundaries. Solid lines represent migratory interchange between Eastern Australia and Oceania breeding sites or Antarctic feeding grounds with numbers indicating the total number of matches detected between any two sites.

TABLE 1: Number of sloughed skin samples collected from 3 sites in Eastern Australia (Byron Bay, Hervey Bay and Ballina) between 1996-2004.

Site	Season	# Samples Collected
Byron Bay	**1996**	1
	1998	11
	1999	8
	2000	86
	2001	139
	2002	169
	2003	83
	2004	116
Hervey Bay	**1997**	2
	2000	140
	2001	279
	2002	239
	2003	169
Ballina	**2003**	29
	2004	74
Total	**1996-2004**	1545

Table 2: Microsatellite loci used in eastern Australia for this study including locus name, forward (F) and reverse (R) primer sequences, fluorescent dye label, genotyping set, size range (in base pairs) and number of alleles.

Locus		Primer sequence	Label[1]	Set[2]	Size Range	# Alleles
EV1	**F**	CCCTGCTCCCCATTCTC	TET	B	119-125	4
	R	ATAAACTCTAATACACTTCCTCCAAC				
EV14*	**F**	CAAAGCAGACCCCAGAGACCAT	HEX	A	126-144	9
	R	GAAGGAGAGCCAGAGCCAAGG				
EV21*	**F**	TTTCCTTAAATGCCTGGGACCA	FAM	A	95-105	6
	R	CTGAGCGCTGAAGGTGTGCC				
EV37*	**F**	AGCTTGATTTGGAAGTCATGAAA	TET	A	163-205	22
	R	CCATAATGGAAAAACCAAAGGC				
EV94*	**F**	TGGCCATCGCTCTTAACAGGA	HEX	A	182-204	11
	R	TCATTGAATTGAACATTTTATAAGGGT				
EV96*	**F**	AATGCCTGAAAAGGCCTGAAGG	FAM	A	146-172	13
	R	TCTTGACCACTCAATTCTTGCCC				
EV104*	**F**	TGGAGATGACAGGATTTGGGAA	TET	A	120-130	5
	R	GGTCCAATGCCTGCTTTCG				
GATA28	**F**	AAAGACTGAGATCTATAGTTA	TET	B	144-200	11
	R	CGCTGATAGATTAGTCTAGG				
GATA417*	**F**	CACCTTGGAACTGCTTGGGAT	FAM	A	216-316	18
	R	ATTTTCAAGTTTGCTTTCCATTTTG				
TAA31*	**F**	GCCGCATCGGACAGCCA	HEX	B	251-277	4
	R	GGATTGTTCTGTGGCTTTTGGAGC				
464/465	**F**	GGGGTTTCTCCTCTA	HEX	B	128-144	6
	R	TGATCTGCCAATAAGA				
GT211	**F**	GGCACAAGTCAGTAAGGTAGG	FAM	B	192-212	10
	R	CATCTGTGCTTCCACAAGCCC				
GT575	**F**	TATAAGTGAATACAAAGACCC	FAM	B	134-174	15
	R	ACCATCAACTGGAAGTCTTTC				

* Re-designed primers were used for these loci instead of the original published primer sequences to increase PCR amplification and reduce non-specific amplification.

[1] ABI fluorescent dye labels were attached to each forward primer (FAM, TET or HEX)

[2] Two separate sets of pooled loci were genotyped so that no two loci overlapped in both dye label and size range

Table 3: Number of unique individual microsatellite genotypes from each site and years in which sampling was undertaken.

Sampling Sites	**Years of Sampling**	**Number unique genotypes**
New Caledonia	1995-2005	385
Tonga	1991-2005	368
Cook Islands	1996-2005	109
French Polynesia	1997-2005	224
Antarctic Peninsula (Area I)	1989-1999	73
Antarctic unknown	2001	3
Antarctic Area I	1994, 2001	13
Antarctic Area II	2005	1
Antarctic Area III	1992-2005	11
Antarctic Area IV	1999	46
Antarctic Area V	1991-2004	9
Antarctic Area VI	1990, 2001	19
Total Eastern Australia	1996-2004	734
Total Oceania	1991-2005	1086
Total Antarctic	1989-2005	175

Table 4: Summary of samples with genotype matches between Eastern Australia and Oceania breeding sites or Antarctic feeding grounds including sample code for each site, sampling site, year sampled at each site, molecular sex identification of each sample, mtDNA haplotype sequence (as per Olavarria *et al*., 2007), number of loci that match exactly between samples with number of mismatching loci shown in brackets ( ), probability of identity as per Paetkau *et al.* (1995) and P*sibs*.

Sample Name	Site	Year	Sex	mtDNA	Number of matching loci	PI PIsibs
98NC025	New Caledonia	1998	Male	SP11	8	2.92×10^{-7}
01BBG09	Byron Bay, EA	2001	Male	unknown		2.29×10^{-3}
01NC074	New Caledonia	2001	Male	unknown	11	3.64×10^{-12}
04BB062	Byron Bay, EA	2004	Male	unknown		7.29×10^{-5}
97NC013	New Caledonia	1997	Male	SP017	11	3.30×10^{-12}
02HBP03	Hervey Bay, EA	2002	Male	SP017		1.13×10^{-4}
01NC009	New Caledonia	2001	Male	SP073	11	3.64×10^{-12}
02BBJ04	Byron Bay, EA	2002	Male	SP073		7.29×10^{-5}
02BBO10	Byron Bay, EA	2002	Male	SP052	10	4.68×10^{-13}
03NC040	New Caledonia	2003	Male	SP052		1.73×10^{-4}
99NC003	New Caledonia	1999	Male	SP063	11	2.42×10^{-13}
00HBB02	Hervey Bay, EA	2000	Male	unknown		1.03×10^{-4}
01BBH04	Byron Bay, EA	2001	Male	unknown	11	9.05×10^{-13}
05NC020	New Caledonia	2005	Male	SP14		5.45×10^{-5}
03HBM08	Hervey Bay, EA	2003	Male	SP011	11	4.88×10^{-12}
05NC090	New Caledonia	2005	Male	SP011		1.05×10^{-4}
95NC001	New Caledonia	1995	Female	SP002	11	5.85×10^{-18}
01BBE04	Byron Bay, EA	2001	Female	unknown		2.44×10^{-5}
01NC066	New Caledonia	2001	Female	SP046	12	1.07×10^{-15}
02HBB10	Hervey Bay, EA	2002	Female	SP046		1.59×10^{-5}
01NC096	New Caledonia	2001	Male	SP014	10	3.64×10^{-11}
04BB068	Byron Bay, EA	2004	Male	unknown		1.81×10^{-4}
00HBD03	Hervey Bay, EA	2000	Male	unknown	11	7.72×10^{-14}
03Tg100	Tonga	2003	Male	SP091		5.64×10^{-5}
91H002	Antarctic Area V	1992	unknown	SP011	10	1.94×10^{-13}
01HBT03	Hervey Bay, EA	2001	Female	unknown		1.17×10^{-4}
99A51519	Antarctic Area V	1999	Male	SP115	10 (1)	3.35×10^{-11}
01BBE01	Byron Bay, EA	2001	Male	unknown		1.84×10^{-4}
91H001	Antarctic Area V	1992	unknown	unknown	12	3.85×10^{-14}
02BBQ03	Byron Bay, EA	2002	Female	SP076		1.89×10^{-5}

Table 5: Microsatellite genotypes of all matching samples detected in this study.

Sample	**464/465**		**Ev1**		**Ev14**		**Ev21**		**Ev37**		**Ev94**		**Ev96**		**Ev104**		**GATA28**		**GATA417**		**GT211**		**GT575**	
MnoIWC91H001	139	139	123	125	131	137	111	113	196	210	212	214	161	163	149	151	147	155	199	222	104	116	145	153
02BBQ03	139	139	123	125	131	137	111	113	196	210	212	214	161	163	149	151	147	155	199	222	104	116	145	153
Mno01NC066	139	143	123	125	129	131	111	111	200	206	210	212	159	163	149	151	147	155	210	218	108	108	137	153
02HBB10	139	143	123	125	129	131	111	111	200	206	210	212	159	163	149	151	147	155	210	218	108	108	137	153
00HBD03	139	143	123	127	131	133	111	111	192	214	206	218	153	165	147	147	147	147	199	226	106	118	151	153
Mno03Tg100	139	143	123	127	131	133	111	111	192	214	206	218	153	165	0	0	147	147	199	226	106	118	151	153
Mno97NC013	139	143	123	125	133	135	111	111	198	210	208	214	153	169	149	149	147	147	195	199	114	116	151	153
02HBP03	139	143	123	125	133	135	111	111	198	210	208	214	0	0	149	149	147	147	195	199	114	116	151	153
Mno99NC003	139	143	123	123	131	131	111	111	198	200	214	216	169	171	149	149	147	159	0	0	108	112	145	161
00HBB02	139	143	123	123	131	131	111	111	198	200	214	216	169	171	149	149	147	159	195	218	108	112	145	161
Mno95NC001	139	139	123	123	135	141	111	117	190	192	214	214	153	155	0	0	147	155	222	226	110	114	151	151
01BBE04	139	139	123	123	135	141	111	117	190	192	214	214	153	155	149	151	147	155	222	226	110	114	151	151
03HBM08	139	143	123	123	131	133	109	111	208	212	214	216	161	169	149	151	147	147	199	218	108	112	151	153
Mno05NC090	139	143	123	123	131	133	109	111	208	212	214	216	161	169	149	151	147	147	199	218	108	112	0	0
MnoIWC91H002	137	139	123	125	131	131	109	109	198	202	206	212	147	165	147	149	147	147	0	0	108	112	153	161
01HBT03	137	139	123	125	131	131	109	109	198	202	206	212	0	0	147	149	147	147	0	0	108	112	153	161
02BBO10	139	139	0	0	133	133	111	113	196	198	208	214	0	0	149	149	147	147	195	238	110	112	147	151
Mno03NC040	139	139	123	123	133	133	111	113	196	198	208	214	147	169	149	149	147	147	195	238	110	112	147	151
Mno01NC009	143	143	123	123	131	131	111	113	192	210	210	214	161	163	149	149	147	147	211	222	110	116	151	157
02BBJ04	143	143	123	123	131	131	111	113	192	210	210	214	0	0	149	149	147	147	211	222	110	116	151	157
Mno01NC096	133	139	123	123	0	0	111	111	196	206	210	212	161	163	151	151	156	183	199	203	104	118	153	153
04BB068	133	139	123	123	131	133	111	111	196	206	210	212	0	0	151	151	156	183	199	203	104	118	153	153
Mno01NC074	139	139	123	127	131	141	109	115	202	208	210	214	159	161	149	149	147	203	199	207	106	108	145	159
04BB062	139	139	123	127	131	141	109	115	202	208	210	214	0	0	149	149	147	203	199	207	106	108	145	159
Mno99A51519	139	139	123	123	131	133	111	111	200	216	216	216	161	163	149	151	147	155	207	218	108	110	151	151
01BBE01	139	139	123	123	131	133	111	111	200	216	214	216	161	163	149	151	147	155	207	218	0	0	151	151
01BBH04	139	139	123	127	133	141	0	0	204	210	212	214	149	169	149	149	147	183	187	199	106	108	145	153
Mno05NC020	139	139	123	127	133	141	109	115	204	210	212	214	149	169	149	149	147	183	187	199	106	108	145	153
Mno98NC025	139	139	127	127	131	133	111	113	214	214	202	218	163	171	147	149	147	187	199	222	108	110	145	151
01BBG09	139	139	127	127	131	133	111	113	0	0	202	218	0	0	147	149	147	187	0	0	0	0	145	151